IN VITRO ANTICANCER ACTIVITY STUDIES OF DIINDOLYLMETHANE (DIM) CONJUGATE OF BIARYLS IN HUMAN CERVICAL AND BREAST CANCER CELLS

THESIS SUBMITTED TO **AcSIR** FOR THE AWARD OF THE DEGREE OF
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CERTIFICATE

This is to certify that the work incorporated in this Ph. D. thesis entitled ***In vitro* anticancer activity studies of diindolylmethane (DIM) conjugate of biaryls in human cervical and breast cancer cells**" submitted by **Ms. Shilpa. G.** to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Biological Sciences embodies original research work carried out by her under my supervision. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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DECLARATION

I hereby declare that the matter embodied in the thesis entitled: "***In vitro* anticancer activity studies of diindolylmethane (DIM) conjugate of biaryls in human cervical and breast cancer cells**" is the result of the work carried out by me at the Agro-Processing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram, under the supervision of Dr. Priya. S. and the same has not been submitted elsewhere for any other degree.

Shilpa. G.

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I am humbled before the universe and its infinite miracles.

Shilpa G

…TO MY MOTHER, DALPHINE

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List of Abbreviations

%	percentage
µm	micrometer
µM	micromolar
2D	two-dimensional
2-NBDG	2-[N-(7-nitrobenz-2- oxa-1, 3-diazol-4-yl) amino]-2 deoxy-D-glucose
3D	three-dimensional
6-CFDA	6-carboxyfluorescein diacetate
ADT	Autodock tools
AEG-1	Astrocyte Elevated Gene-1
AFC	7- amino- 4 trifluoromethyl coumarin
AhR	Aryl hydrocarbon Receptor
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
AMPK	Adenosine Monophosphate-Activated Protein Kinase
AO/EB	Acridine orange/Ethidium bromide
APL	Alkylphospholipid
APS	Ammonium persulfate
AR	Androgen Receptor
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
Bad	Bcl-2-associated death promoter
Bax	BCL2-Associated X Protein
BCA	Bicinchoninic acid
Bcl2	B-cell lymphoma 2
BH3	Bcl-2 Homology 3
CAF	Cancer-associated fibroblasts
CAM	Cell adhesion molecule
CCNS	Cell cycle-Nonspecific
CCS	Cell cycle-specific
CDK	Cyclin-dependent kinases
CFC	Chlorofluorocarbon
c-FLIP	cellular FLICE-inhibitory protein
c-PARP	cleaved Poly ADP (Adenosine Diphosphate)-Ribose Polymerase
CYP	Cytochrome P450
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamidine
DCFDA	2,7-dichlorofluorescein diacetate
DIM	3,3'-diindolylmethane

DIMIRC	DIM Information Resource Centre
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyl Transferase
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Triacetic acid
EGCG	Epigallocatechin-3-Gallate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen receptor
ERK	Extracellular Regulated Kinase
FADD	Fas-Associated Death Domain
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FOXM1	Forkhead Box M1
GAP	GTPase activating proteins
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine 5'-Diphosphate
GI_{50}	Growth inhibition of 50 percentage of cells
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GLUT	Glucose Transporter
GPCR	G protein-coupled receptor
GSK	Glycogen Synthase Kinase
GTP	Guanosine 5'-Triphosphate
h	hour
HDAC	Histone Deacetylases
HER-2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia-Inducible Factor 1 alpha
HK2	Hexokinase
hpf	hours post fertilization
HPV	Human papillomavirus
HR-ESI-MS	High resolution electrospray ionization-mass spectroscopy
HRP	Horseradish peroxidase
HUVEC	Human Umbilical Vascular Endothelial Cell
I3C	Indole-3-Carbinol

IAP	Inhibitor of apoptosis
IARC	International Agency for Research on Cancer
IC_{50}	Median inhibition concentration
IFN	Interferon
IGF	Insulin Growth Factor
IgG	Immunoglobulin G
IP	Immunoprecipitation
kDa	kilodalton
LHRH	Luteinizing Hormone-Releasing Hormone
MAPK	Mitogen-Activated Protein Kinases
MCTS	Multicellular tumor spheroid culture
MET	Mesenchymal to epithelial transition
MMP	Matrix Metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
MSC	Mesenchymal stem cells
mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NCCS	National Centre for Cell Science
NF-κB	Nuclear Factor Kappa Beta
NGS	Normal goat serum
NK	Natural killer
NMR	Nuclear Magnetic Resonance
nM	nanomolar
Nrf2	Nuclear factor E2 p45-related factor 2
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pCR	pathologic complete response
PDGF	Platelet Derived Growth Factor
PFK	6-phosphofructokinase
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
PIC	Protease inhibitor cocktail
PKM2	Pyruvate kinase muscle isozyme M2
PMSF	Phenylmethanesulfonyl Fluoride
poly-HEMA	poly 2-hydroxyethylmethacrylate
PR	Progesterone Receptor
PS	Phosphatidylserine
PTEN	Phosphatase and Tensin Homolog
P-TsOH	P-toluenesulfonic acid
PVDF	polyvinylidene fluoride
Rb	Retinoblastoma
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic Acid

ROS	Reactive oxygen species
RPM	Revolutions per minute
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SERD	Selective Estrogen Receptor Downregulators
SERM	Selective Estrogen Receptor Modulator
SF	Sulforaphane
STAT	Signal Transducer and Activator of Transcription
TEMED	N,N,N,N’ tetramethylethylenediamine
TF	Transcription factor
TGF	Transforming Growth Factor
TIL	Tumor infiltrating lymphocytes
TMB	5, 5' tetramethylbenzidine
TNBC	Triple-negative breast cancer
TNM	Tumor, nodes, and metastasis
TRAIL	Tumor Necrosis Factor-Alpha-Related Apoptosis-Inducing Ligand
TSG	Tumor suppressor genes
uPAR	urokinase-Type Plasminogen Activator Receptor
VAP-1	Vascular adhesion protein 1
VCAM	Vascular CAM
VEGF	Vascular Endothelial Growth Hormone
ZFET	Zebrafish Emryo Toxicity Test

Introduction

Cancer is a severe genetic/metabolic syndrome that claims millions of lives every year and is still on the rise regardless of the modern advancements in drug discovery. The disease progresses by deregulating multiple cellular pathways, and these abilities are popularly known as hallmarks of cancer (Hanahan *et al*., 2012). Effective attenuation of cancer requires inhibition of the multiple hallmarks of cancer. The anticancer drugs currently available in the market incite many undesirable side effects in the body, and hence natural resources are currently being investigated for potential molecules with selective anticancer activity and less toxicity (Ratovitski 2017). 3,3'-diindolylmethane (DIM) is a natural compound present in cruciferous vegetables, that has been widely investigated for anticancer activity in multiple cancer types. DIM inhibits cancer progression at various stages by a pleiotropic mode of action (Thomson *et al*., 2016). DIM Information Resource Centre (DIMIRC) gives a consolidated overview of the various molecular targets regulated by DIM in anticancer studies.

The current study investigates the *in vitro* anticancer activity of novel derivatives of DIM conjugate of biaryls in cervical and breast cancer cells. The DIM compounds were synthesized by a one-step condensation reaction that was economical and time-saving. The compounds were investigated for the detailed anticancer activity studies in cervical and breast cancer cells. The thesis is divided into six chapters.

Chapter 1 is the introductory chapter which comprehensively reviews the literature regarding cancer, its causative factors, progression stages, anticancer therapy, anticancer targets, and major anticancer drugs. It discusses the anticancer properties of various natural products, their targets with specific reference to diindolylmethane in multiple types of cancer and its important molecular targets. It also provides supporting evidence and justifications for the investigation of novel synthetic ortho-biaryl diindolylmethane derivatives for anticancer properties against human cervical and breast cancer cells as potential anticancer compounds.

Chapter 2 provides comprehensive details regarding the materials and methods utilised to investigate the multiple anticancer properties of the compounds in two-dimensional and three-dimensional culture.

Chapter 3 investigates the anticancer potential of the parent compound DIM-biaryl derivative (DIM-1) and its 11 derivatives in human cervical cancer cells (HeLa). Based on the preliminary screening by cytotoxicity evaluation, effective compounds were identified for further studies. The compounds were investigated for various apoptotic parameters such as DNA fragmentation, plasma membrane integrity loss, mitochondrial depolarization, caspase activity and expression of apoptosis-associated proteins. The compounds were also studied for activity against multiple hallmark properties of cancer such as anti-migration, anti-angiogenesis, and cell cycle arrest. The effects of the compounds on prominent cancer associated proteins were also investigated in this chapter.

Chapter 4 studies the anticancer effects of the novel DIM compounds in metastatic breast cancer cells (MDA-MB-231). The compounds were initially screened for cytotoxicity which was followed by studying the ability of the potential compounds to induce apoptosis using multiple assays including Annexin V assay, caspase activity assay and western blot analysis of apoptotic proteins. The anti-migration and anti-angiogenic properties of the compound were also evaluated. The mechanism of action of the compounds was investigated by studying multiple parameters including ROS generation, cell cycle progression, and metabolic fate. For further investigation on the mechanism of action of the compounds, pathways usually found deregulated in cancer were studied. The effect of the compounds on p53, FAK/RAS and PI3K/AKT/mTOR pathways were also investigated.

Chapter 5 deals with the comparative study of the potential compounds in MDA-MB-231 cells cultured in 2D and 3D conditions. The study mainly involves the translation of the anticancer effects obtained for the DIM compounds in monolayer culture to a more complex 3D system, as the latter system more faithfully represents the response of the cells towards a drug, in an *in vivo* system. The parameters subjected to comparison were cytotoxicity, caspase activity, expression of apoptotic proteins, MMP activity, and VEGF expression. The differential expression of p53, integrin/FAK, and PI3K/AKT/mTOR pathway proteins were also investigated. The interaction of the potential compounds with various receptors were investigated using autodock analysis, which was followed by focussed studies on the effect of the compounds on EGFR expression and activation.

Chapter 6 gives a summary on the *in vitro* anticancer activity exhibited by the potential DIM compounds in cervical and breast cancer cells. It also discusses the positive results from 2D and 3D comparative studies that indicate the possible translation of the anticancer activity of the selected DIM compounds from an *in vitro* to an *in vivo* system.

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Chapter 1

Review of literature

1.1. Cancer

Cancer is a term used to include a group of diseases which is characterized by uncontrolled division of abnormal cells which gains the ability to invade other tissues. The International Union against Cancer defines cancer as "a disruption of growth characterized primarily by an excessive proliferation of cells without apparent relation to the physiological demands of the organ involved". In normal cells, all functions including development, division, differentiation, and death are carefully regulated, but the tumor cells gain the ability to divide without responding to any regulation, producing a groups of cells, which can expand indefinitely. Benign tumors are localized and do not invade or spread and are mostly harmless. But malignant tumors, on the other hand, are usually fatal as they invade distant locations through the circulatory system and form secondary tumors in a process known as metastasis (Cooper, 2000). Metastasis is the main reason responsible for the mortality associated with cancer. An important characteristic of cancer is clonality, which is the development of a tumor mass from a single abnormal cell. And according to literature, the initiation and development of cancer is a complex multistep process.

According to the American Joint Committee on Cancer (AJCC), most types of cancer are classified into different stages of progression based on the TNM system. Stage 0 describes cancer which are *in situ* and has not spread to nearby tissues. Stage I comprises of small cancer/tumor which has not spread extensively into adjacent tissues, lymph nodes or distant tissues. But stage II and III comprise of large tumors that have deeply invaded the nearby tissues and may have affected the lymph nodes. And stage IV includes metastatic cancer, which has spread to other organs of the body (www.cancer.net).

In the year 2000, Hanahan and Weinberg described the six hallmarks of cancer: self-sufficiency in growth signals, unresponsive to inhibitory signals, escape from apoptosis, unlimited proliferation, sustained angiogenesis, and tissue invasion and metastasis which is shown in Figure 1.1. In later reports, metabolic

rewiring of pathways, an abetting microenvironment, and immune modulation was further added to the existing hallmarks (Fouad *et al*., 2017).

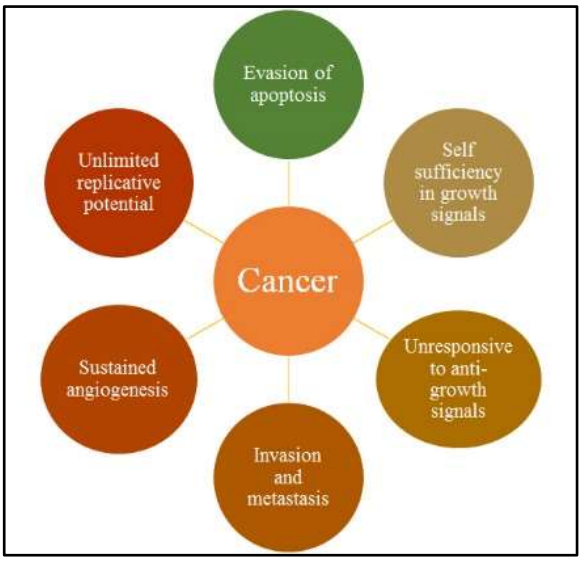


Figure 1.1. Hallmarks of cancer (Hanahan et al., 2000).

1.2. Cancer- global statistics

Cancer is the prominent cause of death worldwide after cardiovascular diseases. International Agency for Research on Cancer (IARC) has postulated estimated incidence and mortality of cancer worldwide using GLOBOCAN 2018. The study estimated 18.1 million new cases and 9.6 million deaths in 2018. With 11.6% of total cases, lung cancer was the most commonly diagnosed and lethal cancer (18.6%), which was closely followed by breast cancer, prostate cancer, and colorectal cancer. Figure 1.2. shows the cancers with top incidence and mortality rates according to GLOBOCAN 2018. In males, lung cancer is the most commonly diagnosed cancer followed by prostate and colorectal cancer. However, in females, breast cancer was the most incident cancer followed by colorectal, lung and cervical cancer (Bray *et al*., 2018). According to Cancer Atlas, the number of new cases of cancer incidence was estimated to be 14.1 million while mortality was estimated at 8.2 million in 2012. So it can be seen that the estimated incidence has increased by 4 million and mortality by 1.4 million cases within 4 years. Hence it can be stated that cancer is a global menace with rapidly growing incidence and mortality rates.

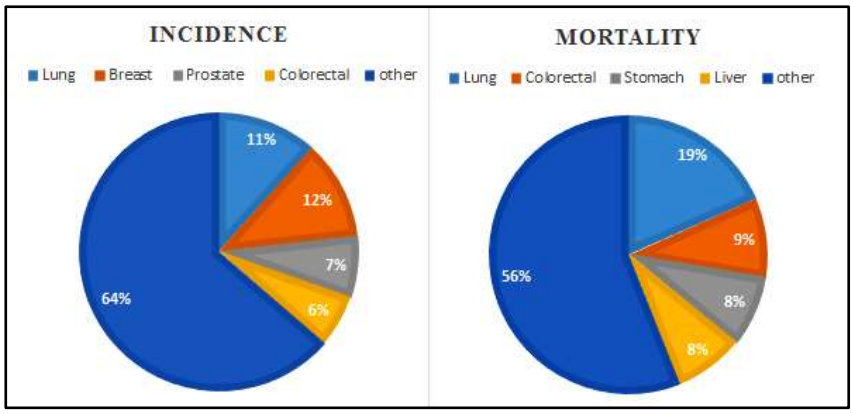


Figure 1.2. Estimates for cancer incidence and mortality in 2018 reported by GLOBOCAN: *The figure displays the five types of cancer with the highest rates of incidence and mortality.*

1.3. Different steps in cancer development

The root cause of cancer is usually genetic or epigenetic alterations, but the progression of cancer involves multiple steps associated with a complex interplay between the tumor cells and its environment. Figure 1.3. gives an overview about the various steps involved in cancer development.

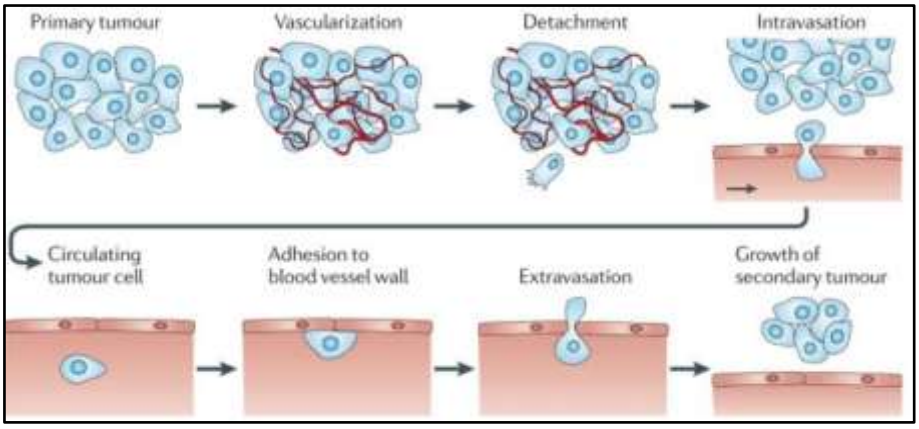


Figure 1.3. Multiple stages of tumor development: *The transition of a primary tumor into a metastatic tumor through a series of steps namely recruitment of blood vessels, EMT, intravasating into the circulatory vessel, extravasation to a secondary location, MET and formation of secondary tumor (Wirtz et al., 2011).*

1.3.1. Formation of primary tumor

The clonal evolution model proposed by Nowell 1976, states that a normal cell becomes neoplastic due to a genetic or epigenetic mutation in a critical gene which leads to uncontrolled proliferation giving rise to a clone of dividing neoplastic cells (hyperplasia). Followed by this, some of the dividing cells accumulate additional mutations that provide a selective advantage over the other

cells in the process known as clonal selection. These alterations typically promote progression from a relatively benign group of proliferating cells to a mass of cells with abnormal morphology, cytological appearance, and cellular organization. This continues throughout the development of the tumor endowing it with many properties thereby making it immortal (Cooper, 2000). The process is shown in Figure 1.4. According to Vogelstein *et al*. 2013, among the thousands of mutations present in tumor cells, only a few driver mutations are responsible for the development of cancer.

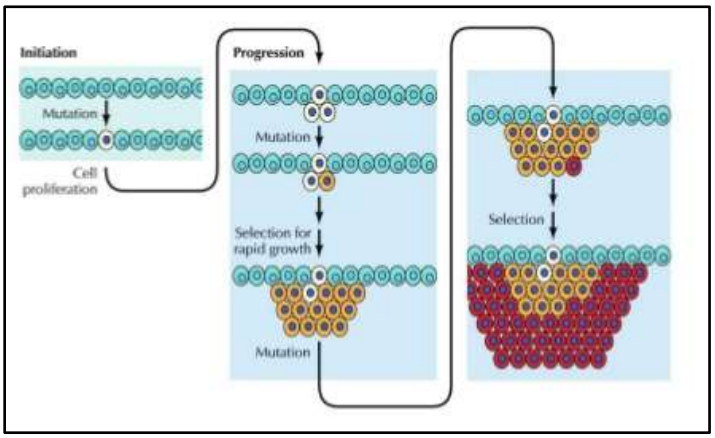


Figure 1.4. Clonal selection in tumor development (Cooper, 2000).

1.3.2. Vascularization

For the unimpeded growth of tumors beyond a certain size and for its metastasis, close proximity to vasculature is very essential. As the tumor size increases, the core loses access to oxygen and nutrients, and this leads to the recruitment of new blood vessels towards the tumor in the process known as angiogenesis (Sever *et al*., 2015). It involves the formation of new vasculature from existing blood vessels through a process of sprouting, division, and assembly of endothelial cells. The process is strictly controlled by the balance of anti-angiogenic and pro-angiogenic factors and in cancer, the switch is tipped towards the pro-angiogenic factors (Carmeliet *et al*., 2000). One of the main triggers for angiogenesis in cancer is the hypoxic condition. Endothelial cells and tumor cells possess many oxygen sensing mechanisms which interact with the hypoxia-inducible transcription factor (HIF) family proteins. During hypoxic conditions, HIF gets stabilized and transcribes many proteins that turn the angiogenic switch

on. VEGF signalling is a key effector of this process that triggers the proliferation and assembly of new vasculature (Hicklin *et al*., 2005; Fouad *et al*., 2017).

1.3.3. Epithelial-mesenchymal transition (EMT)

The invasion and migration of cancer cells can only be possible when the adherent epithelial cells are able to detach and invade the surrounding tissues. This is made possible by the transformation of the polarized primary tumor cells into motile and invasive mesenchymal cells in the process known as epithelial-mesenchymal transition (EMT). The transition is triggered by the reactive tumor microenvironment which produces EMT-inducing signals, notably HGF, EGF, PDGF, and TGF-β (Kalluri *et al*., 2009). These factors trigger a cohort of transcription factors (EMT-TFs) including those of Snail, Slug, Twist and Zeb families which activate the EMT program. During EMT the cells gain biochemical markers of the mesenchymal phenotype while renouncing epithelial markers through the respective transcription programs. The expression of cell-cell adhesion proteins and cell-ECM adhesion proteins are found suppressed in mesenchymal cells. A major hallmark is the replacement of E-cadherin by N-cadherin during EMT (Ye *et al*., 2015). Figure 1.5. depicts EMT and markers associated with the transition. The mesenchymal cells are anoikis-resistant and can enter the circulatory system resulting in metastasis.

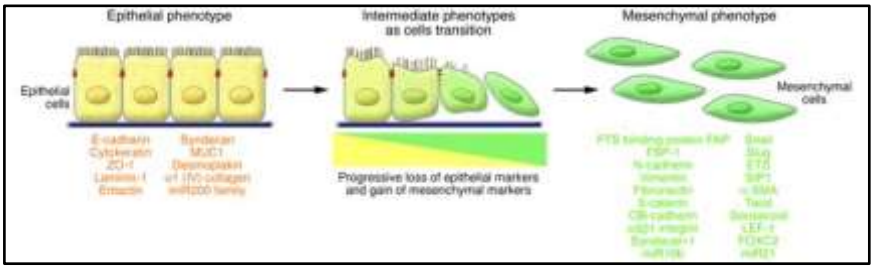


Figure 1.5. Epithelial-mesenchymal transition (EMT): *The figure was adopted from Ye et al., 2015.*

1.3.4. Anoikis resistance

When integrins on the cells, bind to ECM, they form focal adhesions and activate signalling through focal adhesion kinase (FAK) and adaptor proteins to induce pro-survival pathways (PI3K/AKT signaling). But when normal cells lose contact with ECM or neighbouring cells, cell death is induced via intrinsic and

extrinsic pathways and is known as anoikis. The schematic representation of anoikis is depicted in Figure 1.6. Anoikis resistance and anchorage-independence allow tumor cells to invade adjacent tissues, and survive the vascular system, giving rise to metastasis. Tumor cells have devised variety of strategies to overcome anoikis. Some strategies consist of adaptive cellular changes while other strategies aim at hyper-activating survival and proliferative cascades (Guadamillas *et al*., 2011). The upregulation of FLIP inhibits extrinsic apoptotic pathway (Kim *et al*., 2012). EMT-promoting proteins such as N-cadherin are associated with anoikis resistance (Li *et al*., 2001). Loss of E-Cadherin also induces anoikis resistance. Abnormal regulation of growth factor receptors in cancer cells, activates pro-survival signaling pathways, such as the PI3K/AKT, RAS/MAPK, etc. thereby resisting anoikis. This can be either through receptor overexpression or autocrine signalling of growth factors (Sharma *et al*., 2007). Increased cellular ROS in cancer cells were also found to be responsible for anoikis resistance through activation of src dependant pro-survival signalling (Loza-Coll *et al*., 2005).

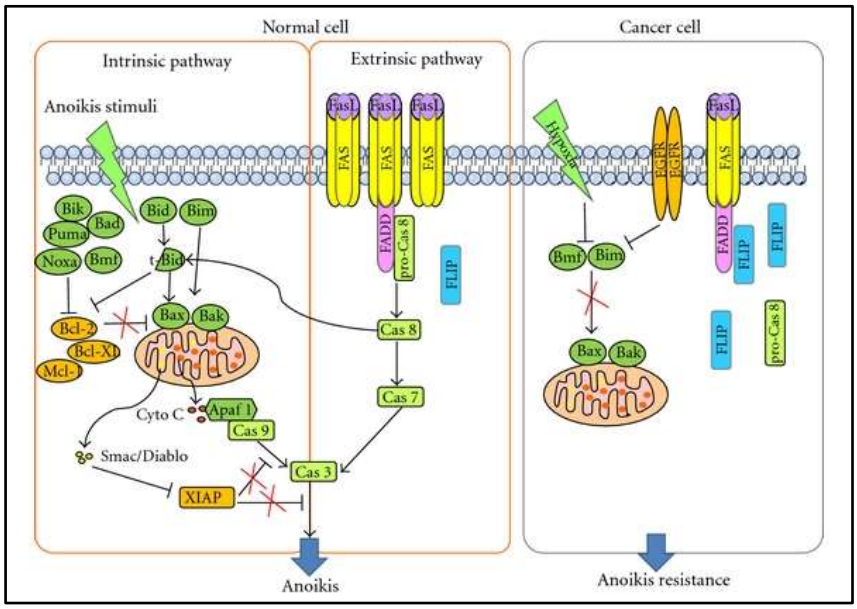


Figure 1.6. Anoikis and anoikis resistance: *In normal cells, detachment triggers both intrinsic and extrinsic apoptotic pathways (anoikis). But in cancer cells anoikis is evaded through the manipulation of various factors (Kim et al., 2012).*

1.3.5. Intravasation

The metastatic cascade involves multiple steps such as invasion, intravasation, and extravasation, leading to secondary tumor formation at a distant site. Intravasation is the process by which tumor cells cross the endothelial barrier

of the blood vessels and enter the circulation. Many factors affect intravasation, and some are microvessel density and diameter of the vasculature, tumor microenvironment and its components (proteases, signaling molecules). Many molecules and pathways responsible for the process have also been identified based on *in vivo* and *in vitro* studies. TGF-β and its target genes were found to enhance the process through EMT cascade. Besides, a number of studies have demonstrated that activation of EGF receptor family and downstream signaling, induce invadopodia aiding intravasation. Many proteases, including uPA/uPAR, and MMPs have been found to assist intravasation (Chiang *et al*., 2016).

1.3.6. Intravascular survival

The tumor cells upon entering the circulatory system use various strategies to overcome the immunological and mechanical stress. Many studies have revealed that the formation of tumor cell-platelet micro-aggregates provide physical protection for tumor cells in circulation (Stegner *et al*., 2014). Studies have shown that tumor cells shed ligands (NKG2D) that are usually recognised by immune cells (Chitadze *et al*., 2013). Tumor cells escape immune responses by secreting immune-modulatory molecules (TGF), physical shielding, expression of VCAM 1 and VAP-1 and the resultant pro-survival signalling (Strilic *et al*., 2017).

1.3.7. Extravasation

Tumor cell extravasation involves adhesion of the circulating tumor cell to the endothelium, modulation of the endothelial barrier, and transendothelial migration to reach the underlying tissues. Paracellular migration was found to be the predominant mode of extravasation, where tumor cells migrated between endothelial cells. But studies have also shown transcellular migration where tumor cells cross the endothelium directly. The adhesion of tumor cells to endothelium is assisted by various molecules like selectins, cadherins, and integrins (Reymond *et al*., 2013). Apart from this, Strilic *et al*. reported that tumor cells can directly promote their own extravasation through many ways. The secretion of proteins such as angiopoietin-like 4, and osteonectin which interacts with endothelial cell junction proteins is one method, while apoptosis induction in the endothelial cell is another. Once the tumor cells have crossed the endothelial cell barrier, they form secondary tumors at the parenchyma of the distant site.

1.3.8. Formation of secondary tumor

In the new stromal environment, an even smaller subset of tumor cells establish micrometastases with the potential to proliferate into fully malignant, secondary tumors. Studies strongly argue that reversion of EMT is essential for metastasis colonization. The mesenchymal-to-epithelial (MET) transition is associated with inhibition of EMT-activators and upregulation of EMT inhibitors. MET is said to be triggered by the new microenvironment which results in the formation of a secondary tumor which has regained its primary characteristics. Many findings suggest that it might be the contact with normal parenchymal cells that contributes to the MET in tumor cells (Yao *et al*., 2011).

1.4. Mutations in cancer

The initial event that leads to the development of cancer is the induction of mutation in a critical gene resulting in continuous cell proliferation. The factors that cause cancer-related mutation can be broadly divided into physical factors (UV rays, γ rays, X rays), chemical factors (carcinogens), viral infection (HPV), inherited mutation (BRCA1/BRCA2), and endogenous factors (replication errors, ROS). IARC has identified major factors responsible for cancer formation namely tobacco, alcohol consumption, occupational exposure (asbestos, silica, coal tar), environmental pollution (CFC), food contaminants (aflatoxins, pesticides), and medicinal contaminants. Sloan *et al*. 2007, also quotes additional risk factors of cancer such as viral infection and physical inactivity. IARC has classified carcinogens based on their potential for carcinogenicity into four groups: Group 1 being the most carcinogenic in humans, Group 2 showing limited evidence of carcinogenicity, Group 3 containing chemicals associated to cancer with insufficient evidence and Group 4 contains chemicals which are not carcinogenic to humans (Kufe *et al*., 2003).

1.4.1. Genes deregulated in cancer

The accumulation of mutations in genes that play an important part in cell proliferation is the primary cause of cancer. These genes were found to be associated with the regulation of cell proliferation, apoptosis or DNA repair. They are categorized into two broad classes of genes, namely tumor suppressor genes (TSG) and proto-oncogenes. Proto-oncogenes play a main role in the control of cell

proliferation and mainly encodes for growth factor kinases, growth factor receptors, cell cycle proteins, transcription factors, etc. A gain of function mutation converts proto-oncogenes into activated oncogenes that are dominant in nature. The viral counterpart of oncogenes present in many viruses can also activate cellular oncogenes. A few well-known oncogenes found deregulated in cancer are RAS, HER-2, MYC, ErbB2, PI3KCA, etc. (Lee *et al*., 2010).

TSG are genes that encode proteins which controls/limits proliferation and any loss-of-function mutations in these genes lift the checkpoints in cell proliferation. They mainly include checkpoint control proteins, cell cycle inhibitors, DNA repair enzymes, enzymes that promote apoptosis, and growth factor receptors. A few of the commonly deregulated genes in cancer are p53, Rb, BRCA1/BRCA2, PTEN, etc. (Lodish *et al*., 2000). Recent evidence also points to epigenetic deregulation as a cause of the abnormal expression of these genes.

1.5. Current cancer therapeutic strategies

The treatment of cancer mainly depends on the type, location, and stage of development. The different types of current treatment strategies are summarised in Figure 1.7.

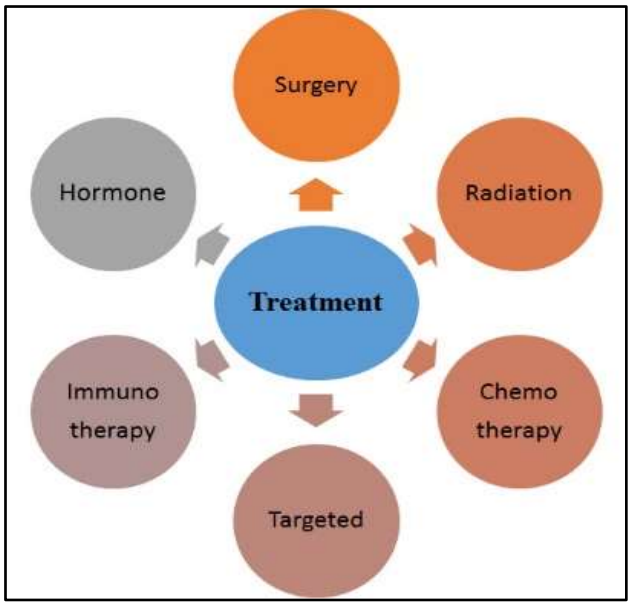


Figure 1.7. Different types of therapeutic strategies currently employed for cancer.

1.5.1. Surgery and radiation

Surgery and radiation are the first lines of treatment for localised tumors. When cancer is diagnosed at the initial stage when the tumor has not spread to other sites, surgical removal of the tumor can usually cures the disease. The concept of surgery has broadened to include laser surgery, electrosurgery, cryosurgery and high-intensity focused ultrasound (Kleinsmith, 2006). Surgery in cancer are of different types, such as curative, debulking, palliative, restorative, and preventive to name a few (www.cancer.org).

Radiation therapy involves the use of high energy ionizing radiations (X rays) such as external beams or internal implants. These radiations kill cancer cells by generating ROS that damage the DNA of cancer cells, or by chromosomal damage resulting in cell death. Radiations can be given as the single modality treatment, palliative or in combination with other therapies (Baskar *et al*., 2012). But when the stage of the cancer is advanced, and there is a high risk of recurrence, systemic treatment strategies are used in combination with surgery and radiation.

1.5.2. Chemotherapy

Chemotherapy involves the use of drugs or chemicals that circulate in the blood stream to reach cells. Chemotherapeutic drugs are usually cytotoxic or cytostatic towards cells with increased cell division like cancer cells. This is the reason behind the side effects associated with chemotherapy, as fast dividing normal cells are also affected. Despite this, it has been successfully applied for the treatment of various cancer cells. So far, there are several different classes of anticancer drugs based on their mechanisms of action, and they include alkylating agents, anti-metabolites, antibiotics, topoisomerase inhibitors, mitotic inhibitors, DNA crosslinking agents, monoclonal antibodies, etc. (Huang *et al*., 2017).

1.5.3. Targeted therapy

Targeted therapy differs from the cytotoxic drugs such that it inhibits specific antigens present or overexpressed in cancer cells. They mainly consist of hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules. A well-known example is trastuzumab (Herceptin) which is a monoclonal antibody that blocks EGFR-2/HER2 which is a receptor found

overexpressed in primary and metastatic breast cancer. Bevacizumab was the second FDA approved monoclonal antibody that targets ligand A of VEGFR in metastatic breast cancer. The small molecule inhibitors like lapatinib and sorafenib targets the tyrosine kinase enzymes (Schutz *et al*., 2008).

1.5.4. Immunotherapy

Immunotherapy bolsters the body's immune system to fight cancer cells either by modifying the host immune system or activating the existing immune cells. They include the use of monoclonal antibodies, cancer vaccines, and immune checkpoint inhibitors. The currently used strategies are the activation of effector immune cells by vaccination or augmentation of antigen presentation, administration of immune cells, antibodies to inhibit regulator T cells, antibodies to inhibit immune-checkpoint molecules, etc. A few of the examples are anti-CLA4, and anti-PD1 monoclonal antibodies (Farkona *et al*., 2016).

1.5.5. Hormone therapy

Hormone therapy is mainly directed against cancers of breast and prostate which are hormone related and are dependent on specific hormone receptors such as estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). Tamoxifen is a selective estrogen receptor modulator (SERM) which competes with estrogen and binds to ER with high affinity. Flutamide (FLU) and bicalutamide are anti-androgens that block the binding of androgen to its receptor (Abdulkareem *et al*., 2012).

1.5.6. Stem cell therapy

Currently, stem cells are also considered as an upcoming cancer therapeutic strategy. They are modified to express enzymes that can convert a pro-drug into a cytotoxic one in the vicinity of the tumor (enzyme-prodrug therapy). Stem cells are also being modified to carry oncolytic viruses, nanoparticle delivery systems and growth inhibitors towards tumor cells (Zhang *et al*., 2017).

1.6. Anticancer drug discovery-major targets

Cancer progression can be inhibited through various mechanisms, mainly by targeting the different hallmarks of cancer. The major hallmarks as already discussed include apoptosis inhibition, uncontrolled proliferation, angiogenic activity, and metastasis. Currently existent anticancer drugs attenuate cancer growth through multiple mechanisms.

1.6.1. Apoptosis pathway

Apoptosis is programmed cell death, activated by both intracellular and extracellular stress signals. Two different pathways lead to induction of apoptosis: the intrinsic (mitochondrial-mediated) and extrinsic (death-receptor) pathways that correlate with the signal type. The extrinsic apoptosis pathway is initiated through the binding of a ligand (Fas-associated death domain) to death receptors, and the intracellular signals include hypoxia, growth factor deprivation, cell detachment, stress signals, and DNA damage. Both the pathways converge at caspase enzymes which are the main effectors of apoptosis. They cleave functional and structural proteins resulting in chromatin condensation, nuclear fragmentation, cell shrinkage, apoptotic body formation which is followed by phagocytic removal of cell debris. The intracellular signals activate the BH3-only members of the Bcl2 family (Bim and Puma) that binds to the pro-survival proteins (including Bcl2 and Bcl-xL) resulting in the release of Bax / Bak which promotes loss of mitochondrial outer membrane potential, cytochrome c release and activation of caspase-9, caspase-3 and intrinsic apoptosis. In the extrinsic pathway, the activation of death receptors (Fas), results in the formation of a death-inducing signaling complex (DISC) which cleaves the initiator caspase, caspase-8 activating it. These events trigger the caspase cascade and hence apoptosis. The mechanism of both apoptotic pathways are summarised in Figure 1.8.

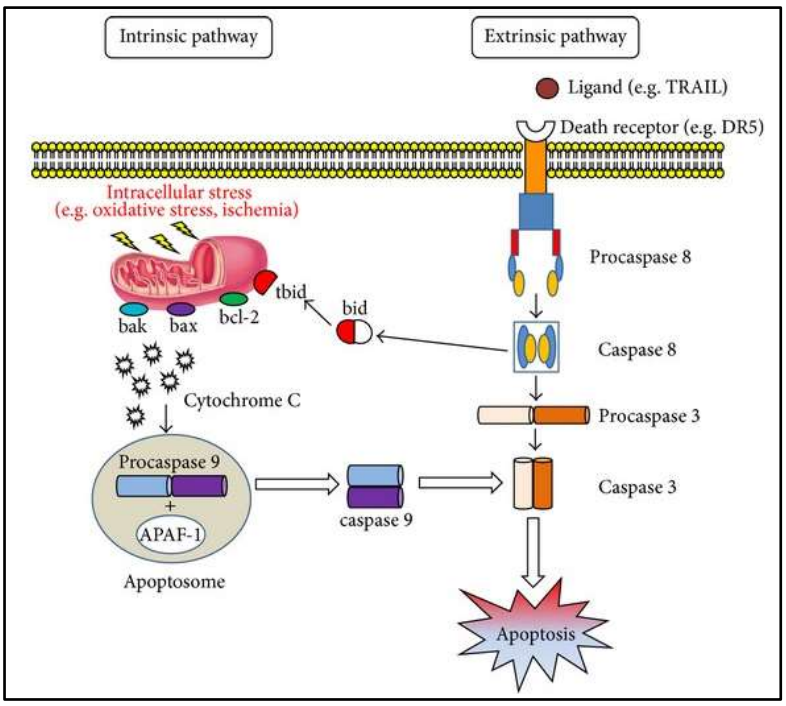


Figure 1.8. The intrinsic and extrinsic pathways of apoptosis (Loreto et al., 2014): *Apoptosis induction is carried out through two pathways, intrinsic and extrinsic pathway. External stress signals activate the death receptor mediated pathway, activating caspase 8 which activates caspase 3.While intracellular stress signals such as DNA damage or hypoxia causes the release of mitochondrial cytochrome c due to MOMP, activating caspase 9 which subsequently activates executioner caspase 3, which cleaves cellular proteins, resulting in cell death.*

Cancer cells evade apoptosis through many mechanisms including upregulation of pro-apoptotic factors, deregulated p53 expression, upregulated survival signalling etc. Most of the current anticancer drugs target diverse cellular functions to mediate apoptosis. Although the primary targets of anticancer drugs are rather distinct, it has been found that drug-induced cytotoxicity converges ultimately to apoptosis induction. Most of the cytotoxic drugs such as DNA intercalating agents, DNA synthesis inhibitors, topoisomerase inhibitors, transcription regulators, etc. inhibit cancer cells by inducing apoptosis. Anticancer drug research, targets include ligands for death-receptors, inhibitors for Bcl2, IAP inhibition and alkylphospholipid analogs (APL) which trigger apoptotic signals. Strategies to induce extrinsic pathway in tumors include the down-regulation of c-Flip by metabolic inhibitors, promotion of caspase-8 activation by interferon and by TRAIL-induced cell death. Many BH3 mimetics that release Bax and Bak proteins which trigger intrinsic pathway are currently under clinical trial. DNA damaging agents, upregulate the tumor suppressor p53 resulting in the transcription of pro-apoptotic BH3 proteins triggering mitochondrial-mediated apoptosis

(Pistritto *et al*., 2016). Interestingly p53 has also been found to be responsible for transcription independent upregulation of pro-apoptotic factors (Le Pen *et al*., 2016). In the presence of BH3 mimetics, p53 acts to activate Bax and Bak. Hence apoptosis is a major target for anticancer drugs.

1.6.2. Cell cycle arrest

Cell cycle progression is strictly regulated by cyclin-dependent kinases (CDK), cyclins, checkpoint kinases, and CDK inhibitors. The CDKs are rational targets for cancer therapy because their expression in cancer cells is often aberrant and their inhibition can induce cell death. As malignant cells evolve, both genetic and epigenetic mechanisms commonly affect the expression of cell cycle regulatory proteins, causing overexpression of cyclins and loss of expression of CDK inhibitors. Another hallmark of the transformed state is incompetent checkpoint control, resulting in aberrant responses to cellular damage. And due to this, despite cellular damage, mutated DNA is replicated that might result in the formation of malignant cells. Several cell cycle inhibitors have been used for the treatment of cancers. They include DNA damaging agents (S phase), microtubule regulators (vinca alkaloids, taxanes), DNA synthesis inhibitors (S phase), CDK inhibitors, etc. Several drugs targeting the cell cycle that are currently in clinical trials include indisulam, bryostatin-1, palbociclib, abemaciclib, ribociclib, roscovitine and flavopiridol (Bai *et al*., 2017). Figure 1.9. shows few established anticancer drugs and CDK inhibitors under clinical trial.

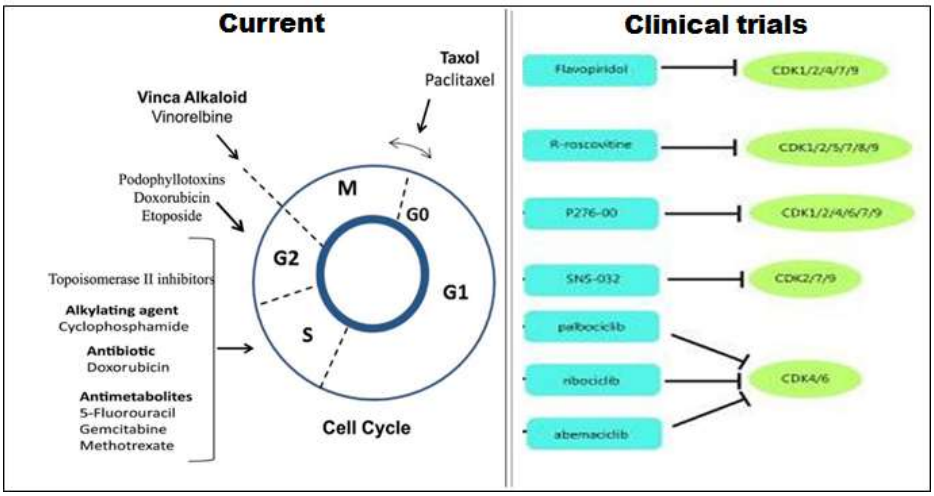


Figure 1.9. Anticancer drugs that induce cell cycle arrest

1.6.3. Angiogenesis inhibition

The primary requirement for angiogenesis is the binding of growth promoting signaling molecules, such as VEGF, to the receptors on the surface of endothelial cells. Angiogenesis inhibitors block the vascular supply to tumours by inhibiting the growth of blood vessels. These drugs interfere with various steps in angiogenesis by several mechanisms. Some are monoclonal antibodies that specifically recognize and bind with VEGF stopping it from activating the VEGF receptor. Other angiogenesis inhibitors bind to VEGF, VEGF receptors, other receptors (PDGFR, EGFR) on the surface of endothelial cells, or to proteins in the downstream signaling pathways, blocking their activities (cancerresearchul.org). Endostatin and combretastatin A4, cause apoptosis of the endothelial cells. Some immunomodulatory drugs also exhibit antiangiogenic properties. Some of the FDA approved angiogenesis inhibitors include bevacizumab, sorafenib, pazopanib, sunitinib, and everolimus. Such drugs do not necessarily kill cancer cells, but instead prevents the tumor from growing (El-Kenawi *et al*., 2013). Figure 1.10. shows the major classes of angiogenesis inhibitors and their molecular targets.

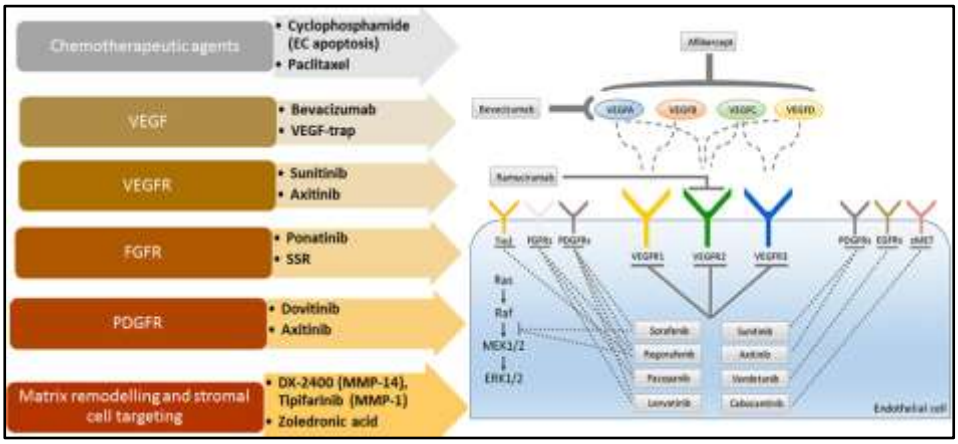


Figure 1.10. Angiogenesis inhibitors and their molecular targets (El-Kenawi et al., 2013).

1.6.4. Epigenetic manipulation

Epigenetics is the alterations in the gene expression without changing the genome structure**.** Accumulating evidence emphasizes the role for epigenetic alterations leading to inactivation of cancer suppressor genes, overexpression of oncogenes, and modification of transcription factors, which leads to deregulation of multiple intracellular signaling cascades, and finally to cancer development.

Epidrugs are the new class of drugs that target enzymes that induce epigenetic changes, and several epigenetic targets are currently under validation for new anticancer therapies. The prominent targets include histone deacetylases (HDAC) and DNA methyltransferases (DNMT), along with several other classes of enzymes which are able to operate post-translational modifications to histone tails. DNA hypermethylation and histone deacetylation in promoter regions is often associated with downregulation or silencing of gene transcription. Epigenetic silencing of tumor suppressor genes plays an important role in malignant transformation. DNMT and HDAC inhibitors induce DNA demethylation and histone acetylation, respectively, leading to reactivation of silenced genes, and dramatic morphological and functional changes in cancer cells (Verma *et al*., 2018). Figure 1.11. gives examples for FDA approved epigenetic drugs.

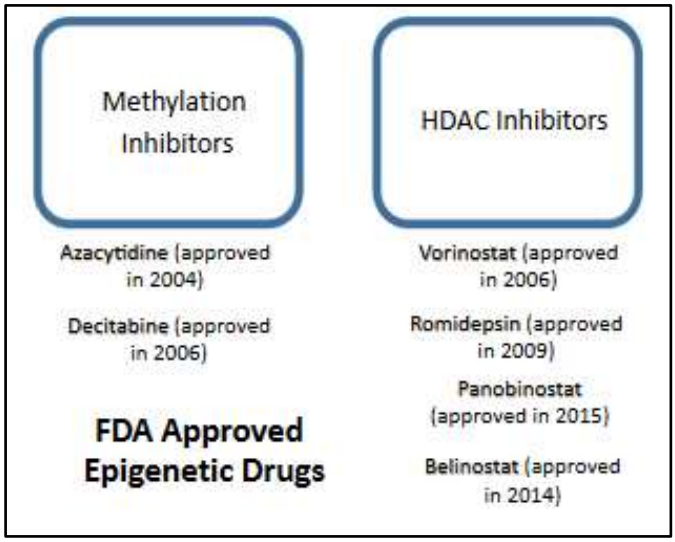


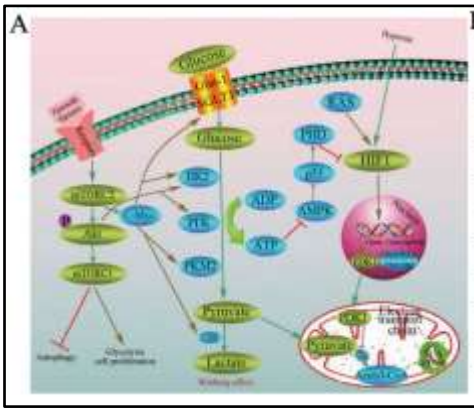
Figure 1.11. FDA approved DNMT and HDAC inhibitors (Verma et al., 2018).

1.6.5. Targeting cancer cell metabolism (Warburg effect)

Energy metabolism in most cancer cells differs remarkably from that of normal cells. Cancer cells have increased dependence on glycolysis (2 ATP) for ATP production, instead of oxidative phosphorylation (36 ATP), and this was first reported by Dr. Warburg and is hence known as the Warburg effect. This adaptation in the energy metabolism was found to be critical for tumor cell growth and proliferation, and many cancer-associated proteins, including the PI3K/AKT/mTOR signaling pathway, Myc, hypoxia-inducible factor and p53, are

involved in its regulation. Figure 1.12. depicts, the molecular regulation of the Warburg effect.

Thus, altered energy metabolism is now appreciated as a hallmark of cancer and a promising target for cancer treatment. Ritonavir, fasentin, genistein, STF-31 and WZB117 are designed to target GLUT1 transporter and exert anti-tumor effects by inhibiting glucose uptake which leads to cell death through glucose deprivation. Lonidamine inhibits HK2 enzyme in the glycolytic pathway thereby inhibiting the process. 2-Deoxy-*D*-glucose is a glucose analogue that blocks the glycolytic pathway. The glycolytic enzymes, PFK-2 and PKM2, have also gained attention as important molecular targets for anticancer compounds (Chen *et al*., 2016).



B

Drug (s)	Target
Ritonavir, Fasentin	GLUTI inhibitor
2-DG, 3-BrPA, Ionidamine	HK inhibitor
3PO, PFK15	PFKFB3 inhibitor
OA, TT-232, VK3, VK5	PKM2 inhibitor
Rapamycin	mTORC1 inhibitor
DCA	PDK1 inhibitor
Hydroxy-chloroquine	Autophagy inhibitor
Metformin	OXPHOS inhibitor
Halofuginone	Akt/mTORC1 inhibitor

Figure 1.12. (A) Molecular regulators of Warburg effect. (B) Drugs targeting Warburg effect (Chen et al., 2016).

1.7. Anticancer drugs

With the advancement of cancer research more and more anticancer drugs are being discovered owing to the need for more potent, selectively toxic, chemosensitive agents. Classically anticancer drugs are classified mainly into three groups namely chemotherapy, hormonal therapy, and immunotherapy. Chemotherapy mainly includes cytotoxic drugs that have been further classified based on their structure and function. They also include target-based drugs that act on a specific tumor antigen. Although various criteria exist for the classification of anticancer drugs, the most commonly used system is represented in Figure 1.13.

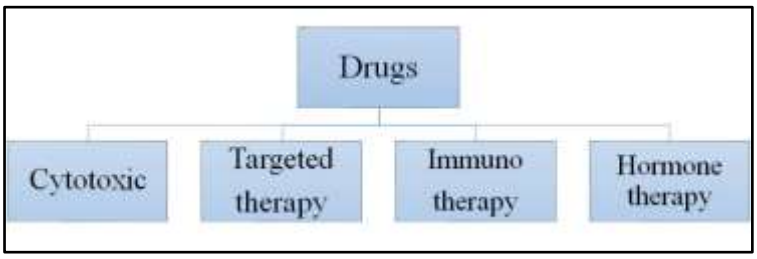


Figure 1.13. Traditional classification of anticancer drugs based on mechanism of action.

1.7.1. Cytotoxic drugs

Cytotoxic drugs are broadly classified into cell cycle-specific (CCS) and cell cycle-Nonspecific (CCNS) agents and these are further classified based on function and structure (Katzung *et al*., 2012). The classification of cytotoxic drugs are shown in Figure 1.14.

Cell cycle-specific drugs	Antimetabolites
	Epipodophyllotoxins
	Taxanes
	Vinca alkaloids
	Anti microtubule inhibitor
	Antitumor Antibiotics
Cell cycle-Nonspecific drugs	Alkylating agents
	Camptothecins
	Antitumor Antibiotics
	Platinum analogs
	Anthracyclins

Figure 1.14. Classification of cytotoxic anticancer drugs

- Alkylating agents: Cross-linking of DNA appears to be of major importance to the cytotoxic action of alkylating agents resulting in the inhibition of DNA synthesis and function, and replicating cells are most susceptible to these drugs. Cyclophosphamide, chlorambucil, and busulfan are a few of the drugs from this class.
- Antimetabolites- These drugs target the metabolism of cancer cells by inhibiting the DNA synthesis. They include antifolates (capecitabine), purine antagonists (6-Mercaptopurine), deoxycytidine analogs (cytarabine), and Fluoropyrimidines (5-Fluorouracil).

- Topoisomerase enzyme inhibitors: Epipodophyllotoxins inhibit DNA topoisomerase II enzyme thereby interfering with DNA replication. Examples are Etoposide and teniposide. Camptothecins inhibit topoisomerase I, the critical enzyme required for cutting and joining single-stranded DNA which ultimately results in DNA damage. Topotecan and irinotecan are the two camptothecin analogs currently used in clinical practice.
- Antitumor antibiotics: Most of these microbial antibiotics bind to DNA through intercalation between specific bases and block the synthesis of RNA, DNA, or both; causing DNA strand breaks; hence interfering with replication. Anthracyclins are widely used cytotoxic drugs that act through four major mechanisms: inhibition of topoisomerase II, Inhibition of the synthesis of DNA and RNA through intercalation, generation of semiquinone free radicals, and oxygen free radicals and binding to cellular membranes to alter fluidity and ion transport. Doxorubicin and idarubicin are a few of the currently used anthracyclins. Mitomycin is a DNA cross-linking agent while bleomycin binds to DNA releasing oxygen free radicals to cause single and double-stranded DNA breaks.
- Platinum analogs: They form intrastrand and inter-strand DNA cross-links, binding to nuclear and cytoplasmic proteins and interrupting DNA synthesis. They include cisplatin, carboplatin, etc.
- Microtubule inhibitors: Vinca alkaloids inhibit tubulin polymerisation thereby disrupting the mitotic spindle assembly and includes vincristine, vinblastine, and vinorelbine. Taxanes inhibit mitosis and cell division by enhancing microtubule polymerisation and include drugs like paclitaxel and docetaxel.

1.7.2. Targeted therapy drugs

These agents are pre-designed to regulate specific molecular markers considered important in cancer growth, progression, and spread. Many studies have shown that targeted therapeutic drugs, induced comparably fewer side effects than cytotoxic drugs. They are mainly divided into two types which are small molecule inhibitors that regulate targets inside the cells and monoclonal antibodies that target surface proteins. Monoclonal antibodies are synthetic versions of antibodies that

are more specific and exclusively target only one protein (Baudino *et al*., 2015). A few of the well-known monoclonal antibody drugs are:

- Angiogenesis inhibitors: Bevacizumab and Ziv-aflibercept target VEGF receptor.
- EGFR inhibitors: Cetuximab and panitumumab target EGFR receptor.
- HER2 inhibitors: Trastuzumab and pertuzumab target HER2.
- CD 20 and CD 52 inhibitors: Monoclonal antibodies raised against CD 20 (Rituxan) and CD 52 (Campath) antigens in lymphomas.

  Small molecule inhibitors prevent the activation of pathways that are dysregulated during cancer and can be further classified into:
- Tyrosine kinase inhibitors- Erlotinib blocks the action of EGFR tyrosine kinase. Imatinib blocks the action of BCR-Abl cytoplasmic tyrosine kinases as well as Kit and PDGFR receptor tyrosine kinases. Gefitinib, is an EGFR inhibitor.
- mTOR inhibitors- These drugs block mTOR, an enzyme that sends signals for growth and spread of cancer cells. Everolimus and sirolimus are mTOR inhibitors.
- Others- Other small molecule inhibitors such as PARP inhibitors (olaparib) and Hedgehog pathway inhibitor **(**vismodegib).

1.7.3. Immunotherapy drugs

They aim towards initiating a novel or boosting an existing immune response against neoplastic cells. While some immunotherapies specifically target a specific tumor-associated antigen, others act in a non-specific manner to boost immune responses of the body. According to a classification carried out by Galluzzi *et al*. 2014, currently approved immunotherapies include dendritic cell based immunotherapy (sipuleucel-T), immunogenic cell death inducers (epirubicin), cytokines (IFN-α2a), PRR agonists (mifamurtide), monoclonal antibodies (cetuximab) and others.

1.7.4. Hormone therapy drugs

- Selective Estrogen Receptor Modulators (SERMs): They are analogs that compete with estrogen to bind with estrogen receptor. Tamoxifen, raloxifene, lasofoxifene are a few SERM drugs.
- Selective Estrogen Receptor Downregulators (SERD): They function as high affinity competitive antagonists and targets the receptor for degradation. Eg. Fulvestrant.
- Aromatase Inhibitors: They block aromatase enzyme that converts androgen into estrogen. Anastrazole, exemestane, letrozole are a few examples.
- Luteinizing hormone-releasing hormone (LHRH) agonists: They lower the production of estrogen or testosterone production. Triptorelin and histrelin are the currently used LHRH agonists.
- Antiandrogens- These drugs bind to the androgen receptor blocking it. Examples of this class of drugs are flutamide and bicalutamide.

1.8. Cancer chemotherapy and side effects

A major source of consternation associated with cancer chemotherapy is the short and long-term side effects that diminish the standard of life of the patients. Some of the well-investigated symptoms include nausea, vomiting, hair loss, muscle toxicity, neurotoxicity, gastrointestinal ulceration and associated anaemia, fatigue, weight loss, etc. Various drugs and methods are employed currently to alleviate many of the side-effects, but they are often ineffective, thereby demanding the need for new approaches to reduce sequelae associated with chemotherapy. Studies have shown that the use of natural bioactive compounds along with standard drugs increases the chemosensitivity and lowers the adverse effects (Nurgali *et al*., 2018).

1.9. Plant compounds with anticancer properties

Plants and their bioactive compounds have been used in medicinal practices since ancient times. Many existing anticancer drugs in the market have been derived from natural resources such as plants, microorganisms, and marine life forms (Ratovitsky 2017). A few of the compounds with anticancer activity of natural origin are shown in Figure 1.15. A recent review stated that only 10% of the

available plant species had been investigated for drug discovery. According to many studies, anticancer activity has been ascribed to bioactive molecules found naturally in plants and also to compounds that were derived from phytochemicals.

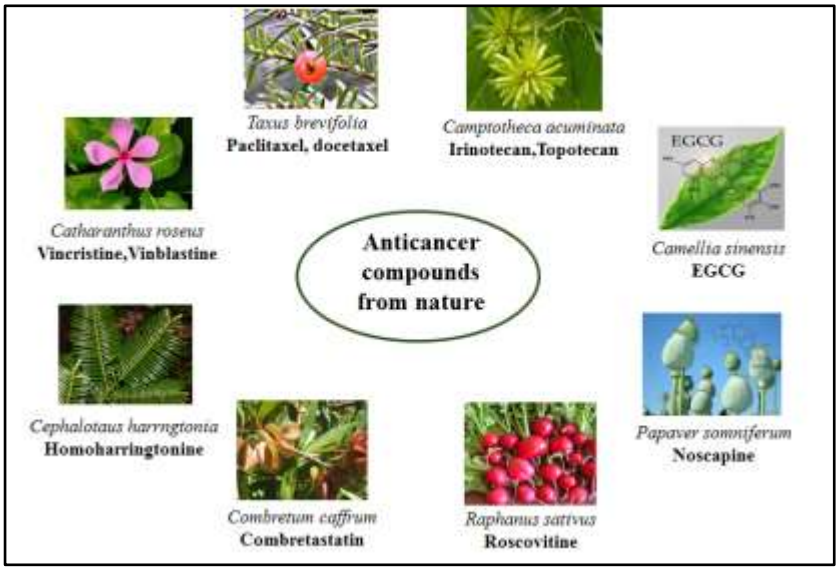


Figure 1.15. Anticancer compounds of natural origin.

1.9.1. Natural anticancer compounds

Polyphenols include a complex assortment of chemicals such as flavonoids, tannins, curcumin, resveratrol, and gallocatechins. Resveratrol is usually found in dietary products such as peanuts and red wine while gallocatechins are present in green tea. Even the consumption of such polyphenol-rich food in the diet was reported to be chemopreventive. Purified flavonoids extracted from *Erythrina suberosa*, namely 4'-Methoxy licoflavanone and alpinum isoflavone induced apoptosis in HL-60 cells (Kumar *et al*., 2013). Polyphenols attenuate cancer through several mechanisms including oxidative DNA fragmentation. Studies by Gupta *et al*. 2014, showed that polyphenols inhibited cancer cell growth through downregulation of tumor necrosis factor (TNF) and other biomarkers.

Brassinosteroids are another category of compounds that exhibit chemopreventive properties in the natural state in multiple cancer cells. Two brassinosteroids that have been studied and identified to possess chemotherapeutic potential are 28-homocastasterone and 24-epibrassinolide. Many natural compounds of this category have been found to inhibit cancer growth by interfering with the cell cycle (G1 phase) (Steigerova *et al*., 2010). These compounds can

interact with receptors of proteins such as ER, AR, EGFR, and HER2, inhibiting the growth of both hormone-sensitive and hormone-insensitive cancer cells (Steigerova *et al*., 2012). They were also found to be selectively cytotoxic to cancer cells which advocates them as strong candidates for cancer treatment.

Naturally occurring indoles from Brassicaceae family have also been widely investigated for its chemopreventive activity (Wattenberg *et al*., 1978). Studies by Higdon *et al*. 2007, is one among many reports that provide evidence for the inverse relationship between cancer risk and dietary consumption of Brassica vegetables. Many compounds of chemo protective and therapeutic potential have been found in this particular family.

1.9.2. Plant-derived anticancer drugs

Plant-derived drugs are considered to be more tolerated and less toxic towards normal cells. According to a review by Amin *et al*. 2009, plant-derived anticancer drugs fall under four major classes with the following mechanisms; inhibition of methyltransferase, prevention of DNA damage, inhibition of HDAC, and inhibition of mitosis.

Derivatives of vinca alkaloids from *Catharanthus roseus* such as vincristine, vinblastine, vinorelbine, vindesine, and vinflunine are drugs which on binding to β-tubulin, inhibit its polymerization and hence mitosis. Derivatives of taxanes from *Taxus* genus, namely paclitaxel and docetaxel are microtubule-targeting agents that bind to polymerized microtubules and prevent tubulin de-polymerisation, leading ultimately to cell death by apoptosis.

Compounds including sulforaphane, isothiocyanates, isoflavones, and pomiferin are considered to be HDAC inhibitors. They reactivate epigenetically-silenced genes in cancer cells, leading to cell cycle arrest and apoptosis. Studies suggest that dietary constituents, such as the isothiocyanates can act as HDAC inhibitors. Sulforaphane is an isothiocyanate found in various cruciferous vegetables like broccoli or its sprouts that inhibit HDAC activity in cancer cells (Nian *et al*., 2009). Pomiferin is another compound which is an HDAC inhibitor. HDAC inhibitors have also been found to increase the chemosensitivity of cancer cells.

The antioxidant activity of green tea is attributed to its most abundant catechin, epigallocatechin-3-gallate (EGCG). Amin *et al*. discusses the various anticancer effects exhibited by EGCG in multiple cell lines. This includes apoptosis induction,

inhibition of telomerase activity, prevention of chemical carcinogenesis, inhibition of angiogenesis (VEGF, MMP, etc.), and inhibition of methyltransferases.

Many plant-derived drugs are still under investigation in clinical trials for chemotherapeutic treatment. Epipodophyllotoxin is an anticancer compound derived from *Podophyllum peltatum* that has shown pro-apoptotic effects and cell cycle inhibition towards lymphoma and testicular cancer. Combretastatin A-4 phosphate from *Combretum caffrum* shuts down vascular support of the tumors. Roscovitine from Raphanus sativus inhibits cell cycle progression. Flavopiridol from *Dysoxylum binectariferum* and noscapine from *Papaver somniferum* are in Phase II clinical trials (Greenwell et al., 2015).

1.10. Diet and cancer

Many studies have been reported regarding the association between dietary habits and cancer risk. In a study conducted in 1975, dietary consumption of meat and fat were strongly correlated to the development of cancers including colon and breast (Armstrong *et al*., 1975). In fact, numerous reports have been published on the association between various dietary factors and cancer incidence (Correa, 1981). Due to these studies, the dietary agents that showed chemoprotective properties have been widely investigated. Wattenberg and team described two main classes of chemopreventive agents namely anti-initiators and anti-promotional agents. The first criteria include indoles, iso-thiocyanates, and dithiolethiones in cruciferous vegetables, terpenes from citrus fruits, carotenes, coumarins, curcumins, EGCG, etc., while anti-promotional agents include retinoids, vitamin D analogs, monoterpenes, and selenium (Wattenberg 1980; Wattenberg *et al*., 1994). The dynamics between diet and cancer is still a hot area of investigation (Weber *et al*., 2018).

1.10.1. Anticancer activity of cruciferous vegetables

Cruciferous vegetables belong to the Brassicaceae family and are named for their cross-shaped flowers. They include commonly consumed vegetables like cabbage, broccoli, cauliflower, horseradish, radish, turnip, etc. which are shown in Figure 1.16. Many epidemiological studies have shown that consumption of cruciferous vegetables is associated with lowered cancer risk. Studies by Verhoeven *et al*. 1996, showed an inverse association with brassica vegetable consumption and cancer risk (Lung, breast). Another cohort study indicated a lowered colon cancer risk with high

intake of cruciferous vegetables (Voorrips *et al*., 2000). A clinical trial by Walter *et al*. 2004, revealed that the consumption of these vegetables led to an increased elimination of carcinogen found in overcooked meat. A review by Higdon *et al*. 2007, furnishes more studies associated with dietary consumption of cruciferous vegetables and cancer risk.

Figure 1.16. Cruciferous vegetables belonging to the Brassicaceae family.

Many vegetables, including cruciferous vegetables, contain phytochemicals with cancer chemopreventive properties, such as folate, fiber, carotenoids, and chlorophyll. But the cruciferous families are unique, as they are rich sources of sulphur-containing compounds known as glucosinolates which are responsible for their characteristic pungent flavour (Drewnowski *et al*., 2000). During maceration of these vegetables, myrosinase enzyme catalyzes the hydrolysis of glucosinolates, yielding glucose and an unstable product, which undergoes spontaneous rearrangement to form a number of products. Different glucosinolate precursors hence give unique hydrolytic products. For example, sulforaphane (SF) is a product of glucoraphanin (broccoli), indole 3 carbinol (I3C) from glucobrassicin (broccoli), phenethyl isothiocyanate from gluconasturtin (watercress), etc. There are mainly four major classes of glucosinolates from Brassica family: 1) Isothiocyanates (SF); 2) Indoles (I3C); 3) Nitriles (Propionitrile); and 4) Thiocyanates (Cavernothiocyanate). Studies have shown that isothiocyanates and indoles are the

main compounds that show chemoprotective properties. Zhang *et al*. 2007, presents SF as an anticarcinogenic and anticancer compound that modulates cancer inhibition by affecting cell death, cell cycle, angiogenesis, and metastasis. SF was initially noticed as an anticancer compound due to its ability to induce anti-carcinogenic phase II enzymes. A recent report by Royston *et al*. 2015, points out that SF was an HDAC inhibitor and downregulated DNA methyltransferases.

Another prospective cruciferous compound is I3C which combats cancer progression through many pathways. It is primarily known to be a negative estrogen regulator where it inhibits the transcription of estrogen responsive genes (Ashok *et al*., 2001). Cell cycle arrest, growth inhibition, and apoptosis was another consequence induced by I3C in cancer cells (Chinni *et al*., 2001). Although many studies have confirmed the antitumor activity of I3C against multiple types of cancer, I3C instability and the toxicity associated with P-450 enzyme induction have limited the use of I3C as a chemoprotective agent (Bradlow, 2008). In the acidic pH of the stomach, I3C molecules are unstable and are converted into a complex mixture of biologically active compounds, collectively known as acid condensation products out of which one of the most prominent by-product is the dimer 3,3′-diindolylmethane (DIM). This conversion is shown in Figure 1.17. Hence the effects induced by I3C *in vivo* could be attributed to its condensation products. Studies have shown that I3C and DIM both exhibited antagonistic effects towards invasion and angiogenesis (Chang *et al*., 2005). Weng *et al*. 2008, discusses the pleiotropic nature of I3C and DIM as they target multiple aspects of cancer cell-cycle regulation and survival. This incude stress response signaling, caspase activation, cyclin-dependent kinase (CDK) activities, estrogen metabolism, ER signaling, endoplasmic reticulum stress, BRCA gene expression and, AKT-nuclear transcription factor-κB (NF-κB) signaling which plays critical roles in the control of cell proliferation, survival, tumor invasion, metastasis, and drug resistance. Due to its broad spectrum of anticancer effects combined with low toxicity, DIM has gained precedence as a potential cancer therapeutic agent and has been extensively investigated. To date, DIM has been studied more extensively than any other I3C metabolite. The multiple aspects of DIM induced effects are summarised in the consecutive section.

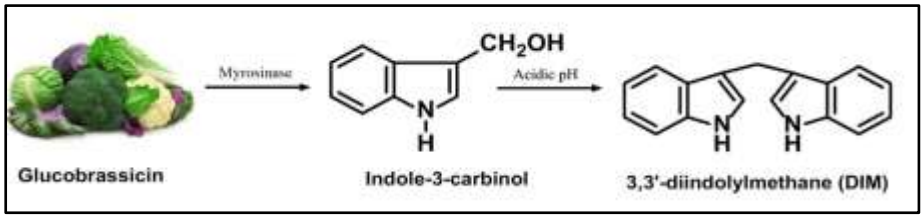


Figure 1.17. Formation of DIM from glucosinolates by acid condensation in cruciferous vegetables.

1.10.2. Anticancer activity of DIM-cellular effects

1.10.2.1. A brief history

In the 1970s, Wattenberg first described the chemo protective abilities exhibited by DIM in crucifers, through many studies. The studies found that DIM had a role in aryl hydrocarbon hydroxylase induction, carcinogen metabolism, and inhibition, and chemical neoplasia inhibition (www.diindolylmethane-dim.com). Further studies revealed the instability of I3C *in vivo* and the acid-condensation products that were responsible for I3C induced effects (Kruif *et al*., 1991). Various successive studies were conducted in both I3C and DIM until the instability of I3C was revealed, which highlighted DIM as the prime target for anticancer investigations. Since then, DIM has been found to target multiple proteins and pathways for the attenuation of cancer progression. The major molecular targets of DIM are shown in the Figure 1.18.

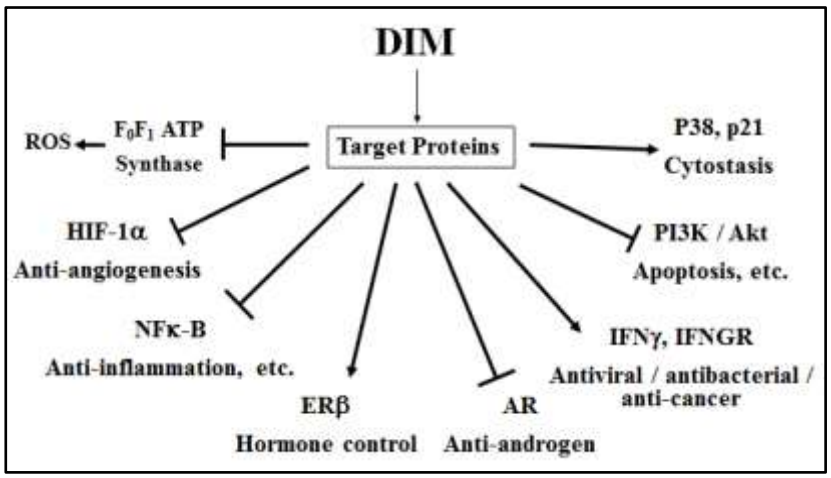


Figure 1.18. Molecular targets of DIM (DIMIRC).

1.10.2.2. Effect of DIM on phase I and phase II enzymes

Phase I (cytochrome P450s (CYP) enzymes) and phase II (GST isoenzymes etc.) are responsible for the detoxification and excretion of carcinogens and xenobiotic substances that enter the body. Anti-carcinogenic property of DIM can be ascribed to its effect on the detoxification enzymes. For instance, DIM inhibits the catalytic activity of many CYP enzymes which are responsible for the activation of many pro-carcinogens (Streser *et al*., 1995). Consolidated information on the effect of DIM on detoxification enzymes by Banerjee *et al*. 2011, reports increased detoxification and inhibition of carcinogen activation. DIM also has a role in the estrogen detoxification, where it is reported to convert estrogen into less estrogenic forms.

1.10.2.3. Regulation of cell cycle

The cell cycle progression is under the strict regulation of cyclin-dependent kinases (CDK) and associated CDK inhibitors (CKI). DIM predominantly induces a G1 phase cell cycle arrest in many types of cancer, which can be attributed to the upregulation of p21 (CDKI) resulting in the resulting in the suppression of CDK2 (Gong *et al*., 2006). Studies by Vivar *et al*. 2009, showed that DIM inhibited the phosphorylation of Rb by CDK4 and CDK2 leading to cell cycle arrest. A review by Zhang *et al*. 2014, provides additional evidence on cell cycle inhibition by DIM through downregulation of cyclin D1, cyclin E, CDK4 and CDK6 and upregulation of cell cycle inhibitors p15 and p27. DIM also has been found to induce cell cycle arrest in phases other than G1 such as G2/M phase via DIM's inhibitory effect on the expression of cyclin B1 and cdc25c. Reports by Zhang *et al*. 2014 presents evidence that cell cycle arrest by DIM is associated with p38 MAPK, Sp1 pathway, and ROS stress. The effects of DIM on cell cycle regulation summarised by Banerjee *et al*. 2011 is shown in Figure 1.19.

HDAC regulates the acetylation status of histones thereby restricting the accessibility of many transcription factors to DNA. In a study by Li *et al*. 2009, DIM selectively induced proteasomal degradation of class I HDAC lifting the transcriptional inhibition of p21 and p27 triggering cell cycle arrest in the G2 phase of the cell cycle (Li *et al*., 2010).

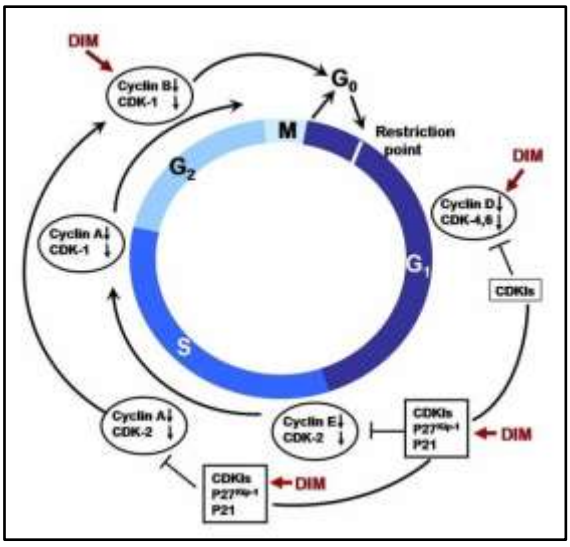


Figure 1.19. Effect of DIM on key regulators of cell cycle (Banerjee et al., 2011).

1.10.2.4. DIM and apoptosis

DIM induces apoptosis in various cancer cells and literature has revealed that it is by two mechanisms, either by downregulation of anti-apoptotic proteins such as Bcl2, Bcl-xL, survivin, inhibitor of apoptosis protein (IAP), and Fas-associated death domain (FADD) proteins or by upregulating proapoptotic protein Bax, release of mitochondrial cytochrome C, p53, *N*-myc downstream regulated gene-1, activation of caspase-9, and caspase-3 (Zhang *et al*., 2014). An interesting finding is that DIM induced apoptosis is selective towards cancerous cells. Induction of apoptosis by DIM has been reported in many types of cancer cells including breast, prostate, colon, pancreatic, and hepatic (Banerjee *et al*., 2011). DIM-induced apoptosis has been associated with regulation/inhibition of tumor-associated proteins such as AKT, FOXM1, NF-κB, p38 mitogen-activated protein kinase (p38 MAPK), and survivin (Jin *et al*., 2015; Rahman *et al*., 2007; Rahman *et al*., 2006; Weng *et al*., 2012).

1.10.2.5. DIM and oxidative stress

Studies showed that DIM induced reactive oxygen species (ROS) generation in many cancer cells. This initiated a cascade of events including activation of DNA damage checkpoint signaling, and generation of mitochondrial ROS. The ROS production led to the activation of stress-activated pathways such as p38MAPK. Studies have shown that oxidative stress has a major role in mediating the cellular

activity of DIM. Studies by Gong *et al*. 2006, showed that DIM-induced hyperpolarisation in mitochondria is due to the inhibition of mitochondrial H^+-ATPase by DIM leading to decreased cellular ATP level resulting in the generation of mitochondrial ROS which ultimately triggers apoptosis. Another study correlated the ROS generation by DIM to cell cycle arrest (Kandala *et al*., 2010). Surprisingly DIM also plays a chemoprotective role by protecting cells against oxidative stress (BRCA1 mediated) by stimulation of nuclear factor E2 p45-related factor 2 (Nrf2) activity (Gopalakrishnan *et al*., 2008).

1.10.2.6. DIM induces anti-angiogenesis

There are many reports about the anti-angiogenic effect of DIM in cancer cells. Chang *et.al.* 2005, reported the effect of DIM on angiogenesis *in vitro* and *in vivo* conditions. DIM suppressed the proliferation, invasion and capillary tube formation of HUVEC cells and induced a G1 cell cycle arrest. Another study by Chang *et.al*. 2006, showed that DIM inhibits VEGF-induced cell proliferation *via* RAS signaling. DIM also inhibited angiogenesis in PC3 cells by inactivation of both mTOR (mammalian target of rapamycin) and AKT (Kong *et al*., 2008). Riby *et al*. 2008, documented the suppression of HIF activity in hypoxic tumor cells leading to the suppression of hypoxia-responsive factors.

1.10.2.7. Effect of DIM on EMT and metastasis

Urokinase-type plasminogen activator (uPA) and uPA receptor are found overexpressed in metastatic cancer. DIM inhibited growth and migration of cancer cells by downregulating uPA and uPAR expression (Ahmad *et al*., 2009). DIM inhibited metastatic modulators and MMP-2 and MMP-9 in thyroid cancer (Rajoria *et al*., 2011). In a study by Wu *et al*. 2014, DIM inhibited nasopharyngeal carcinoma metastasis by regulation of EMT. The study revealed the downregulation of key proteins associated with EMT both *in vitro* and *in vivo.* DIM treatment reversed EMT in pancreatic cancer cells by increasing the expression of E-cadherin and decreasing the expression of transcription factors- ZEB1, slug, and mesenchymal marker- vimentin (Li *et al*., 2009). The above studies portray the ability of DIM in attenuating metastasis.

1.10.3. Molecular targets responsible for anticancer activity of DIM

1.10.3.1. Transcription factors

NF-κB signaling plays important roles in the regulation of cell proliferation, survival, tumor invasion, metastasis, drug resistance, and stress response and it is usually found overexpressed in cancer cells. There are many studies that provide evidence for the NF-κB inhibition in cancer cells induced by DIM that ultimately leads to apoptosis induction (Rahman *et al*., 2007). Studies by Banerjee *et al*. 2009, also revealed that DIM increased the chemosensitivity of many anticancer drugs, and this involved NF-κB inhibition.

Aryl hydrocarbon receptor (AhR) is a transcription factor, which on activation transcribes genes encoding phase-I and II xenobiotic metabolising enzymes and DIM has been found to be an effective AhR modulator, which is responsible for the xenobiotic metabolism shown by DIM (Vivar e*t al*., 2010). Nrf2 is a transcription factor that is responsible for the transcription of many phase II enzymes, and DIM has been found to stimulate several Nrf2-regulated promoters like NQO1 and GSTα1 (Fan *et al*., 2009).

1.10.3.2. Regulation of AKT and mTOR pathway

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway in most cancers are frequently activated due to the loss of AKT inhibitor, PTEN. AKT signalling interacts with many other pathways including receptor tyrosine kinase signaling, NF-κB signaling, and extracellular regulated kinase (ERK) signaling cascade. Once activated it regulates proteins involved in cell proliferation, migration, and invasion. Cheng *et al. 2006*, reported that DIM, induced apoptosis in cancer cells by downregulating AKT activity. Various other studies have also reported that DIM-induced apoptosis was modulated through AKT signalling (Jin *et al*., 2015; Weng *et al*., 2012). Mammalian target of rapamycin (mTOR) controls many cellular processes, e.g. cell growth, proliferation, and cell division. Studies by Kong *et al*. 2008, showed that DIM-induced mTOR and AKT repression, inhibited invasion and angiogenesis in cancer cells.

1.10.3.3. Effect of DIM on MAPK pathway

Mitogen-activated protein kinase (MAPK) cascade controls the expression of various genes. Studies have shown that DIM induces interferon-gamma (IFNγ)

secretion which has a role in preventing tumor development, through JNK and p38 pathway activation (Xue *et al*., 2005). Studies by Gong *et al*. further supplied proof for the activation of these pathways through mitochondrial ROS generation in breast cancer cells. Studies by Khwaja *et al.* 2009, underscored that DIM is capable of inducing p75 (oncogene) dependent apoptosis *via* the p38 MAPK pathway in prostate cancer cells.

1.10.3.4. Androgen receptor and estrogen receptor

Androgen and AR have been implicated in the development of prostate cancer. Studies by Chinnakannu *et al*. 2009, has shown that DIM acts as a novel AR antagonist, which represses AR through competitive binding and alters AR nuclear accumulation and degradation by the ubiquitin-proteasome pathway. DIM suppresses cell proliferation, endogenous PSA transcription, and suppresses androgen-induced AR translocation into the nucleus (Nachson-kedmi *et al*., 2004). Studies by Li *et al*. 2007, revealed that DIM regulates known AR regulators such as FOXO3a, GSK-3β, and β-catenin to induce apoptosis in cancer cells.

Estrogen is a major breast cancer risk factor, and DIM is a phytoestrogen, which plays chemopreventive roles. The regulation of breast cancer progression by DIM is mediated through ERα. Reports showed that in the absence of estradiol, concentrations of DIM at 10 μM activated ERα signalling, resulting in cellular proliferation, but growth arrest was achieved at higher concentrations of DIM (50 μM) (Marques *et al*., 2014). DIM supplementation enhanced the 2-hydroxylation of estrogen, resulting in the selective activation of estrogen receptor β target genes, responsible for the anti-inflammatory effects (Vivar *et al*., 2010). DIM was also found to regulate estrogen metabolism via CYP enzymes, negatively (Szaefer *et al*., 2012).

1.10.4. DIM derivatives and their anticancer activity

In search of potentially more effective anticancer activity than natural DIM, many modified derivatives of DIM have been synthesized and examined for properties including increased potency and pharmacological profile (i.e., improvement in their specificity, bioavailability, toxicity, and stability). Studies on dihaloDIMs carried out by McDougal *et al*. 2000, revealed that they significantly inhibited carcinogen-induced mammary tumor growth in female Sprague-Dawley

rats. The halogenated derivatives namely, 4,4'-Dichloro-, 5,5'-dibromo-, and 6,6'-dichloroDIM, were found to be more active than DIM. Further on Cho *et al*. 2008, developed a series of synthetic ring-substituted DIM analogues including 5,5′-dibromoDIM which showed more potent anticancer activity than DIM. 5,5′-dibromoDIM inhibited the growth of colon and oral cancer cells with more potency than DIM. It was found to induce cell cycle arrest through p21 induction in both *in vivo* and *in vitro* conditions. Another study from the same laboratory reported that this halogenated DIM analogue, induced caspase-mediated apoptosis *via* downregulation of Bcl2 protein expression through p38 MAPK in oral carcinoma cells (Choi *et al*., 2010). Many studies have been published by Dr. Safe's lab on the promising anticancer activity of DIM analogues. In these studies, DIM was used as a starting substrate to synthesize a series of 1,1-Bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes (C-DIMs) which are triarylmethane derivatives of DIM. DIM was conjugated to different aromatic moieties in a *para* conformation which included compounds containing *p*-CF_3 (DIM-C-pPhCF_3), *p*-*t*-butyl (DIM-C-pPhtBu), and *p*-phenyl (DIM-C-pPhC_6H_5). All these compounds were found to be cytotoxic towards cancer cells. Nuclear receptor 4A1 (NR4A1) belongs to the nuclear receptor superfamily of transcription factors and is found highly expressed in many types of cancer. Studies by Lee *et al*. 2011, reported that C-DIMs act strictly through NR4A1 (TR3 and nur77) and induce apoptosis in cancer cells. Studies on nur77-independent apoptosis induction was investigated by Cho *et al*. 2008, which revealed that 1,1-Bis(3'-indolyl)-1-(p-methoxyphenyl)methane (DIM-C-pPhOCH(3)), induced apoptosis in cancer cells through both intrinsic and extrinsic pathways. Another study revealed the ability of C-DIMs in activating the extrinsic apoptotic pathway by inducing ER stress. The said study proved the receptor independent mechanism of C-DIMs (ER stress), while they have also been found to act through the AhR and peroxisome proliferator-activated receptor gamma (Abdelrahim *et al*., 2006). Studies by Shin *et al*. 2011, also reported the ER stress pathway mediated apoptosis induction by C-DIMs in cancer cells

Hence it can be concluded that many novel modified analogs of DIM have been identified to exhibit improved anticancer activity than the natural counterpart, by manipulating different pathways. Reports have even provided conclusive evidence regarding the superior activity of multiple DIM derivatives when compared to natural DIM.

1.11. Novel ortho biaryl DIM compounds

Studies from Dr. Safe's lab provides extensive evidence regarding the improved anticancer activity of many DIM derivatives. Interestingly, most of the modified derivatives with reported anticancer activity were *para*-substituted derivatives of DIM. Studies by Lee *et al*. 2011, Abdelrahim *et al.* 2008, and Shin *et al*. 2011, are some examples that advocate the anticancer activity of *para*-substituted derivatives of DIM. But surprisingly, there has been no evidence in the existing literature regarding *ortho*-substituted DIM analogues. And as an answer to this query, novel *ortho*-substituted DIM compounds were synthesized by Jamsheena *et al*. 2016, using a condensation process, where the DIM was conjugated to biaryl moieties in an *ortho* conformation. The study aimed at the synthesis of a novel compound by the conjugation of two potential chemotherapeutic compounds, i.e. DIM and biaryls. The compounds were synthesized in a single step condensation process of indoles and biaryl carbaldehydes, which was both economical and time-saving. The reaction for the synthesis is shown in Figure 1.20. Hence the anticancer activity of novel *ortho* substituted derivatives of DIM were the focal point of this study.

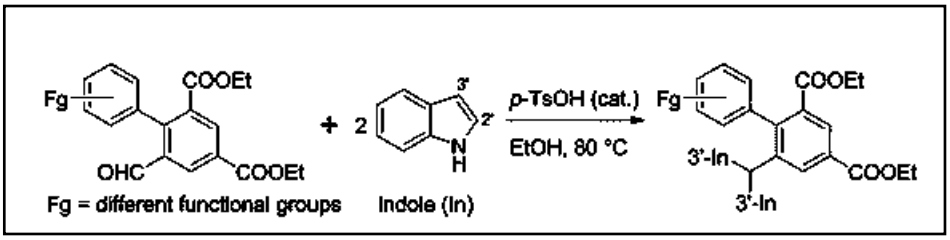


Figure 1.20. Single step condensation reaction for the synthesis of novel ortho-biaryl DIM compounds (Jamsheena et al., 2016).

1.11.1. Anticancer activity of biaryls

There are many compounds in nature with a biaryl structure that present a plethora of pharmaceutical activities such as anti-inflammatory, antifungal, and antimicrobial activity (Jain *et al*., 2013). Some of these compounds exhibit antimitotic activity deeming them as potential anticancer agents. Such compounds interfere with tubulin or other elements involved in cell cycle progression**.** Many of the microtubule-targeting natural anticancer compounds are composed of a biaryl structural component. A review by Baudoin *et al*. 2003, discusses the antimitotic activity of three axially chiral natural biaryl compounds, allocolchicinoids, steganes, and rhazinilam-type compounds. A study by Santhoshi *et al*. 2014,

showed that biaryl inserted noscapine analogues had a higher affinity to tubulin compared to the parent compound and that the biaryl substitution impacted their therapeutic potential towards multiple cancer types. Another study by Mcnulty e*t al*. 2015, showed that biaryl analogs of colchicine and combretastatin A4 induced apoptosis in cancer cells by inhibiting tubulin polymerisation while being non-toxic towards normal cells. The immense potential displayed by biaryl pharmacophore as anticancer agents justifies the choice of using the molecule to improve the anticancer activity of DIM structure.

1.12. Cancer risk in women

One of the main reason responsible for mortality in women across the world is cancer. But the burden of cancer is more pronounced on the low and middle-income countries due to the increasing life expectancy and the prevalence of risk factors associated with an economic transition. With economic development, the risk factors associated with it also increases which included weight increase, pollution, fewer childbirths, and later age at first childbirth (Franceschi *et al.*, 2013). According to Torre *et al*. 2017, the three most frequently diagnosed cancers in women are breast, colorectal, and lung cancers. They are also responsible for the three leading causes of cancer-related death in women, globally. But in developed countries it is breast, cervical and lung cancer that ranks the highest. Breast cancer is the most commonly incident cancer among women (140 countries), whereas cervical cancer is the most common in low-income countries (39 countries), all of which are low-income countries. Breast cancer is responsible for highest cancer mortality in 103 countries, which is closely followed by cervical cancer in 43 countries and lung in 27 countries. According to Ferlay *et al*. 2012 and GLOBOCAN 2012 statistics, breast cancer accounts for 25% of cancer incidence and 15% of cancer-related deaths. In developing countries cervical cancer is the second most commonly diagnosed cancer and the third leading cause of cancer associated mortality.

1.13. Objectives

From the review of literature, it can be understood that chemotherapy is the most preferred among the various available cancer therapeutic strategies. But the undesirable side effects of the currently available anticancer drugs, led to the search for superior, non-toxic drugs from natural resources. Studies have revealed that nature is an abundant repository for potential anticancer compounds and that many approved anticancer drugs are of natural origin. DIM is a natural compound that displays the potential to be an excellent anticancer drug due to its multi-targeting effect towards the attenuation of cancer. Various modifications of DIM have provided exceptionally potential compounds. Breast and cervical cancer are two of the leading causes for mortality due to cancer in women globally and studies pertaining to the anticancer activity of DIM in these types of cancer have been reported.

The major objectives of the present study was, to investigate the anticancer activity of the novel synthetic *ortho*-biaryl derivatives of DIM in two predominant female cancers namely cervical and breast cancer. The analysis will be carried out in the following aspects.

- To conduct the preliminary screening of the 12 compounds by cytotoxicity evaluation and identify potential compounds.
- To carry out apoptotic studies on the cytotoxic compounds
- To investigate the effect of the compounds on cell cycle progression.
- To investigate anti-migration and anti-angiogenic potential of the compounds
- To investigate the effect of the compounds on ROS generation and cancer cell metabolism.
- To study the effect of the compounds on PI3K/AKT/mTOR pathway and integrin/FAK pathway
- To reproduce the anticancer effects of DIM compounds obtained *in vitro* 2D culture into 3D condition which more accurately represents the *in vivo* system.
- To assess the toxicity of the potential compounds in a living system.

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Chapter 2

Materials and methods

2.1. Materials

2.1.1. Cells and cell culture consumables

HeLa, (human cervical adenocarcinoma cell line) and MDA-MB-231, (human triple negative breast carcinoma cell line) were purchased from National Centre for Cell Science (NCCS), Pune, India. Embryonic rat cardiomyoblast cell line, H9C2, was obtained from American Type Culture Collection (ATCC), Manassas, USA.

The constituents required for the cell culture namely, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin-EDTA (10X), and Antibiotic and Antimycotic solution (100X) containing 100 µg/mL streptomycin and 100 units/L penicillin, were purchased from HiMedia (Mumbai, India). Sterile cell culture flasks, multiwell plates, and cryo-vials were purchased from ThermoFisher Scientific (Waltham, MA, USA). Centrifuge tubes, microcentrifuge tubes, cell scraper, and cell strainer were bought from Tarsons Product Pvt. Ltd. (Kolkata, India).

2.1.2. Chemicals, biochemicals, assay kits and antibodies

Solvents and chemicals required for the synthesis of DIM compounds including Indole, aldehyde, and P-toluenesulfonic acid (P-TsOH) were purchased from Spectrochem Pvt. Ltd., India. Methanol and glacial acetic acid were acquired from Merck (Merck & Co, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), normal goat serum (NGS), and dimethyl sulfoxide (DMSO), were purchased from HiMedia (Mumbai, India). 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI), acridine orange (AO), ethidium bromide (EB), 2,7-dichlorofluorescein diacetate (DCFDA), propidium iodide (PI), RNase A, glycine, tris base, gelatin, RIPA buffer, protease inhibitor cocktail (PIC), poly 2-hydroxyethylmethacrylate (poly-HEMA), and 2-[N-(7-nitrobenz-2- oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) were acquired from Sigma Aldrich (St Louis, MO, USA). Magnetic affinity nanoparticles for immunoprecipitation was purchased from MagGenome Technologies Pvt. Ltd.,

India. Epidermal growth factor (EGF) was acquired from Cell Signalling Technology Inc. (CST, USA). Hoechst stain was purchased from ThermoFisher Scientific.

Nuclear/Cytosol fractionation kit, cytochrome c releasing apoptosis assay kit, caspase-3 fluorometric kit, caspase-9 fluorometric kit, and anoikis assay kit, were bought from BioVision Inc. (San Francisco, USA). JC-1 mitochondrial staining kit and annexin V CY3^{TM} apoptosis detection kit were purchased from Sigma-Aldrich. Active RAS detection kit was purchased from CST. ELISA kit was acquired from BD Biosciences USA. BCA protein estimation kit was purchased from ThermoFisher Scientific.

Primary antibodies for Bax, Bcl2, Bad, cleaved PARP (c-PARP), VEGF, fibronectin, p38, pp38, NF-κB, AKT, pAKT, PI3K, pPI3K, HIF-1α, and GAPDH were purchased from Santacruz Biotechnology (USA). Primary antibodies for p53, Ras, FAK, pFAK, mTOR, pmTOR, and Integrin αVβ3 and Horseradish peroxidase-conjugated polyclonal secondary antibodies specific for mouse and rabbit IgG were obtained from CST. FITC conjugated anti-rabbit IgG was also purchased from CST.

2.2. Methods

2.2.1. Synthesis of ortho-biaryl diindolyl methane (DIM) derivatives

Biaryl-2-carbaldehydes were synthesised via a multicomponent reaction of dienaminodioate, cinnamaldehyde, allyl amine and trifluoroacetic acid (Challa *et al*., 2014). The simple biaryl carbaldehydes so obtained were utilised for the synthesis of the ortho biaryl DIM derivatives using the protocol published earlier (Jamsheena *et al*., 2016). The biaryl carbaldehyde used for the synthesis of parent compound DIM-1 was diethyl 6-formyl-[1, 1'-biphenyl]-2,4-dicarboxylate. Various substituted versions of this carbaldehyde were employed for the synthesis of the remaining DIM derivatives (DIM-2 to DIM-12). DIM-ortho-biaryls were synthesized in one step condensation of pertinent biaryl-2-carbaldehydes with two equivalents of indole in the presence of a catalytic quantity of p-TsOH (To a solution of biaryl-2-carbaldehyde (1 equivalent) in ethanol, indole (2 equivalent) and a catalytic amount of para-toluenesulfonic acid were added). The reaction mix was heated up to a temperature of 80 °C until the starting material was completely

consumed as indicated over TLC. The crude product was subjected to purification by column chromatography (20% EtOAc/Hexane). The final products were purified by column chromatography, and structural confirmation was carried out by ^{1}H, ^{13}C NMR, and HR-ESI-MS. The synthesis, purification, and structural elucidation of DIM compounds were carried out by the Organic Chemistry division of CSIR-NIIST, Trivandrum.

2.2.2. Cell culture and maintenance

Human cervical cancer cell line, HeLa and human breast cancer cell line, MDA-MB-231 were cultured and maintained in DMEM containing 1.0 g/L glucose, 3.7 g/L sodium bicarbonate and 110 mg/L sodium pyruvate supplemented with 10% FBS and penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified incubator supplied with 5% CO_2 at 37°C. H9C2 (rat cardiomyoblast) was maintained in DMEM containing 4.5 g/L glucose. 3.7 g/L sodium bicarbonate and 110mg/L sodium pyruvate supplemented with 10% FBS and penicillin (100 units/mL) and streptomycin (100 µg/mL) in a CO_2 incubator. The medium was replaced with fresh maintenance medium on alternate days until the cells attained confluency, followed by which the cells were subcultured.

The cells were subjected to passage on reaching 70 to 80% confluence. The media (DMEM) supplemented with 10% FBS and 1X Antibiotics solution, 1X Phosphate Buffered Saline (PBS) and 1X Trypsin-EDTA were brought to room temperature. The new cell culture flasks/plates were prepared under sterile conditions, and the required volume of prepared media was poured into the dishes. The cells to be passaged were washed with 2 mL of 1X PBS to remove any residual FBS and then incubated with 2 mL of 1X Trypsin for 2 minutes in CO_2 incubator. After 2 minutes, 5 mL of complete media was added to the flask, so that the FBS quenches the Trypsin function. The media with the detached cells were aspirated and centrifuged at 3000 rpm for 3 minutes. The cell pellet was suspended in fresh media, counted using a hemocytometer and distributed into the new prepared culture dishes for cell maintenance or experiments.

2.2.3. Cell culture treatments

The cell culture experiments require a uniform density of cells for ensuring reproducibility of the results. The cells stained with 0.4% Trypan blue (1:1 ratio) were counted using a hemocytometer and diluted according to the requirement of the experiments. The cell density was decided according to the requirement of each experiment, and after seeding, the cells were left for 24 h in the incubator to ensure proper attachment to the culture plates. The cells were then exposed to varying concentrations of the compounds to be studied for a period of 24 – 48 h. The stock solutions of the compounds were prepared in DMSO, and the working concentrations of 2 μM, 5 μM, 10 μM, 20 μM and 50 μM for the treatment were prepared in DMEM supplemented with 2% FBS. In all experiments, each concentration was performed in triplicates to ensure reliability. Standard anti-cancer agents were taken as the positive control and introduced to the cells at concentrations ranging from 0.1 μM to 50 μM. The effective concentrations of the compounds and positive control to be used regarding each cell line was decided based on the cytotoxic potential. After the required incubation period (24 - 48 h) the cells were investigated for varying parameters. Some of the parameters investigated could be measured directly in the adherent cells and some required the harvesting of cells.

2.2.4. Cytotoxicity assay

MTT **(**3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used to estimate the percentage of cytotoxicity induced by the compounds in cell lines. It was developed by T. Mosmann as a quantitative colorimetric assay for cell proliferation and survival (Mosmann, 1983). The yellow tetrazolium salt is reduced by metabolically active cells (mitochondrial dehydrogenases) into insoluble purple formazan crystals that can be dissolved and measured by spectrophotometric means. The protocol used for this study was based on the original method by Mosmann 1983 with minor modifications.

Reagents:

1) MTT reagent-0.5 mg/mL MTT in DMEM.
2) DMSO.

3) 1X PBS (pH 7.5)-

8 g of NaCl, 0.2 g of KCl, 1.78 g of Na_2HPO_4, and 0.24 g KH_2PO_4 was added to 500 mL of distilled water and pH was adjusted to 7.5. The final volume was made up to 1L with distilled water.

Procedure: The experiment was conducted at standard conditions of 5% CO_2 and 37°C. Cells were harvested and seeded at a density of 1×10^4 cells/well in a 96 well plate and incubated for 24 h in a CO2 incubator. Varying concentrations of DIM compounds (2 μM - 50 μM) were prepared in DMEM supplemented with 2% FBS. The media was aspirated from the wells, and the cells were washed gently with 1X PBS. 100 μL of the prepared compounds and positive controls were introduced into the wells for 24 h. The medium was removed and the wells were exposed to 200 μL of MTT reagent for 4 h. The MTT solution was replaced with 100 μL of DMSO and the plate was gently agitated in a shaker for 30 minutes at room temperature for the dissolution of the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Synergy^{TM4}, BioTek Instruments, Inc. Vermont). The absorbance is directly proportional to the number of viable cells and was used to estimate the percentage of growth inhibition.

Percentage of growth inhibition = [1-(absorbance of treated cells/absorbance of untreated cells)] x100.

2.2.5. Morphological analysis

Phase contrast light microscopy was utilized to investigate the morphological changes induced by the compounds at varying concentrations. Key indicators of cell death (apoptosis or necrosis) such as shrinkage, blebbing, bloating, apoptotic bodies etc. can be identified with phase contrast imaging.

Procedure: The cells were seeded at a density of 1×10^4 cells/well in a 96 well plate and incubated for 24 h at standard conditions of 5% CO_2 and 37°C. The cells were treated with 100 μL of varying concentrations of compounds and positive control for 24 h. The morphological changes induced by the compounds were observed and recorded using a phase contrast microscope with an attached camera (Nikon Eclipse ^{TS} 100).

2.2.6. Nuclear fragmentation analysis

4, 6-Diamidino-2-phenylindole (DAPI) is a DNA specific probe that binds selectively to the adenine-thymine rich regions (minor groove) of double-stranded DNA (dsDNA) and forms complexes that fluoresces 20 times more than DAPI alone. DAPI stains both live and dead cells. The excitation minimum for DAPI bound to dsDNA is 358 nm, and the emission maximum is 461 nm. The application of DAPI as a biological stain for the visualization and quantitation of DNA was described by Kapuscinski (Kapuscinski, 1985). The staining reveals the morphology of the nucleus and hence can be utilized to study nuclear damage. The protocol described here was standardized in our lab from the procedure specified by the manufacturer (Sigma).

Reagents:

1) 1X PBS.

2) DAPI (14.3 mM) –

10 mg DAPI dissolved in 2 mL of ultrapure water (working concentration of DAPI solution was 300 nM).

Procedure: Cells were seeded at a density of 1×10^4 cells/well in a 96 well black plate and incubated for 24 h at standard conditions in an incubator. The medium was aspirated, and cells were incubated with 100 µL of compounds and positive control at varying concentrations for 24 h. The medium with the compounds was aspirated, and replaced with 50 µL working solution of DAPI for 5-10 minutes in the incubator. The staining solution was removed, and the cells were gently washed thrice with 100 µL of 1X PBS. Cells were suspended in 100 µL of 1X PBS and visualized under a spinning disc fluorescent microscope (BD Pathway^{TM}).

2.2.7. Membrane damage analysis

Acridine orange/ethidium bromide co-staining method helps distinguish between live, dead and necrotic cells. It combines the differential uptake of both dyes with the morphological changes of chromatin to achieve this. Acridine orange (AO) is a nuclear stain that stains live and dead cells, and fluoresces green when it intercalates with double stranded DNA. Ethidium bromide (EB) can be only taken up by cells with membrane damage (dead cells or necrotic cells) and emits red fluorescence on intercalation with DNA. Hence late apoptotic cells with membrane

damage will have bright orange areas of condensed chromatin in the nucleus, while early apoptotic cells will have bright green patches. The protocol for the co-staining method was adopted with minor modifications from a standard protocol (Leite *et al*., 1999).

Reagents:

1) 1X PBS.

2) AO stock (1 mg/mL).

3) EB stock (1 mg/mL).

4) Working AO/EB solution (1 µg/mL) -

Mix 1 µL of AO stock and 1 µL of EB stock with 998 µL of distilled water.

Procedure: For the experiment, cells were plated at a density of 1×10^4 cells/well in a black 96 well plate, and left for attachment for 24 h. The medium was aspirated and cells were treated with effective concentrations of compounds and positive control for 24 h. The media was removed, and the cells were exposed to 100 µL working solution of AO/EB for 10 minutes. This was followed by a gentle wash with 1X PBS for three times to remove the background stain. The cells were suspended in 100 µL of 1X PBS and analyzed using a spinning disc fluorescent microscope (BD Pathway^{TM}).

2.2.8. Mitochondrial membrane potential assay

JC-1 (3,30-tetraethylbenzimidazolylcarbo-cyanine iodide) is a cationic lipophilic dye that is used for the convenient detection of changes in the electrochemical potential of the inner mitochondrial membrane. The dissipation of mitochondrial electrochemical potential gradient is an early event in apoptosis. JC-1 is taken up by active mitochondria and hence can be used to distinguish between apoptotic and healthy cells. In normal cells, the positive JC-1 dye concentrates in the higher potential of the mitochondrial matrix to form red fluorescent aggregates (J-aggregates). In a cell where the mitochondrial potential is lost JC-1 is dispersed as monomers (J-C1-monomers) throughout the entire cell giving off green fluorescence. The protocol specified in the assay kit manual by the manufacturer (Sigma Aldrich) was carried out for determination of mitochondrial membrane depolarization (Reers *et al*., 1991).

Reagents:

1) JC-1 stock solution (1 mg/mL DMSO).

2) JC-1 staining buffer (assay kit component).

3) JC-1 staining solution-

25 µL of the JC-1 Stock Solution was added to 4 mL of ultrapure water and incubated at room temperature for 2 minutes. To this 1 mL of 1X staining buffer was added.

Procedure: For this assay, cells were plated at a density of 1 x 10^4 cells/well in a black 96 well plate and incubated at standard conditions for 24 h. After attachment, the cells were treated with 100 µL of active concentrations of compounds, and positive control for 24 h. The staining mixture was freshly prepared by adding an equal volume of JC-1 staining solution to an equal volume of medium. The medium was aspirated and cells were incubated in 100 µL of staining mixture for 20 minutes at 37°C in a humidified atmosphere containing 5% CO2. Staining mixture was aspirated, and cells were washed with growth medium twice to remove the background stain. The cells were then overlaid with 100 µL growth medium, and visualised under a fluorescent microscope. The JC-1 red fluorescence (525 nm excitation/ 590 nm emission) was visualised using standard broad pass filters for propidium iodide while the green fluorescence (490 nm excitation/530 nm emission) was analysed using broad pass filters for FITC by employing a live cell imager (BD pathway TM Bioimage System, BD Bio-sciences).

2.2.9. Phosphatidylserine translocation assay

Phosphatidylserine (PS) is an aminophospholipid present in the inner phase of the plasma membrane that is translocated to the outer membrane during apoptosis. PS acts as a beacon for macrophages signalling phagocytosis of apoptotic cells. PS exposure seems to last from an early phase of apoptosis until the final stage at which the cell has broken up into apoptotic bodies. Annexin V-Cy3 kit from Sigma uses Cy3.18, a strong fluorochrome conjugated to annexin V and allows the detection of PS exposed on the outer membrane of apoptotic cells This assay also utilizes non-fluorescent compound 6-carboxyfluorescein diacetate (6-CFDA) which is hydrolyzed by the esterases in live cells into fluorescent 6-carboxyfluorescein for identifying viable cells. Annexin V binds exposed PS on the cell surface, fluorescing red while 6-CFDA stains viable cells green. Early

apoptotic cells are seen to be annexin V positive and 6-CFDA positive while normal cells stain positive for 6-CFDA and negative for annexin V. The protocol used for the current study was adapted from the manufacturer's protocol (Pigault *et al.*, 1994).

Reagents:

1) Annexin V Cy3.18 conjugate (100 µg/mL) -

   10 µg of annexin V was dissolved in 50 mM Tris HCl, pH 7.5, containing 100 mM NaCl.

2) 50 mM 6-CFDA-

   2.32 mg 6-CFDA dissolved in 0.1 mL acetone.

3) Double staining solution-

   1 µg/mL Annexin Cy3 (20 µL) and 500 µM 6-CFDA (20 µL) in 1.96 mL 1X Binding Buffer.

4) 1X PBS.

Procedure: The assay required the seeding of cells at a density of 1 x 10^4 cells/well in a black 96 well plate and was left for attachment for 24 h. The medium from the wells was replaced with 100 µL of medium containing DIM compounds at various concentrations. After 24 h of treatment, the medium was aspirated and the cells were gently washed thrice with 1X binding buffer. The cells were incubated with 50 µL of double staining solution for 10 minutes at standard conditions in the dark. The wells were washed thrice with 50 µL of 1X binding buffer, and overlaid with 35 µL of binding buffer before visualization under a fluorescent microscope.

2.2.10. Caspase activity assay

Caspases are cysteine-aspartic endoproteases that are the primary effectors of apoptosis. Caspase 3 is the executor caspase in both extrinsic and intrinsic apoptotic pathways. The enzyme recognizes and cleaves proteins with DEVD sequence. Caspase 8 is the initiator caspase which has a major role in the extrinsic pathway via death receptors and cleaves proteins with IETD sequence. Caspase 9 is responsible for the formation of apoptosome in intrinsic or mitochondrial-mediated pathway and cleaves substrates with the sequence LEHD. For the estimation of caspase activity, fluorescent based assay kits were employed. The substrates provided for caspase 3, caspase 8 and caspase 9 in the assay kit were DEVD-AFC (7- amino- 4 trifluoromethyl coumarin), IETD-AFC, and LEHD-AFC respectively.

On the cleavage of substrates conjugated to AFC, free AFC is released that emits a yellow-green fluorescence that can be detected by fluorescent microtitre plate reader. The fold increase in the caspase activity of an apoptotic sample can be determined by comparison of fluorescence with a control sample. The protocol specified by the manufacturer (BioVision Inc) was standardized in the lab for performing this assay (Gurtu *et al*., 1997).

Reagents:

1) Cell lysis buffer.

2) 2X Reaction buffer-

10 µL of 1.0 M DTT was added to 1 mL of 2X reaction buffer.

3) Substrate-

DEVD-AFC (caspase 3), IETD-AFC (caspase 8), LEHD- AFC (caspase-9).

Procedure: Cells were counted and 5 x 10^6 cells/well were plated in a 96 well plate and was left for attachment for 24 h. The medium was removed and replaced with 1.5 mL of growth medium with different concentrations of compounds and positive control for 24 h. The medium was aspirated, and cells were resuspended in 50 µL of cold cell lysis buffer for 10 minutes in ice. To each sample, 50 µL of 2X reaction buffer and 5 µL of 1mM substrate was added and incubated for 1-2 h at 37°C. Samples were analysed in a fluorescent microplate reader equipped with a 400-nm excitation filter and 505-nm emission filter.

2.2.11. Measurement of reactive oxygen species (ROS) generation

ROS are highly reactive molecules that contain oxygen, which includes oxygen ions, free radicals and peroxides. They are the by-products of normal metabolic processes in the body and are involved in various signalling processes including the induction of apoptosis**.** DCFDA (2',7' –dichlorofluorescin diacetate) is a fluorogen used for the detection of ROS (Jakubowski *et al*., 1997). The cell permeable dye is deacetylated by cellular esterases into a product that is oxidized by cellular ROS into a highly fluorescent DCF which can be visualized by standard broad pass filters for FITC. The current protocol was carried out according to the manufacturer's manual.

Reagents:

1) 20 mM DCFDA stock.

2) Working DCFDA solution (20 µM) –

10 µL from DCFDA stock was made up to 1 mL using 1X PBS.

3) 1X PBS.

Procedure: For the assay, cells were seeded at a density of 1-3 x 10^4 cells/well in a 96 well black plate and left for attachment for 24 h in a CO_2 incubator at standard conditions. The medium was aspirated, and wells were washed twice with 1X PBS. 100 µL of DCFDA working solution was added to the wells and incubated for 20 minutes in dark at 37°C in a CO_2 incubator. The stain was aspirated and the wells were gently washed twice with PBS. The wells were layered with 100 µL of PBS, and fluorescence was visualized using a fluorescent microscope (ex 495 nm/ em 529 nm).

2.2.12. Cell migration assay

Scratch wound assay is a cost-effective, simple and established method to measure cell migration *in vitro*. It helps to study the regulation of cell migration by interactions between cells and extracellular matrix (ECM). The method has the advantage of mimicking the wound healing process *in vivo* to an extent. The method involves the introduction of a gap or wound in a monolayer of cells and observe the wound at regular intervals and capture images, to detect the rate of cell migration. This method was used by McCormack in his studies on mucosal healing in 1992 and later adapted for migration studies on cells *in vitro*. (McCormack *et al*., 1992; Liang *et al*., 2007).

Procedure: Cells were subcultured and seeded in 6 well plates supplemented with 1.5 mL of growth medium and incubated at standard conditions for attachment. The cells were monitored under a microscope for determining the growth of cells. Growth medium was replaced daily until a monolayer was formed. A wound was introduced in a straight line in the middle of each well with the help of a sterile 1 mL micropipette tip. The medium with the unattached cells was removed and the wells were washed with 1X PBS. Growth medium containing varying concentrations of compounds were introduced into the cells, and images of the wound was captured immediately. The treatment was carried out for the requisite amount of time until the wound closed. Images were taken at regular intervals by a camera attached to a microscope to visualize the extent of migration.

2.2.13. Cell cycle analysis

Different phases of the cell cycle can be differentiated by its DNA content, and this property can be manipulated for the detection of phases of cell cycle and any alterations associated. To state an example S phase will have double the DNA content of G1 phase. Propidium Iodide (PI) is a fluorescent, stoichiometric, nuclear stain usually employed for cell cycle analysis using flow cytometry. PI fluorescence is directly proportional to the DNA content present in cells, and flow cytometry can be utilized for assessing the measure of cells in different stages of the cell cycle. The analysis of cell cycle by PI stain was successfully carried out by Crissman *et al*. in 1973, and further established by A. Krishan in 1975 (Crissman *et al*., 1973; Krishan 1975). The protocol provided by the manufacturer (Sigma Aldrich) was standardized according to the lab requirements.

Reagents:

1) 70% ethanol.

2) RNase A (10 mg/mL).

3) Propidium Iodide (1 mg/mL).

4) 1X PBS.

Procedure: For performing cell cycle analysis, 1-2 x 10^6 cells/well were seeded in 6 well plates and incubated for 24 h to ensure attachment. The cells were exposed to treatment with compounds and positive control for 24 h in 1.5 mL growth medium. The cells were harvested mechanically by using a cell scraper and washed in 1X PBS. The cell pellet was resuspended in 0.3 mL of 1X PBS and cells were fixed by drop wise addition of 0.7 mL 70% ethanol. The cells were gently vortexed, and incubated in ice for 30 to 60 minutes. Ethanol was removed by centrifugation at 2000 rpm for 5 minutes followed by washing twice with 5 mL of 1X PBS. The cell pellet was resuspended in 0.25 mL of 1X PBS, and 5 µL of RNase A was added to the cell suspension. The samples were incubated for 30 to 60 minutes, followed by addition of 10 µL of 1 mg/mL PI. The fixed samples were incubated for 10 minutes in the dark at 4°C. The cell suspension was diluted in 0.25 mL of 1X PBS and filtered through a cell strainer to remove clumps. For each measurement, data from 20,000 single cell events were collected using flow cytometry (BD FACS Aria II).

2.2.14. Glucose uptake studies

Cancer cells require energy for the high metabolism associated with uncontrolled cell proliferation. Glucose is the chief energy source of cells and GLUT's are transporters responsible for the transportation of glucose into the cells. The glucose taken up by cells enter the glycolytic pathway and provide energy for the cells. To study the uptake of glucose by cells, a fluorescent D-Glucose analog, 2-NBDG was employed. This analog can be taken up by cells through GLUT transporters, but cannot be fully utilized for energy production due to its modifications. Hence 2-NBDG accumulates inside the cells, and the fluorescence generated by this glucose derivative can be taken as a measure of the glucose uptake by the cells. The direct measurement of glucose uptake using a fluorescent glucose analog by flow cytometry was first reported by Zou *et al*. in 2005 (Zou *et al*., 2005). The protocol specified by the manufacturer was standardized according to lab requirements. Data from 10,000 single cell events were collected for each measurement using flow cytometry.

Reagents:

1) 2-NBDG stock (10 mM).

2) 2-NBDG working solution-

Add 10 µL of the 2-NBDG stock solution to 990 µL of 1X PBS.

Procedure: For conducting this study, 1-5 x 10^5 cells were plated in each well of a 6 well plate and incubated for 24 h in growth medium supplemented with 10% FBS. The wells were washed with 2 mL of 1X PBS. The treatment compounds prepared in growth medium containing 0.5 - 1% FBS (serum starvation) was added to the wells and left for the required duration (2-4 h). Media was removed and cells were washed with 1X PBS. Each well was exposed to 100 µM NBDG for 30 minutes. Cells were trypsinized and neutralized with 1X PBS. Cell pellet obtained by centrifugation at 3000 rpm for 5 minutes was suspended in 1mL 1X PBS and analyzed by flow cytometry.

2.2.15. Anoikis assay

Anoikis is a form of cellular apoptosis encountered by normal cells on detachment from ECM or due to inappropriate adhesion. Adhesion to ECM is essential for the survival of many adherent cells. Metastatic cancer cells can detach by resisting anoikis without compromising its ability to migrate and invade distant

organs. Anoikis detection was carried out using a standard kit assay protocol using calcein AM dye, and a coated chamber that mimics anoikis conditions. Calcein AM is a non-fluorescent, cell-permeable derivative of calcein that emits fluorescence only when hydrolyzed by esterases in live cells. The protocol provided by the manufacturer was followed for carrying out this assay.

Reagents: 1) Calcein AM dye (kit component).

Procedure: To conduct the assay, 100 µL of solution from $1x10^6$ cells/mL cell suspension was added into each well of the anoikis chamber (96-well anchorage resistant plate) and control chamber (96-well adhesive plate) and left for 24 h in a CO_2 incubator. The wells were introduced to 100 µL of treatment compounds and positive control for 24 h. After incubation, the wells were incubated with 2 µL of calcein AM dye for 30-60 minutes in a CO_2 incubator. The cells were monitored by fluorescent microscopy for green fluorescence. The fluorescence was also quantified at 485 nm/ 530 nm using a fluorescence microplate reader (Synergy™ 4, BioTek Instruments Inc., VT, USA).

2.2.16. Preparation of cell lysate

Extraction of cellular proteins without protein degradation requires proficient lysis of the cells. The solubilisation of many nuclear and cytoplasmic proteins is dependent on the presence of detergents and salt, while the extraction is dependent on pH and chelating agents. RIPA was chosen as the buffer for cell lysis as it allows rapid extraction of proteins, efficiently solubilises proteins and the buffer components do not adversely interact with the proteins. To reduce proteolytic activity, inhibitors of proteases were also added to the lysis buffer. The effective denaturation of proteins in the sample is achieved through the use of detergent, reducing agent, and heat. The protocol used for the current studies was standardized from the manufacturer specified procedure.

Reagents:

1) RIPA buffer-

   50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate.

2) 10 X Protease Inhibitor Cocktail (PIC).

3) Triton X 100.

4) 1X PBS.

5) RIPA lysis buffer-

10 mL RIPA buffer, 1 mL PIC, and 10 µL Triton X 100 were mixed together.

Procedure: Prior to cell lysis, the medium was aspirated and the cells were washed twice with cold 1X PBS. An appropriate volume (1 ml for 0.5 to 5 x 10^7cells) of RIPA lysis buffer was added to the cells and incubated on ice for 10-30 minutes. The cells were rapidly scraped with a cell scraper to detach cells from culture plate and lyse the remaining cells. The lysate was subjected to freeze-thaw cycles and clarified by centrifugation at 12000 rpm for 20 minutes at 4°C. The supernatant was collected as cell lysate and stored at -80°C for further use.

2.2.17. Protein estimation

Protein estimation was carried out using BCA Protein Assay which quantifies total protein concentration by colorimetric detection. This assay combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret method) with selective detection of Cu^{+1} by bicinchoninic acid (BCA) (Smith *et al.*, 1985). The chelation of two molecules of BCA with a cuprous cation is responsible for the purple-colored (A^{562}) reaction product of this assay. The molecular structure of the protein, peptide bonds and four specific amino acids namely cysteine, cystine, tryptophan and tyrosine are responsible for the color formation by BCA. Protein concentrations of unknown sample are determined by comparison with the absorbance of a known standard protein such as BSA.

Reagents:

1) BCA Working reagent-

50 parts of BCA Reagent A was mixed with 1 part of BCA Reagent B.

2) BSA stock (2 mg/mL).

Procedure: Protein standard dilutions were prepared in the working range of 25 µg to 2000 µg. For the assay, 25 µL of standard or unknown sample were pipetted into a microplate well. To each well, 200 µL of working reagent was added, and the plate was agitated in a shaker for 30 seconds. The plate was incubated in the dark at 37°C for 30 minutes. The absorbance was measured at 562 nm in a microplate reader. A standard curve was plotted from the known concentrations of the standard protein and the concentration of unknown samples was obtained from the graph.

2.2.18. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a variant of PAGE, developed by Ulrich Laemmli for the separation of proteins by their molecular weight in an electric field (Laemmli, 1970). The SDS coats the proteins at a constant molar ratio, giving them a negative charge relative to their peptide chain length. In SDS-PAGE, the proteins separate by charge and by the sieving effect of the gel matrix. Sharp banding of the proteins is attained by the use of a discontinuous gel system that has stacking and separating gel layers that differ in parameters such as ionic strength, pH, and acrylamide concentration. In the stacking gel, the potential gradient created by the slow-moving glycine ions and rapid chloride ions helps to stack the proteins as a narrow band. And the major separation of proteins occurs in the resolving gel due to its molecular weight. The discontinuous system allows improved resolution of sample separation. The protocol used here was originally taken from Sambrook *et al*. and modified further by Blancher *et al*. in 2001 (Sambrook *et al*., 1989; Blancher *et al*., 2001).

Reagents:

1) Tris-glycine electrophoresis buffer (5X)-

   15.1 g of Tris base, 94 g of glycine, and 5 g of SDS was made upto 1L with distilled water.

2) 30% Acrylamide/bis-acrylamide solution-

   30 g Acrylamide and 0.8 g bis-acrylamide was dissolved and made up to 10mL with distilled water.

3) 1.5 M Tris HCl (pH 8.8)-

   18.171 g of Tris base was dissolved in 50 mL distilled water and made upto 100 mL with distilled water after pH was adjusted.

4) 0.5 M Tris HCl (pH 6.8)-

   6.05 g of Tris base was made up to 100 mL with distilled water after adjusting pH.

5) 10% SDS-

   10 g SDS was dissolved in 100 mL of distilled water.

6) 10% APS (Ammonium persulfate)-

   100 mg APS was dissolved in 1mL of distilled water (freshly prepared).

7) TEMED (N,N,N',N' tetramethylethylenediamine)- used as supplied by the manufacturer.
8) Coomassie staining solution-

   500mL of Methanol, 100 mL of Acetic acid was made up to 1L with distilled water. 2.5 g of Coomassie brilliant blue R250 was added to the solution. The solution was filtered through Whatman filter paper.
9) Destaining solution-

   150 mL Methanol, 100 mL Acetic acid and 750 mL distilled water.
10) Reducing sample buffer (6X)-

    50mM Tris HCl (pH 6.8), 5% Glycerol, 2% SDS, 0.01% bromophenol blue, and 1% β-mercaptoethanol.
11) Prestained molecular weight markers.
12) 5% Stacking gel (5 mL)-

    2.8 mL Distilled water, 0.83 mL 30% Acrylamide mix, 1.25 mL 0.5 M Tris HCl (pH 6.8), 50 µL 10% SDS, 50 µL 10% APS and 5 µL TEMED.
13) Resolving gel-

    Distilled water, 30% Acrylamide mix, 1.5 M Tris HCl (pH 8.8), 10% SDS, 10% APS and TEMED. The reagent volumes for the preparation of different percentages of resolving gel is given in Table 2.1

Solution components (mL)	**5 mL**	**10 mL**	**15 mL**
8% gel			
H_2O	2.3	4.6	6.9
30% Acrylamide mix	1.5	2.7	4.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.003	0.006	0.009
10% gel			
H_2O	1.9	4.0	5.9
30% Acrylamide mix	1.7	3.3	5.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006
12% gel			
H_2O	1.6	3.3	4.9
30% Acrylamide mix	2.0	4.0	6.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006

Table 2.1. Solutions for preparation of resolving gel for SDS-PAGE.

Procedure: Mini gels (7 x 10 cm) were prepared using vertical electrophoresis casting equipment. The gel was prepared in two layers, stacking gel on top and separating gel beneath it. Resolving gel of the pertinent percentage was prepared by mixing the gel components mentioned above, poured into the casting equipment, and left to set for 30 minutes. A thin layer of butanol/water was poured to ensure an even interface between the two layers. Stacking gel was prepared next and poured above the set resolving gel and a teflon comb was inserted on the top. The setup was left for 15-20 minutes to solidify. The cell lysate samples (protein equalized) were diluted in 6X reducing sample buffer, and heated at 95 to 100°C for 5 minutes. The samples (protein concentration of 50-100 µg/mL) were introduced into the wells created by the comb. The sample was subjected to separation in the vertical gel electrophoresis apparatus at 90 V for 1.5 to 2 h. The gel was separated from the sandwich glass plates on either side and treated according to further analysis. The gels were either stained by Coomassie staining

solution for the visualization of separated protein bands or subjected to western blot analysis for detection of specific proteins.

2.2.19. Gelatin zymography

Zymography is a method used for detection of the proteolytic activity of an enzyme using an electrophoretic method such as SDS-PAGE. MMP-2 and MMP-9 are ECM degrading enzymes that can be quantified by gelatin zymography. These enzymes have a central role in assisting the migration of cancer cells during metastasis. This method employs the ability of these enzymes to degrade one of their substrates, gelatin. The procedure involves the co-polymerisation of gelatin to polyacrylamide in the resolving gel, which is cleaved by activated enzymes in the sample. The samples are prepared in SDS-PAGE sample buffer without reducing agent or heat denaturation. The resolved proteinases are renatured by a non-ionic detergent and activated by a Ca^{2+} containing buffer. Zymogram was subsequently stained, and the areas of digestion were identified. Studies on gelatinases by means of electrophoresis was developed by Hibbs 1985. A modified protocol was followed for estimating the activity of MMP enzymes.

Reagents:

1) 5% Stacking gel (5 mL)-

   2.8 mL Distilled water, 0.83 mL 30% Acrylamide mix, 1.25 mL 0.5 M Tris HCl (pH 6.8), 50 µL 10% SDS, 50 µL 10% APS and 5 µL TEMED.

2) 8% Resolving gel-

   30% Acrylamide mix, 1.5 M Tris buffer HCl (pH 8.8), 20 mg/mL gelatin, 10% APS and TEMED.

3) Non-reducing loading buffer-

   50 mM Tris HCl (pH 6.8), 5% Glycerol, 2% SDS, and 0.01% bromophenol blue.

4) Washing buffer-

   2.5% Triton X 100 prepared in distilled water.

5) Incubation buffer-

   50 mM Tris HCl, 0.15 M NaCl and 10 mM CaCl2 in distilled water (pH 7.8-8).

6) Coomassie staining solution. (Refer 2.2.18.)
7) Destaining solution. (Refer 2.2.18.)

Procedure: For conducting this assay**,** cells were plated in 6 well plates and grown till they reached 80% confluence. The medium was aspirated and the cells were exposed to treatment with treatment compounds for 24 h. The medium in which the cells were grown was collected for enzyme assay. Gelatin used in the preparation of resolving gel was dissolved in distilled water by heating. The resolving and stacking gel were prepared and cast in a vertical electrophoretic apparatus. The samples were prepared in a non-reducing loading buffer and loaded into wells and run at 90V for 2 h in ice. The gel was washed for 40 min in 2.5 % Triton X-100 solution at room temperature and incubated for 20 h in incubation buffer at 37°C. The gel was then stained with Coomassie staining solution for 2 h followed by overnight destaining. The clear areas in the blue background indicated areas where gelatin was cleaved and was visualized using a ChemiDoc™ MP System (BioRad, Hercules, CA, USA).

2.2.20 Western blot analysis

It is a widely accepted analytical technique, used for the detection of a specific protein from a sample. Western blotting involves two major techniques namely SDS-PAGE and protein blotting. The proteins in a sample are separated based on molecular weight using SDS-PAGE, and the separated proteins are then transferred onto a matrix such as PVDF (polyvinylidene fluoride) or nitrocellulose membrane. The membrane is then blocked to prevent non-specific binding of antibodies with a blocking solution. The membrane is then probed by a primary antibody specific to the protein of interest. The membrane is then incubated with a secondary anti-immunoglobulin that is either radiolabelled or conjugated to an enzyme. The detection of bands are achieved by colorimetric reaction, chemiluminescence or autoradiography. The position of the band and its intensity can be used to analyze the expression of the protein. Western blot was carried out according to the standard protocol described by Maniatis *et al*. 1983.

Reagents:

1) Reagents required for SDS-PAGE. (Refer 2.2.18.)
2) 1X PBST-

   0.1% Tween 20 was added to 1X PBST.
3) Transfer buffer-

   3.03 g of Tris base, and 14.4 g of glycine added to 700 mL of distilled water. 200 mL of methanol was added and the volume made up to 1L using distilled water.
4) 5% Skim milk solution-

   5 g skim milk was dissolved in 1X PBST.
5) Ponceau S solution (used as supplied by manufacturer).
6) Methanol.
7) Primary antibody solution-

   Primary antibody solution was prepared in PBST or skim milk solution. The dilution was prepared according to the manufacturer's protocol (1:500/1:1000 dilution).
8) Secondary antibody solution-

   Secondary antibody was diluted in PBST skim milk solution according to manufacturer's manual (1:500/1:1000 dilution).
9) ECL substrate-

   Reagent A and Reagent B were freshly prepared in a 1:1 ratio.

Procedure: The samples for western blot were obtained from cells cultured in standard conditions followed by exposure to various experimental procedures. Total protein lysate was prepared from the cells and subjected to protein equalization. The sample was then subjected to separation by SDS-PAGE. The gel obtained after electrophoresis was further processed for western blot analysis.

1) Transfer of proteins from gel to a membrane support

The proteins in the gel was transferred to a PVDF membrane by semi-dry or wet blot method. The PVDF membrane was initially soaked in methanol for 10-20 seconds followed by immersing it in transfer buffer for 5-10 minutes. Whatman filter papers and the gel was also soaked in transfer buffer. The Whatman filter papers are stacked followed by PVDF membrane. The SDS-PAGE gel was placed on top of the membrane and additional Whatman filter papers are stacked on top of it. The sandwich system was placed in the transfer apparatus such that the gel is

near the negative electrode. The semi-dry transfer system was run at 25 V for 10-15 minutes, while wet blot was run at 90 V for 1-2 h in ice. The membrane was stained with Ponceau S to ensure transfer of proteins.

2) Blocking

To prevent non-specific binding of antibodies, the membrane was incubated in skim milk solution for 1 h at room temperature on a shaker. The membrane was washed thrice with 1X PBST for 10 minutes.

3) Probing with antibodies

The membrane was then incubated with primary antibody for 1-2 h at room temperature or overnight at 4°C in a shaker. The membrane was again washed with 1X PBST for ten minutes to remove unbound antibody. The membrane was then kept in a secondary antibody with conjugated enzyme for 1-2 h at room temperature with constant agitation. The membrane was washed thrice with 1X PBST for 10 minutes.

4) Detection

The membrane was incubated with chemiluminescent substrate for 5 minutes in the dark and the bands were visualized using a ChemiDoc™ MP System (BioRad, Hercules, CA, USA).

2.2.21. Active RAS detection assay

RAS superfamily comprise of small GTP- binding proteins (G proteins) that possess GTP/GDP binding and GTPase activities. They regulate signalling of many cellular processes including cell cycle progression and survival. An upstream signal stimulates the dissociation of GDP from GDP-RAS (inactive form) and leads to the binding of GTP to RAS (active form). The activated G protein can activate downstream signalling molecules. The intrinsic GTPase activity of RAS mediated by GTPase activating proteins (GAP), can switch off the activation. In cancer cases, the GTPase based inactivation is inhibited due to point mutation in Ras, leading to continuous activation of the protein.

The detection of GTP-bound RAS (active) was carried out by active RAS detection kit (CST). The kit consists of GST-Raf1-RBD fusion protein which binds to the GTP-bound RAS and can be immunoprecipitated using a glutathione resin. Then employing a RAS antibody, RAS activation levels can be determined using western blotting. The kit protocol is based on the activation assay developed by

Taylor and Shalloway 1996. The protocol provided by the manufacturer was meticulously followed for carrying out this assay.

Reagents:

1) 1X PBS.
2) 1mM PMSF.
3) 1X Lysis buffer (kit component).
4) 0.5 M EDTA.
5) 1M $MgCl_2$.
6) GST-Raf1-RBD.
7) DTT.
8) 2X reducing sample buffer (kit component).

Procedure:

1) Cell lysis

This assay required the seeding of 1-2 x 10^7 cells in a 75 cm^2 flask for each sample. Cells were harvested and rinsed in ice cold PBS. The cell pellets were suspended in 0.5 mL of ice cold 1X Lysis buffer with 1mM PMSF and incubated in ice for 5 minutes. The cells were centrifugeD at 16,000xg for 15 minutes at 4°C and the supernatant was collected as the cell lysate.

2) *In vitro* GTPγS or GDP Treatment

To ensure proper execution of immunoprecipitation, GTPγS (positive control) and GDP (negative control) treatments were included. 500 µL of cell lysate with 10 µL of 0.5M EDTA was mixed by vortexing. To the sample, 5 µL of 10 mM GTPγS or 5 µL of 100mM GDP was added and incubated for 15 minutes at 30°C with constant agitation. The reaction was terminated by placing the samples on ice after adding 32 µL of 1M $MgCl_2$.

3) Affinity precipitation of activated G protein

Spin cups and collection tubes provided by the kit were used to precipitate activated G protein from the sample. 100 µL of glutathione resin in a spin cup was centrifuged at 6000xg for 10-30 seconds and the flow through was removed. The resin was washed with 400 µL of 1X Lysis buffer. 80 µg of the GST-Raf1-RBD fusion protein and 700 µL of cell lysate were added to the glutathione resin in the spin cup. The sample was vortexed, and incubated at 4°C for 1 h with gentle rocking. Then the sample was centrifuged at 6000xg for 30 seconds to remove the flow through. The sample was washed thrice with 1X Lysis buffer. The spin cup

was transferred to a new collection tube, and 50 µL of 2X reducing sample buffer with 200 mM DTT was added to the resin. After incubation for 2 minutes, the sample was eluted by centrifugation at 6000xg for 2 minutes. The resin was discarded, and the eluted sample was heated for 5 minutes at 100°C. The samples were subjected to electrophoresis and western blot for further analysis.

2.2.22. Enzyme linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique used to quantify proteins. An enzyme which can react with chromogenic substrates to give colored products are conjugated to antibodies, and used for the detection of target molecules. The development of color can be measured using a microplate reader. ELISA was introduced by Perlmann and Engvall in 1971. The standard protocol described by the ELISA kit was followed to perform the assay.

Reagents:

1) 1X PBST.
2) 5% BSA solution-

   5 g of BSA was dissolved in 100 mL of 1X PBST.
3) Primary antibody solution-

   Primary antibody was diluted in 1X PBST according to manufacturer's instructions (1:1000).
4) HRP conjugated secondary antibody solution-

   Secondary antibody was diluted in 1X PBST according to company manual (1:1000).
5) 5, 5' tetramethylbenzidine (TMB).
6) 3N HCl.

Procedure: For performing indirect ELISA method, cell lysate or cell culture medium was taken as the sample. Cell lysate or the medium in which the cells were grown was coated in a 96 well ELISA plate, and incubated overnight at 37°C. The remaining sample was aspirated, and the wells were washed three times with 1X PBST for 5 minutes each. The wells were incubated with BSA blocking solution for 1 h in a shaker. The wells were then washed three times with 1X PBST for 5 minutes each. This was followed by incubation with the primary antibody solution for 2 h. The corresponding HRP conjugated secondary antibody solution was then added to the wells and incubated for 1 h. Media was aspirated and the wells were

washed thrice with 1X PBST to remove any unbound antibody. The wells were then exposed to TMB substrate for 15 minutes. The reaction was stopped using 3N HCl and the yellow color was read at 490nm using a microplate reader (Biotek Synergy 4, VT, USA).

2.2.23. Immunoprecipitation (IP)

Immunoprecipitation is a method used to isolate a specific protein from a mixture of proteins, employing antigen-antibody reaction. Small scale affinity purification of antigens is achieved by using bacterial proteins, Protein A or Protein G that are immobilized to a solid matrix (Harlow *et al*., 2006). These bacterial proteins efficiently bind to antibodies. Fundamentally the antibody specific for the target protein, binds to the bacterial proteins in the matrix. Then the sample is incubated with the antibody bound matrix, where the target protein binds to the antibody. The proteins bound to the antibody can be isolated, giving a concentrated sample of the specific protein. It is a widely used protocol for isolation of proteins from cell or tissue lysates. The standard protocol provided by the manufacturer (MagGenome Technologies) was followed to carry out the assay.

Reagents:

1) Protein A or Protein G nanoparticles-
   10 mg nanoparticles/mL in 5 mM PBS.
2) 1X PBS
3) RIPA buffer

Procedure: This protocol was used to precipitate proteins present in low concentrations in cell lysate for further analysis. Lysate from control and compound treated cells with equalized protein concentration was taken as the sample to be immunoprecipitated. The sample was incubated with 2 µL of primary antibody specific to target protein overnight at 4°C. The magnetic nanoparticles were pre-cleared by washing with 1X PBS or lysis buffer, three times. To each lysate-antibody sample, 50 µL of magnetic nanoparticle was added and incubated at 4°C for 2 h with continuous agitation. The nanoparticles were collected with a magnetic stand and after multiple washes, 50 µL of 1X SDS-PAGE reducing sample buffer was added to it. The supernatant was collected after heating the sample at 100°C for 5 minutes. The purified sample was used for subsequent SDS-PAGE and western blot analysis.

2.2.24. Immunofluorescence studies

Immunofluorescence is a technique that allows the detection, and visualization of the distribution of target antigens using fluorescent tagged antibodies specific to the target protein. The basic idea behind the method is to target the protein of interest using fluorochrome-conjugated primary or secondary antibody. The location or distribution of the target molecules can be visualized by means of fluorescent microscopy. The standard protocol for immunofluorescence staining by the manufacturer (CST) was carried out.

Reagents:

1) 1X PBS.
2) 1X PBST.
3) 4% Paraformaldehyde-

   16% paraformaldehyde (manufacturer) was diluted in PBS.
4) Normal Goat serum (NGS).
5) Primary antibody solution-

   2 µL of antibody was diluted with 98 µL NGS (1:50 dilution).
6) Secondary antibody solution-

   1 µL of antibody was made upto 100 µL with NGS (1:100 dilution).
7) Hoechst stain-

   1 drop was added to 2 mL PBS (specified by the manufacturer).

Procedure: For performing this assay 1 x 10^4 cells were seeded in 96 well black plates and allowed for attachment overnight. Treatment with compounds and positive control was carried out for 24 h. Cells were then washed three times with 1X PBS. This was followed by fixation of the cells in 100 µL of 4% paraformaldehyde in each well for 15 minutes. The cells were then permeabilized by incubation in 100 µL of PBST for 10 minutes. The wells were washed with 1X PBS. The cells were blocked with 100 µL of NGS for 30 minutes in a shaker followed again by PBST wash. The wells were then incubated with 100 µL of primary antibody solution for 2 h. After washing the wells with 100 µL of NGS, 100 µL of secondary antibody labelled with FITC was added and left for 1 h. The wells were washed once with PBST and treated with 100 µL of Hoechst stain for 7 minutes. The dye was replaced with PBS and wells were observed under a confocal laser scanning microscope (FV3000, Olympus Life sciences, Pennsylvania, USA).

2.2.25. Three dimensional cell culture

Three-dimensional (3D) cell culture facilitates the translation of *in vitro* studies into a more realistic *in vivo* mimicking model. Cells in spheroids show a higher degree of morphological and functional differentiation, than cells grown in two-dimensional (2D) culture. The cells grown in monolayer or 2D conditions have low resistance for therapy, hence does not include factors like tumor microenvironment. 3D culture for this study was performed using poly 2-hydroxyethylmethacrylate (poly-HEMA) as the scaffold owing to its non-adhesive property. The protocol followed was standardized from given literature (Lawrenson *et al*., 2012; Phung *et al*., 2011).

Reagents:

1) 95% ethanol-

   5 mL of distilled water was added to 95 ml of 100% ethanol.

2) Poly-HEMA stock (10X) –

   150 mg poly-HEMA was dissolved in 10 mL of 95% ethanol 65^{o}C for 8 h.

3) Poly-HEMA working solution-

   1 mL of 10 X poly-HEMA stock was made up to 10 mL with 95% ethanol (freshly prepared).

Procedure:

1) Preparation of plates using poly-HEMA

The fresh working solution of poly-HEMA was prepared and used to coat culture plates. 1 mL of poly-HEMA working solution was pipetted into each well of 6 well plate and left to air dry in the laminar flow chamber overnight at room temperature. For 96 well plates, 100 µL of poly-HEMA working solution is adequate. To ensure uniform coating, the coating process was repeated once again. Before use, the dry coated wells were washed once with PBS. Cells were seeded at a density of 1-5 x 10^6 cells/well for 6 well plate and 1 x 10^5 cells/well for 96 well plate. The plates were incubated for 24 h to guarantee the formation of aggregates without any agitation. This was confirmed by observing under a light microscope.

2) Treatment and analysis

The medium was not aspirated, and the remaining experiment was designed so as not to disturb the aggregates. The cells were then treated with the compounds and positive control for 24 h following which the spheroids were observed under a

phase contrast microscope. Cells grown in 3D condition using this method was subjected to viability assay, caspase activity assays, gelatin zymography, ELISA and western blot as per the protocols furnished above.

2.2.26. Toxicology studies using zebrafish embryo model system

Based on the OECD guidelines, this test (Test no. 236) is intended to determine the acute or lethal toxicity of chemicals on the embryonic stages of fish. Newly fertilized zebrafish embryos are exposed to the test chemical for a period of 96 h. Endpoints such as coagulation of embryos, lack of somites formation, lack of tail detachment and heart beat are observed and the lethality of compounds determined. The work was carried out in the Ecotoxicology Laboratory of CSIR-Indian Institute of Toxicology Research (IITR), Lucknow.

Procedure:

1) Culture of zebrafish embryo

Sexually mature wild-type (ASWT strain) zebrafish (*Danio rerio*) were procured from a commercial supplier with identification certificate. The fish were kept in communities of up to 40 individuals in 8.5 L water with a flow-through standalone system. The physico-chemical parameters such as temperature (26-28ºC), pH (6.5-7.5) and conductivity (400-600 µS/cm) were maintained within the range. Commercially available diet (Grade 400) from zebrafish management systems, U.K. were provided twice daily as feed. The day before the experiment, males, and females in the ratio 2:1 were placed in egg collection chambers before dawn. The chambers were incubated at 28°C with 16 h light and 8 h darkness photoperiod. After the commencement of light the next morning, spawning and fertilization took place within 30 minutes. Fertilization rate of >70% was considered as quality control for further exposures.

The collected embryos were washed with E3 medium four times to prevent any fungal infection. Then the embryos were placed in the embryo medium (60 mg of ocean salt in 1L of reverse osmosis water) for incubation in the incubator at 28°C until treatment. Fertilized embryos at 16 – 64 celled stage (1.5 to 2 h post fertilization; hpf) were selected using a light microscope for exposure.

2) Exposure to compounds

Fertilized embryos were exposed to 250 µM, 500 µM, 750 µM and 1000 µM of the DIM compounds. The samples were dissolved in 0.1% DMSO and treatment

was carried out in triplicates with ten embryos per replicate with control and solvent control groups in 24-well sterile plates. The toxicity potential of drugs on different developmental stages of zebrafish embryos were determined. The embryos were observed for abnormalities regularly at 24 h time intervals upto 96 h post-treatment with compounds using a light microscope (NIKON – Eclipse, Japan) at 4x objective. At the end of the exposure period, acute toxicity is determined.

2.2.27. Molecular docking

It is a key tool for studies on computer-aided drug design where it predicts plausible binding interactions between ligand and known 3D structure of a protein. It uses a scoring function to find the most potential binding. The software Autodock 4.2 was used for investigations between treatment compounds and receptors. The binding energies were used to analyze the most probable binding.

Procedure: 3D structures of the proteins were acquired from NCBI database as '.pdb' files and compound ligand structures were drawn using ChemDraw software. Both the receptor and ligand structures were opened using Autodock Tools (ADT). Water molecules were removed from the structure, and polar hydrogen bonds added. If the molecule was a peptide, Kollman charges were added, or else gastieger charges were added by ADT. Next PDBQT file formats for protein and ligand were prepared. AutoGrid was opened, and the structures of receptor and ligand were selected. Grid parameters were created using ADT and AutoGrid was launched. The program was left to run till results in the form of '.glg' files were obtained. Macromolecule and ligand file for docking run was selected. Docking parameter files were created next using ADT and AutoDock was launched. The output file format of '.dlg' was obtained and analyzed.

2.2.28. Statistical data analysis

Values are given as mean ± standard deviation (n=3). Data were analyzed by one-way ANOVA, and the significance of the difference between means was calculated by Duncan's multiple range tests using SPSS statistical analysis programme (SPSS, Inc.). 'p' value of less than 0.05 was considered to be statistically significant.

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Chapter 3

Anticancer activity studies of Biaryl DIM derivatives in human cervical cancer cells

3.1. Introduction

Cancer is one of the leading causes of death in both developed and developing countries. Cervical cancer is the most common reason for mortality due to cancer among women in developing countries (Denny *et al*., 2012). According to the GLOBOCAN 2012 report, cervical cancer is the fourth most common cancer in women accounting for more than 5 million new cases in 2012, and studies have also reported that human papillomavirus (HPV) infection is a common reason for cervical cancer incidence, worldwide (Ferlay *et al*., 2015; Walboomers *et al*., 1999). The currently practiced treatment options of surgery and radiotherapy have many shortcomings such as recurrence and side effects. The present chemotherapeutic treatment regime for cervical cancer which includes cisplatin, carboplatin, topotecan, paclitaxel, etc. exhibit inefficiency in the treatment of recurring metastatic cervical cancer (Cadron *et al*., 2007; Tao *et al*., 2008; Rose PG, 2003).

Natural products contain relatively non-toxic alternatives with reduced side-effects for treatment of cancer of any origin. Many compounds of natural origin show promise in studies conducted on cervical cancer. Cucurbitacin D (analogue of cucurbitacin), fucosterol (sterol), syringin (glycoside), trichosanthin (protein from Cucurbitaceae), and oblongifolin c (lignan from *Garcinia yunnanensis*), are few of the promising natural compounds that induce antiproliferative or apoptotic effects in cervical cancer cells (Sikander *et al*., 2016; Jiang *et al*., 2018; Xia, 2016; Wang *et al*., 2013). The bioactive compounds of natural origin can interact with multiple biological targets, due to their complex structure, making them pleiotropic compounds. Anticancer compounds act by targeting and inhibiting the hallmark events of cancer such as proliferation, angiogenesis, migration, etc.

Many plant compounds consumed as part of the diet can provide additional benefit of protection against many diseases. Dietary compound indole-3-carbinol (I3C) and its bioactive compound diindolylmethane (DIM) from cruciferous

vegetables have been reported to induce chemotherapeutic effects in various types of cancer cells. In the late 1990s, I3C was found to prevent carcinogen-induced tumorigenesis in the cervix and breast tissue, in multiple animal model studies (Jin *et al*., 1999; Grubbs *et al*., 1995). I3C and DIM have been reported to induce apoptosis in cervical cancer cells (*in vitro*) and in cervical tissue *in vivo* (transgenic mice) (Chen *et al*., 2001). Apart from inducing characteristic indicators of apoptosis such as DNA fragmentation and caspase activation, I3C and DIM possess the ability to attenuate cancer cells by influencing the other hallmarks of cancer. Studies indicated that apoptosis in cancer cells induced by I3C and DIM, involved the inhibition of AKT and NF-κB respectively, where both these molecules play a major role in cancer survival and proliferation (Chinni *et al*., 2002; Rahman *et al*., 2005). DIM and I3C have induced cell cycle arrest (mainly at G1 phase) by regulation of CDK's or cell cycle inhibitors in several cancer types (Cover *et al*., 1998; Hong *et al*., 2002; Chinnakannu *et al*., 2009). DIM was found to downregulate MMP and VEGF in prostate cancer cells and thereby inhibited angiogenesis and invasion (Kong *et al*., 2007). The most aggressive hallmark of cancer is its ability to migrate and metastasize, and DIM has shown inhibitory effect towards this particular hallmark as well. DIM has been subjected to many synthetic functional modifications to improve its bioavailability, potency, and safety.

The current chapter is dedicated to the study of synthetic derivatives of DIM as potential anticancer candidates. The derivatives were synthesized by the novel conjugation of two chemotherapeutic compounds: DIM and biaryl. The compounds were initially screened for cytotoxicity towards cervical cancer cells followed by a detailed investigation of the potential compounds on the probable cause for cytotoxicity induction. Its role in attenuating the major hallmarks such as apoptosis, angiogenesis, cell cycle progression, and migration are studied with appropriate markers.

3.2. Methods

The anticancer properties of novel biaryl conjugates of DIM were investigated in the cervical cancer cell line, HeLa. The conjugates were synthesized based on a previously published protocol (Jamsheena *et al*., 2016). Initially, all the 12 derivatives (DIM-1 to DIM-12) were assessed for cytotoxic potential in HeLa cells

using MTT assay, followed by a comparative study in normal rat cardiomyoblast H9C2 cells.

1. The apoptosis indicating parameters of the potential cytotoxic derivatives were further studied in HeLa cells using the given methodologies.

- Morphological analysis by Phase contrast imaging.
- Detection of nuclear fragmentation by DAPI staining.
- Investigation of membrane damage by AO/EB staining.
- Assessment of mitochondrial membrane potential loss using JC-1 staining.
- Fluorimetric study of caspase 3 activity.
- Investigation of Bax, c-PARP, Bcl2 protein expression by western blotting.

2. The effect of DIM compounds on the migration of HeLa cells was studied using *in-vitro* scratch wound assay and gelatin zymography.
3. The anti-angiogenic potential of DIM derivatives was studied by western blot analysis of VEGF and fibronectin. Autodock analysis of VEGF receptors with potential derivatives was also carried out for the detection of probable interaction.
4. The effect of DIM conjugates on cell cycle arrest was studied by flow cytometry.
5. The expression of major cancer-associated proteins were also investigated by western blot.

3.3. Results

3.3.1. Synthesis of DIM-*ortho*-Biaryl conjugates

The parent biaryl DIM conjugate namely DIM-1 was synthesized using a standardised one-step condensation protocol explained in section 2.2.1 of chapter 2. The derivatives of DIM-1 namely DIM-2 to DIM-12, were synthesized by varying the functional groups of the parent compound. The structures of the DIM compounds are shown in Figure 3.1.

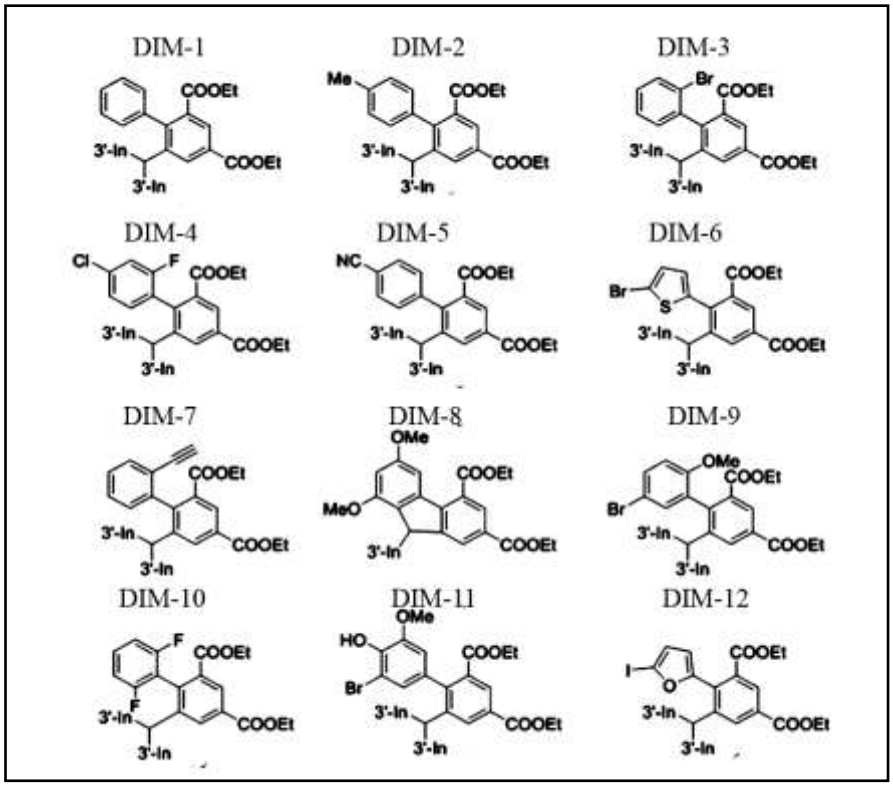

Figure 3.1. The chemical structures of novel DIM-ortho biaryl derivatives: *Novel DIM-ortho biaryl compounds were synthesized by one step condensation of biaryl carbaldehydes and indole with p-TSOH as the catalyst (Jamsheena et al., 2016). DIM-1 is the parent compound and the remaining derivatives, namely DIM-2 to DIM-12 were synthesized with modifications to DIM-1in a single biaryl moiety.*

3.3.2. Cytotoxicity evaluation of DIM compounds in cervical cancer cells

The 12 compounds (DIM-1 to DIM-12) were subjected to cytotoxicity evaluation using MTT assay in HeLa and H9C2 cells. Cells were exposed to varying concentrations of the DIM compounds (0, 2, 5, 10, 20 and 50 µM) for 24 h and the percentage of growth inhibition for each compound was calculated by the formula given in section 2.2.4. The results indicated that DIM-1,4 and 8 exhibited significant cytotoxicity towards HeLa cells. GI_{50} (concentration at which 50% of growth inhibition is achieved) for DIM-1, 4 and 8 were found to be 11.00±0.707 µM, 8.33±0.416 µM, and 1.45±0.180 µM respectively in HeLa cells. DIM-1 and 4 were non-toxic towards normal H9C2 cells (GI_{50} above 50 µM), while DIM-8 induced toxicity at GI_{50} value of 10.00±0.265 µM. Table 3.1. shows the GI_{50} values of all the 12 DIM compounds studied.

The standard anticancer drug, cisplatin was used as the positive control and it induced toxicity in both cancer and normal cells with a GI_{50} of 3.75±0.213 µM and 4.38±0.528 µM respectively.

S.No	Compounds	GI_{50} (µM)	
		HeLa	H9C2
1	**DIM-1**	**11.00±0.707**	**>50**
2	DIM-2	>20	>50
3	DIM-3	>20	>50
4	**DIM-4**	**8.33±0.416**	**>50**
5	DIM-5	>20	>50
6	DIM-6	>20	>50
7	DIM-7	>20	>50
8	**DIM-8**	**1.45±0.180**	**10.00±0.265**
9	DIM-9	>20	>50
10	DIM-10	>20	>50
11	DIM-11	>20	>50
12	DIM-12	>20	>50

Table 3.1. Evaluation of cytotoxicity of DIM compounds in HeLa and H9C2: *HeLa and H9C2 cells were treated with varying concentrations of DIM compounds (DIM-1 to DIM-12) for 24 h and the GI_{50} values were determined using MTT assay. DIM-1 and DIM-1 showed selective cytotoxicity towards cancer cells. DIM-8 also showed promising $GI_{50,}$ but induced toxicity in normal cells. Values are means, with standard deviations represented as ±.*

The concentration dependent effects of DIM-1, DIM-4 and the standard drug cisplatin in HeLa cells are given in Figure 3.2. Out of the twelve compounds chosen for cytotoxicity studies, DIM-1 and DIM-4 induced significant effects in cancer cells without harming normal cells. Hence these compounds were taken for further detailed analysis for mechanism of action in terms of apoptosis, cell cycle arrest and inhibition of migration and angiogenesis.

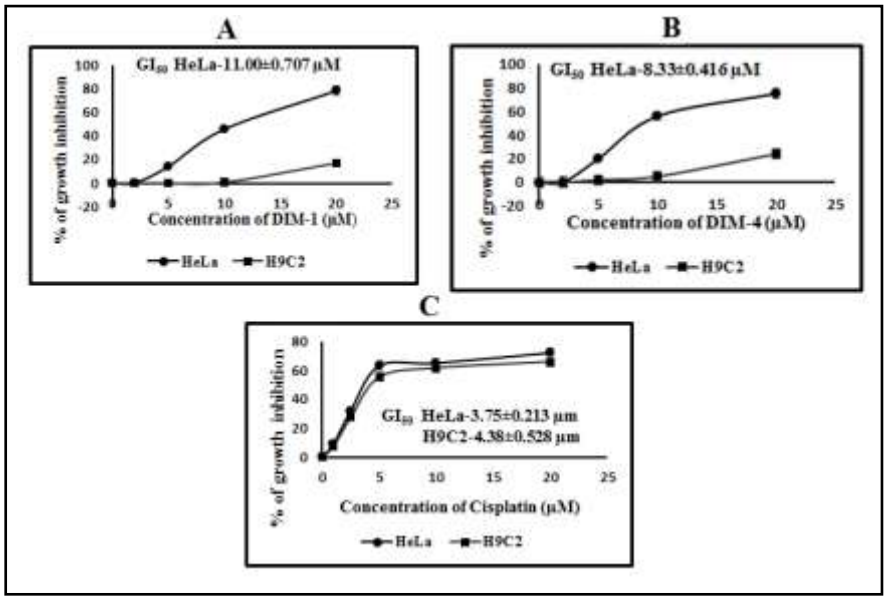

Figure 3.2. Cytotoxicity induced by DIM-1, DIM-4, and cisplatin in HeLa and H9C2 cells: *The graphical representation of concentration dependent cytotoxicity induced in HeLa and H9C2 cells by (A) DIM-1; (B) DIM-4 (C) cisplatin. The percentage of growth inhibition was calculated using MTT assay. DIM-1 and DIM-4 induced concentration dependent inhibition only in cancer cells, while cisplatin showed toxicity towards cancer and normal cells. Data represented are values ± standard deviation (SD) of three independent experiments ($p \leq 0.05$).*

3.3.3. Effects of DIM-1 and DIM-4 in inducing morphological alterations

The cells undergoing apoptosis exhibit significant morphological changes such as cell shrinkage, membrane blebbing and formation of apoptotic bodies. Based on the GI_{50} value obtained in cytotoxicity studies, two different concentrations (10 µM and 20 µM) for DIM-1 and DIM-4 and 5 µM for cisplatin were selected for further treatment. The morphology of HeLa and H9C2 cells were analysed after treatment with DIM-1 and DIM-4 using phase contrast microscopy. Control cells exhibited normal morphology with ellipsoidal shape and good amount of cytoplasm within intact membrane structure while the treated cells showed a more round appearance. DIM-1 and DIM-4 induced severe shrinking and formation of small disintegrating cellular bodies comparable to apoptotic bodies along with a significant decrease in the number of cells. Signs of membrane blebbing (formation of circular bulges on the plasma membrane) was also observed at higher concentrations. DIM-1 and DIM-4 treatments did not induce any noticeable morphological changes in normal H9C2 cells. The standard drug cisplatin was found to be equally toxic in both HeLa and H9C2 cells at treated concentration. The results are given in Figure 3.3. The morphological alterations such as cell shrinkage, alteration in shape, blebbing and

formation of apoptotic body like fragments suggested apoptosis as the cause for cytotoxicity.

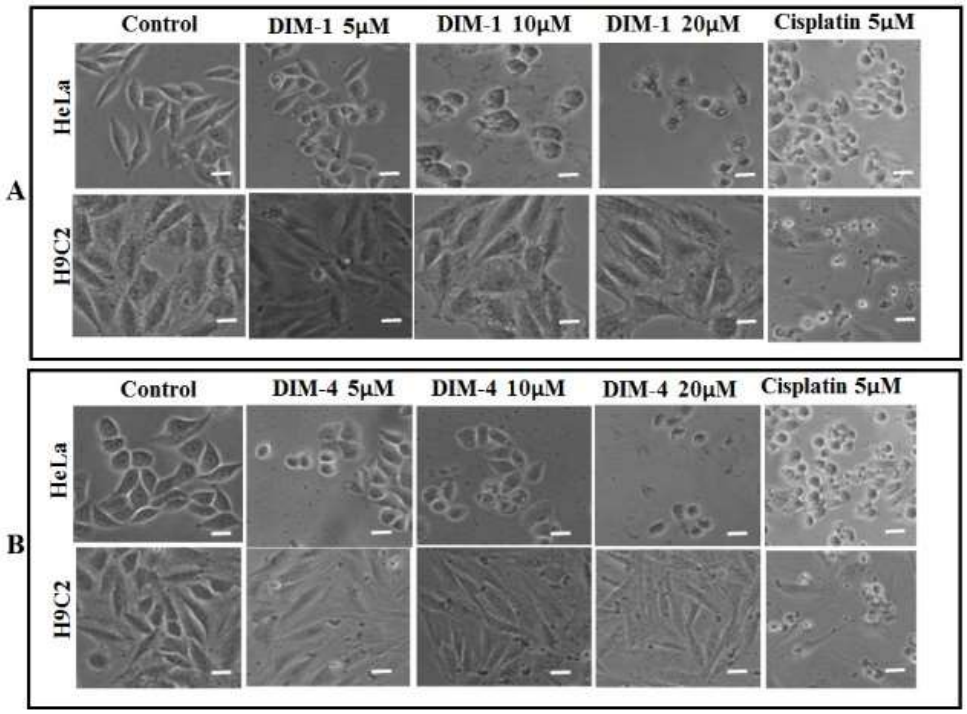

Figure 3.3. Morphological analysis of HeLa and H9C2 cells treated with DIM-1, DIM-4, and cisplatin by phase contrast microscopy: *Phase contrast images of HeLa and H9C2 cells treated with two different concentrations of (A) DIM-1 and (B) DIM-4 and cisplatin are represented in the figure. DIM compounds induced significant morphological changes in HeLa cells, such as cell shrinkage, blebbing and apoptotic body formation, without affecting the morphology of normal cells. Positive control cisplatin (5 µM) induced morphological changes in normal and cancer cells. The scale bar represents a size of 20 µm.*

3.3.4. Effects of DIM-1 and DIM-4 in nuclear fragmentation

DNA fragmentation is an important event in apoptosis and effects of DIM-1 and DIM-4 on nuclear fragmentation was studied by DAPI staining. The results given in Figure 3.4. indicated that DIM-1 and DIM-4 (10 µM and 20 µM) for 24 h induced significant nuclear fragmentation in cervical cancer cells, while the control cells showed firm and undamaged nucleus. Cisplatin, the positive control also induced nuclear damage in cervical cancer cells. Thus DIM-1 and DIM-4 induced nuclear fragmentation in HeLa cells, and this is an indication of apoptosis.

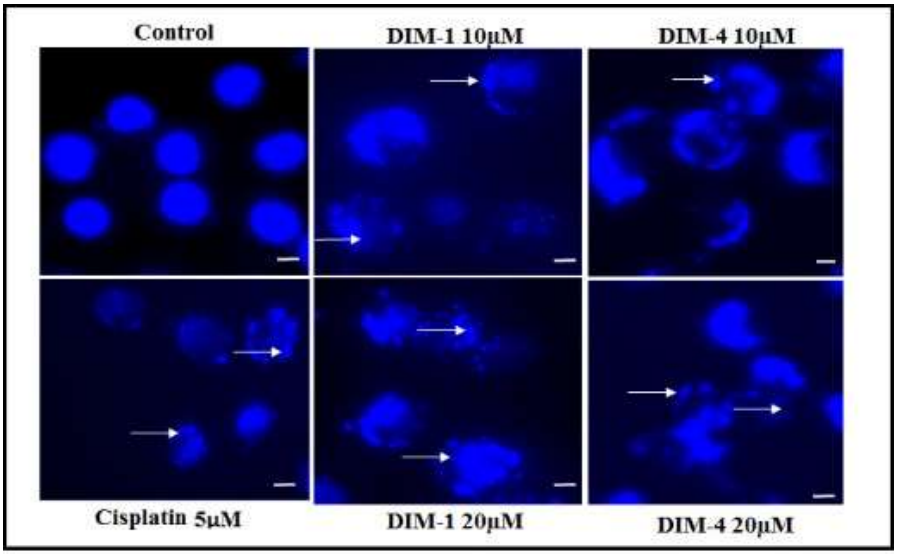

Figure 3.4. Analysis of DNA fragmentation by DAPI staining in HeLa cells: *HeLa cells treated with two different concentrations of DIM-1, DIM-4 (10 µM and 20 µM) and cisplatin (5 µM) showed clear signs of nuclear damage, when compared to the intact nucleus of the control cells. The arrows indicate the nuclear damage and the scale bar denotes a size of 10µm.*

3.3.5. Effects of DIM-1 and DIM-4 on plasma membrane integrity

An intact plasma membrane is the sign of a healthy cell. During late stage of apoptosis, plasma membrane integrity is compromised. AO/EB staining was used to analyse membrane integrity in DIM treated cells (10 µM and 20 µM for 24 h). The control cells show only green fluorescence, which indicates an intact membrane. But the DIM-1 and DIM-4 treated cancer cells showed a yellow fluorescence due to the co-staining of EB with AO which indicated the damage of cell membrane. The results were comparable to the standard drug cisplatin. Hence DIM-1 and DIM-4 are capable of changing the integrity of the plasma membrane which is an indication of apoptosis.

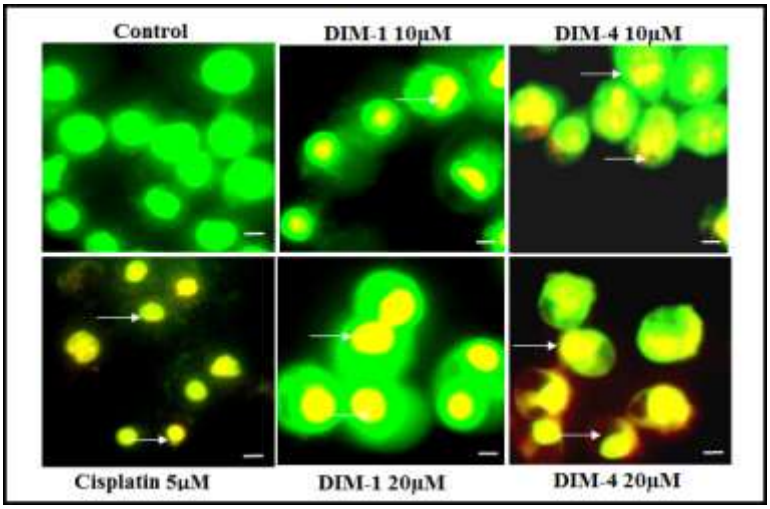

Figure 3.5. Investigation of membrane integrity in HeLa cells by AO/EB staining: *HeLa cells exposed to DIM-1, DIM-4, and cisplatin exhibited yellow fluorescence (EB uptake) which indicates loss of plasma membrane integrity. The control cells showed only green fluorescence indicating an intact plasma membrane. The arrows indicate membrane damage. The scale bar represents a size of 10µm.*

3.3.6. Effects of DIM-1 and DIM-4 on mitochondrial membrane potential

Apoptosis triggering signals cause the depolarization of mitochondria through the release of molecules required for the intrinsic apoptotic pathway. Effects of DIM-1 and DIM-4 on changes in mitochondrial membrane potential was studied by JC-1 staining. The results are depicted in Figure 3.6. A high percentage of red fluorescence in the control cells indicated a negatively charged active mitochondria (JC-1 aggregates give red fluorescence). The red/green fluorescence intensity ratio reduced by almost half in DIM-1 and DIM-4 treated cells to 0.76 and 0.82 respectively ($p \leq 0.05$) from 1.5 in control cells. The results are comparable to that of standard drug cisplatin. On treatment with DIM-1 and DIM-4, a change from red fluorescence intensity to green was observed due to the loss of active mitochondria.

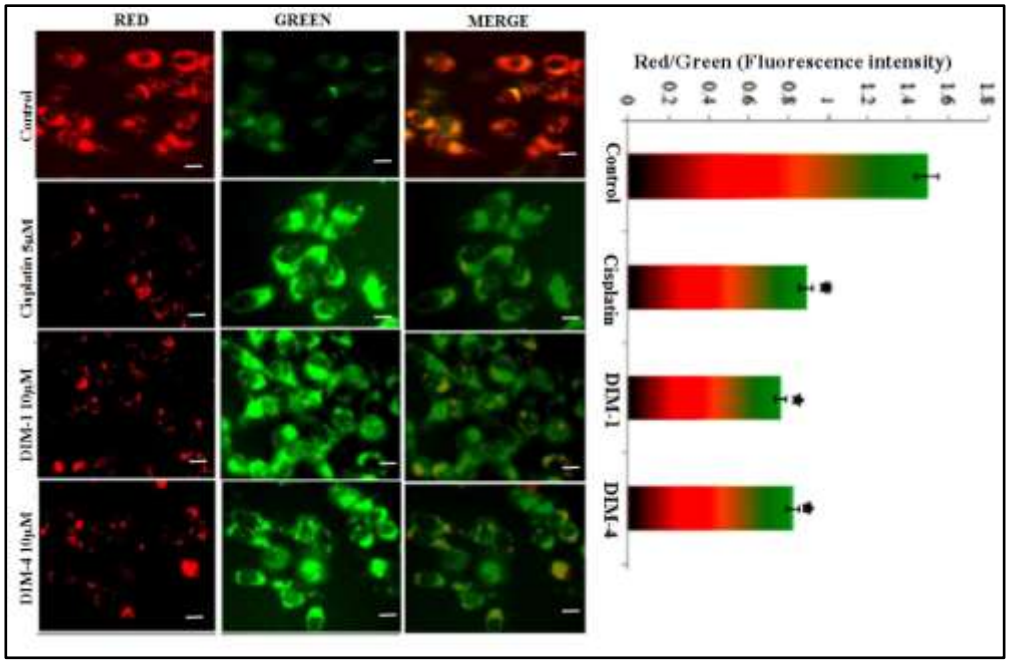

Figure 3.6. Effects of DIM-1 and DIM-4 on mitochondrial membrane potential in HeLa cells: *Fluorescent images of JC-1 stained cells showed reduction in red fluorescence intensity on 24 h DIM treatment, which indicates the loss of mitochondrial membrane potential. The graph shows the reduction of red/green fluorescence intensity in DIM treated cells in comparison to the control cells. The scale bar represents a size of 20 µm. Values are means, with standard deviations represented as vertical bars. * indicates significant difference from control group ($p \leq 0.05$).*

3.3.7. Effects of DIM-1 and DIM-4 in caspase 3 activity and expression of apoptosis related proteins

Activity of caspase 3, and expression of major apoptotic proteins were studied in HeLa cells treated with DIM-1 and DIM-4 for 24 h. Caspase 3 activity was measured by a fluorescent based assay kit as described in the methods. The fluorescence values were compared to determine the variations in caspase 3 activity between control and DIM treated cells and the results are shown in Figure 3.7. The caspase 3 activity of HeLa cells treated with DIM-1 and DIM-4 were found to increase significantly ($p \leq 0.05$) by 3 fold at 10 µM and by about 4 fold at 20µM. The results were comparable to standard drug cisplatin.

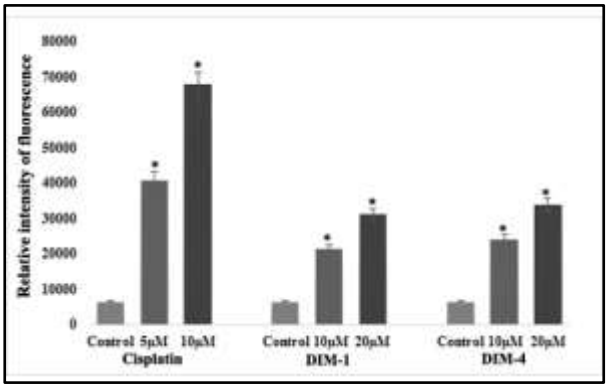

Figure 3.7. Effects of DIM-1 and DIM-4 on caspase 3 activity in HeLa cells: *Caspase 3 activity was determined in the lysates of the control and treated cells using a fluorimetric assay. DIM-1, DIM-4, and cisplatin upregulated caspase 3 activity in HeLa cells. Values are means with standard deviation of three independent experiments. * indicates significant difference from control group ($p \leq 0.05$).*

Western blot studies were employed to detect the expression of major apoptotic proteins namely c-PARP, Bax, and Bcl2. The pro-apoptotic proteins, c-PARP, and Bax when subjected to densitometric analysis and relative density calculation, revealed a notable ($p \leq 0.05$) escalation in protein expression by 1.77 and 1.74 fold on treatment with 20 µM concentration of DIM-1 and DIM-4 respectively when compared to the control cells. DIM-1 and DIM-4 remarkably reduced the expression of antiapoptotic protein Bcl2 in a concentration dependent manner. GAPDH was employed as the house keeping protein for comparing the variations. The results are given in Figure 3.8

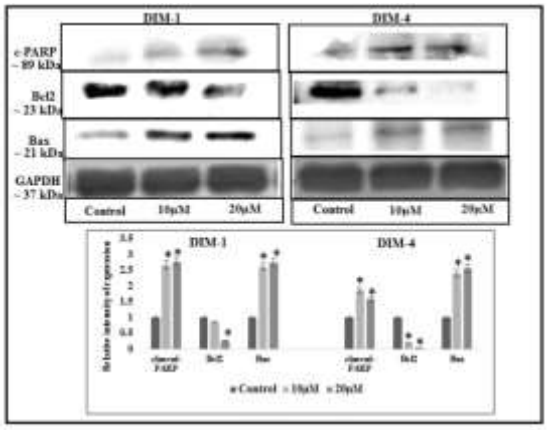

Figure 3.8. Effects of DIM-1 and DIM-4 on apoptotic proteins in HeLa cells: *Variations in protein expression of c-PARP, Bax and Bcl2 was determined by western blot analysis .Western blot was performed in lysate of cells treated with DIM-1 and DIM-4. The densitometric analysis showed upregulation of c-PARP, and Bax, and downregulation of Bcl2 which were also represented in the graphical format. Values represented are means with SD represented by vertical bars.*indicates significant difference from control group ($p \leq 0.05$).*

3.3.8. Effects of DIM-1 and DIM-4 on cell migration and MMP activity

After investigating the role of DIM compounds in apoptosis, its effect on migration was studied by scratch wound assay. The extent of cell migration and hence the wound closure in control cells was remarkable after 24 h, whereas migration in DIM-1, DIM-4 and cisplatin treated cells was significantly reduced and wounds were open even after 24 h. The percentage of wound closure was calculated using Image J software, where the percentage of wound closure in control cells was ~ 91 %, while it was reduced to less than 30% in treated cells. The results are depicted in Figure 3.9.

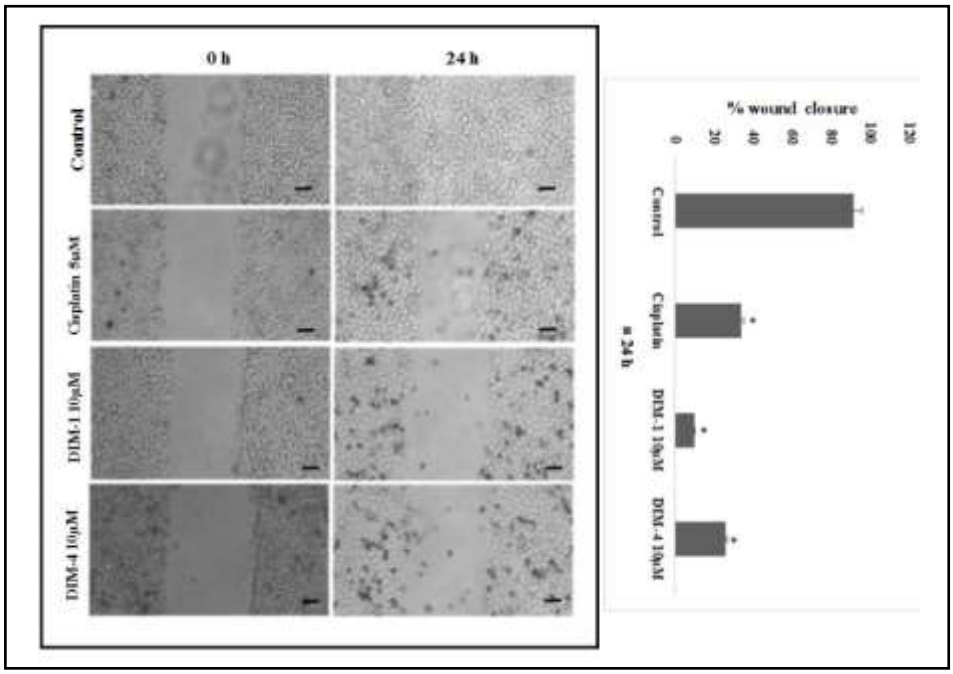

Figure 3.9. Effects of DIM-1 and DIM-4 on migration of HeLa cells: *Scratch wound assay was performed to study the effect of DIM-1 and DIM-4 on migration of HeLa cells. The results clearly showed a decrease in wound closure in treated cells when compared to the control. The graphical representation of the percentage of wound closure also corroborates the fact. The scale bar represents a size of 100 µm. The vertical bars represent SD of values'means.* indicates significant difference from control group ($p \leq 0.05$).*

Studies using gelatin zymography revealed that the activity of matrix degrading enzymes, MMP-2 and MMP-9 was significantly down-regulated on treatment with DIM compounds (10 µM) compared to control cells. DIM- 1 caused the decrease in relative density of MMP-9 to 0.27 and MMP-2 to 0.25 when compared to control, where relative density was taken as 1. The results are given in Figure 3.10. DIM compounds effectively reduced cancer cell migration along with the suppression of migration promoting enzymes.

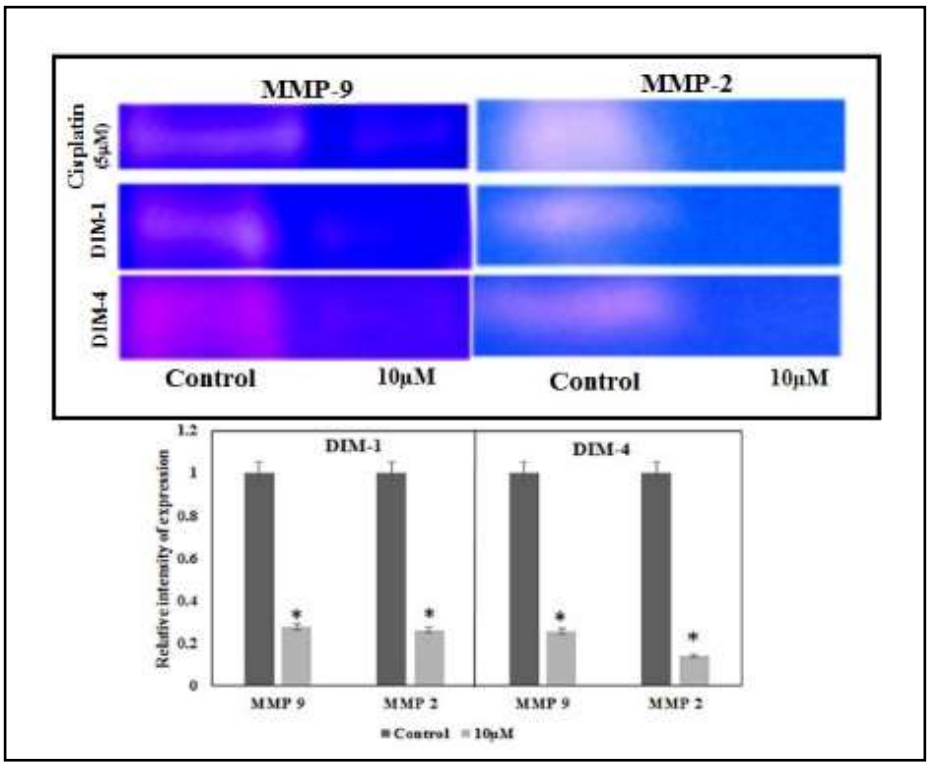

Figure 3.10. Effects of DIM-1 and DIM-4 on MMP-2 and MMP-9 activity: *The gelatinolytic activity of MMP-9 and MMP-2 enzymes, in control and DIM treated cells were determined by zymography. The clear zones represent areas of enzymatic activity. Relative intensity of expression of MMP-2 and MMP-9 is shown in graphical format. The vertical bars represent standard deviation of means. * indicates significant difference from control group ($p \leq 0.05$).*

3.3.9. Effects of DIM-1 and DIM-4 on expression of VEGF and fibronectin

VEGF is a key mediator of angiogenesis, secreted by tumor cells that triggers endothelial cells to form new vasculature to supply nutrients and oxygen for the cancer cells. Fibronectin is an ECM protein that is required for endothelial cell survival and tube formation. The expression of VEGF and fibronectin were studied by western blot. The intensity graphs represented along with each blot shows a reduction in the expression of both angiogenic proteins on treatment with DIM-1 and DIM-4. DIM-1 (20 μM) reduced the intensity of expression for VEGF and fibronectin from 1 (control sample) to 0.54 and 0.37 respectively. DIM-4 reduced the intensity of VEGF and fibronectin expression to 0.51 and 0.46. The results are given in Figure 3.11.

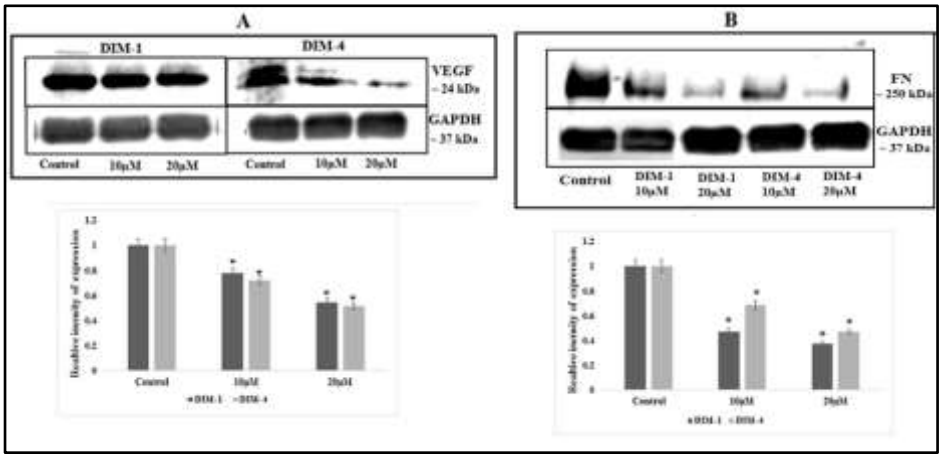

Figure 3.11. Effects of DIM compounds on the expression of VEGF and fibronectin in HeLa cells: *The variations in protein expressions on DIM treatment was investigated in the cell lysates using western blot. (A) The bands represent VEGF expression in control and treated cells (B) The variation in the expression of fibronectin (FN) on DIM -1 and DIM -4 treatment in HeLa cells. The relative intensity graphs for VEGF and fibronectin expression is represented in graphical form. The vertical bars represent SD of the values means. * indicates significant difference of DIM treated samples from control group ($p \leq 0.05$).*

3.3.10. Docking analysis of DIM compounds with VEGF receptors

Since we observed a decreased expression of VEGF on treatment with DIM-1 and DIM-4, we decided to study the interaction of these compounds with VEGF receptors. Docking studies were carried out using Autodock 4.2 and the binding energy signifying the affinity of interaction between VEGF receptors and DIM-1 and DIM-4 was estimated. The best binding conformations between compounds and receptors are shown in Figure 3.12. Autodock has estimated the free binding energy for DIM-1 and DIM-4 with VEGFR1 to be -7.97 and -7.6 respectively. But the free binding energy obtained for DIM compounds with VEGFR2 receptor was considerably higher than VEGFR1 indicating better binding affinity (Table 3.2.).

RECEPTORS	**BINDING ENERGY**	
	DIM-1	DIM-4
VEGFR1	-7.97	-7.60
VEGFR2	-9.15	-8.67

Table 3.2. Binding energy of DIM-1 and DIM-4 with VEGF receptors obtained by Autodock 4.2.

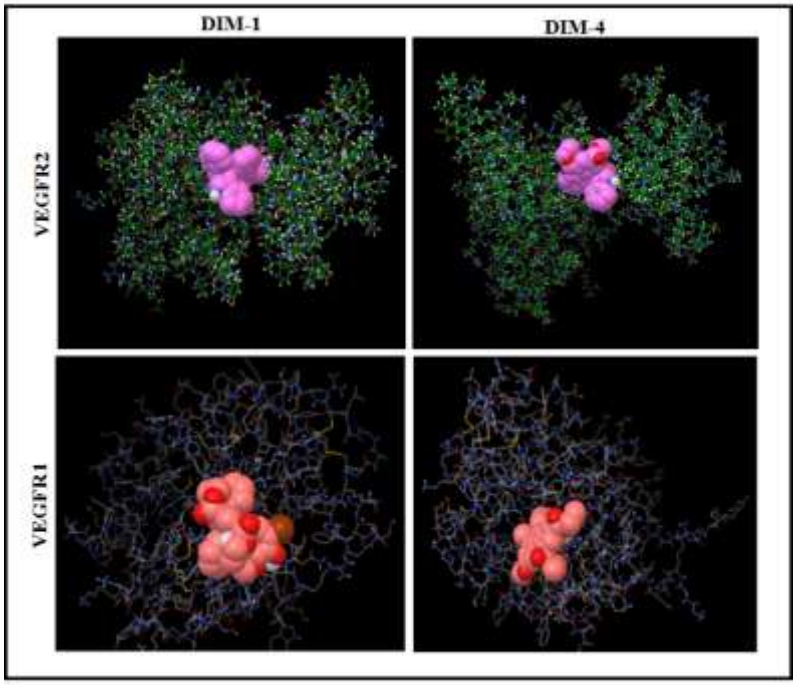

Figure 3.12. Docking of DIM-1 and DIM-4 to VEGF receptors: *The binding affinity of DIM -1 and DIM -4 with VEGF receptors was investigated using Autodock 4.2 .Ligand – protein docking was used to study the interaction of DIM compounds with the receptors. The best binding conformations obtained with each receptor is shown in the figure.*

3.3.11. Effects of DIM-1 and DIM-4 on cell cycle arrest

The role of DIM-1 and DIM-4 in cell cycle progression was analysed by flow cytometry. Figure 3.13. showed the distribution of cells in different phases of cell cycle (Sub G0/G1, G1, S and G2/M) in control as well as in treated conditions. The histogram in the figure represents the distribution of cells in different phases and showed a significant increase in the sub G0/G1 phase along with a decrease in the subsequent G1 phase, in cells treated with DIM-1 and DIM-4 which indicated cell cycle arrest at G1 phase. Positive control cisplatin, induced variations in the cell distribution in all the phases.

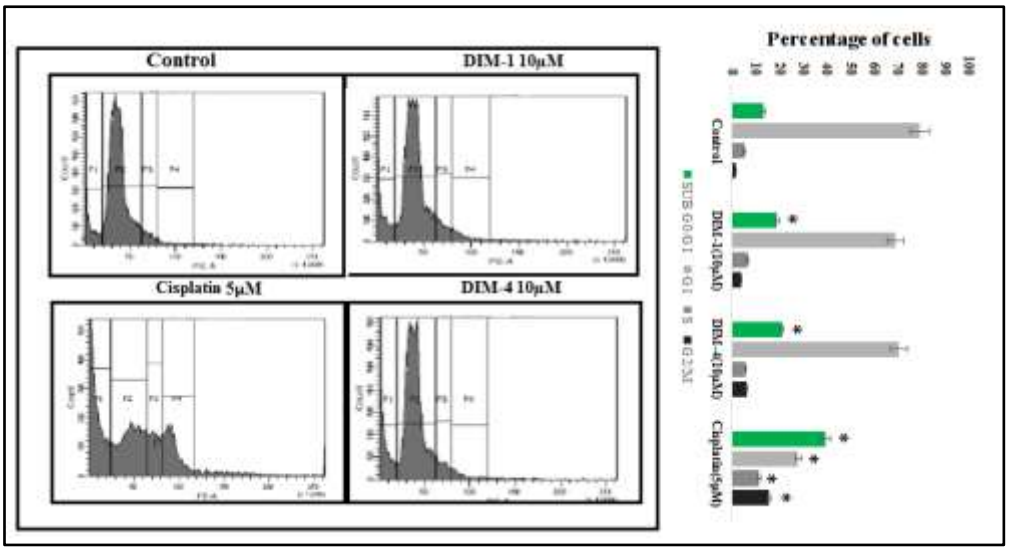

Figure 3.13. The effect of DIM-1 and DIM-4 in cell cycle progression in HeLa cells: *The effects of DIM-1 and DIM-4 on cell cycle progression in HeLa cells was investigated using flow cytometry. The distribution of cells in different phases were identified through DNA content and is represented as a histogram. The bar diagram representing percentage of cells in each phase show a noticeable increase in the sub G0/G1 phase of treated cells compared to the control cells. The vertical bars represent SD of the values means. * indicates significant difference from control group (p≤ 0.05).*

3.3.12. Effects of DIM-1 and DIM-4 on the expression of p53, p38 and NFκB

The effect of DIM-1 and DIM-4 on major cancer regulating proteins were studied by western blotting. p53 and p38 are part of the stress regulator signalling pathway, and have a major role in inducing apoptosis, by triggering either the apoptosis machinery or cell cycle arrest. As evident from the western blot data, DIM compounds upregulated the expression of p53 and pp38 in comparison to the untreated cells. At the same time, NFκB expression was found to be downregulated by DIM compounds, negating the cancer growth promoting role of this protein. So DIM-1 and DIM-4 interfere with the growth of cancer cells by blocking any one of these signalling pathways.

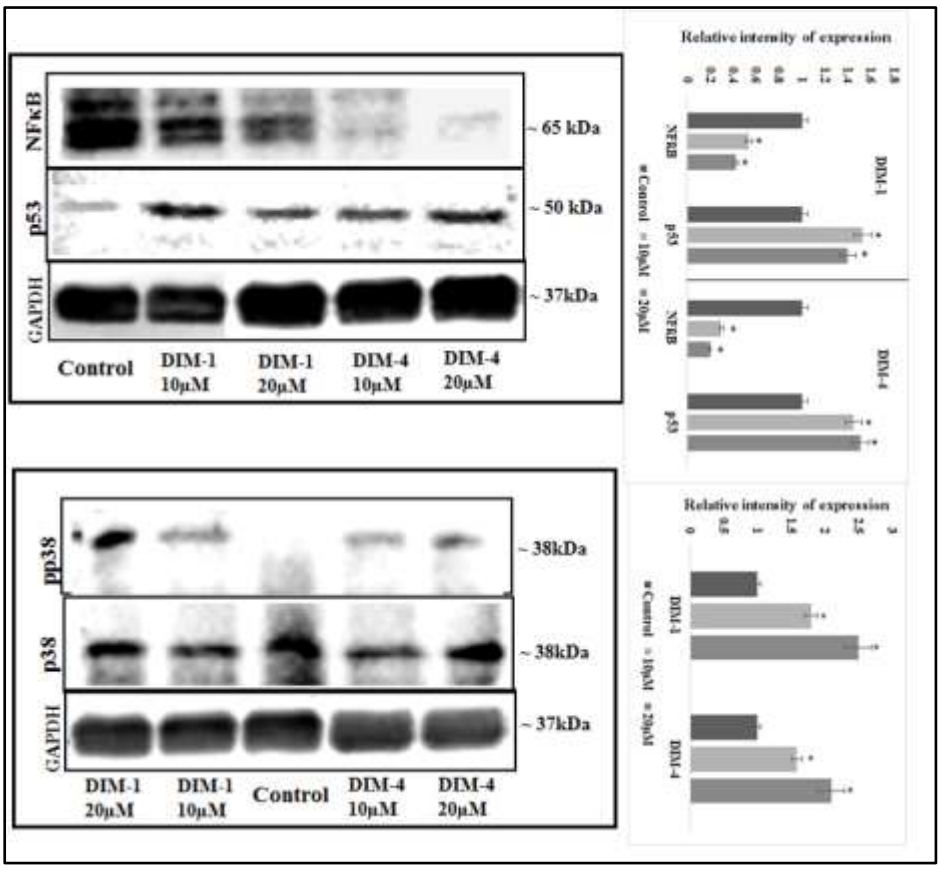

Figure 3.14. Western blot analysis of p53, p38 and NFκB in HeLa cells*: HeLa cells treated with DIM compounds were studied using western blot for variations in the protein expression of p53, p38, pp38, and NFκB. DIM compounds induced the up-regulation of p53and pp38 while downregulating NFκB. The relative intensity of the bands are represented as bar graphs. The vertical bars represent SD of the values means. * indicates significant difference from control group (p≤ 0.05).*

3.4. Discussion

Despite being the focus of extensive research due to its complex and lethal status, cancer still claims lives by millions across the world. The side effects exhibited by current day chemotherapeutics has led to the growing trust and dependence on compounds of natural origin. Among the natural compounds, food products with medicinal properties such as cruciferous vegetables and fruit berries have gained significant attention (Huntley *et al*., 2009; Pledgey-Tracy *et al*., 2007). Many recent studies testify to the role of dietary nutrients in tumor growth attenuation by altering the tumor microenvironment by means of epigenetic alterations (Andreescu *et al*., 2018). The literature presents extensive evidence for the anticancer properties shown by dietary products.

DIM, a bioactive compound from cruciferous vegetables exhibit many promising anticancer properties in multiple cancer cell lines (Zou *et al*., 2018; Draz *et al*., 2017). But due to the poor solubility, absorption, and low potency displayed by un-substituted DIM, modified derivatives of DIM have been extensively studied. Many studies from literature have shown that modified derivatives of DIM exhibited superior potency in cancer cells, compared to natural DIM. The enhancement in activity of DIM was achieved through multiple approaches ranging from structural modifications to delivery systems. Studies carried out by Abdelrahim *et al*. 2006, showed that modified DIM derivatives work through receptor dependant or independent pathways to induce apoptosis in cancer cells. Studies by Draz *et al*. 2017, on DIM and its halogenated compounds, revealed induction of protective autophagy in prostate cancer cells through AEG-1 (oncoprotein) and AMPK. Recent studies show a considerable improvement in the anticancer activity of DIM, achieved through a novel nanobiocomposite delivery system of chitosan and graphene oxide nanoparticles in MCF-7 cells (Deb *et al*., 2018). Knockdown of genes like STAT3, is another strategy that was used to improve the activity of DIM in ovarian cancer, thereby increasing its chemotherapy sensitivity (Zou *et al*., 2018). Recent studies in docetaxel treated breast cancer cells, revealed the importance of DIM as an effective chemo sensitizing agent (Lanza-Jacoby *et al*., 2018). DIM is still a continually investigated drug due to its multi-targeting role in attenuation of cancer. Hence novel synthetic conjugates of DIM and biaryl moiety in an ortho conformation, was investigated for anticancer activity in cervical cancer cells.

Despite the development of novel drug targets, cytotoxic drugs still constitute a major part of the current chemotherapeutic regime (Cozzi *et al*., 2004). Cytotoxicity is the preferred screening standard adopted by pharmaceutical companies to identify potential anticancer compounds. Hence the preliminary screening of DIM compounds namely DIM-1 to DIM-12 for anticancer activity in HeLa cell line was carried out by MTT assay. DIM-1, DIM-4, and DIM-8 induced a concentration dependent growth inhibition in cervical cancer cells with GI_{50} values indicating a high cytotoxic potential. But DIM-8 was deemed unfavourable for further investigations as it induced cytotoxicity in normal H9C2 cells. All the compounds except DIM-1 and DIM-4 were excluded from further investigations due to a high GI_{50} value in cancer cells (low potency) or toxicity towards normal

cells. Cisplatin was selected as the positive control for the study, as it is one of the preferred chemotherapeutic agents currently used for the treatment of cervical cancer (Cadron *et al*., 2007).

In order to investigate the mechanism for cytotoxicity of DIM compounds, the cervical cancer cells were analysed for morphological alterations which revealed the classical signs of an apoptotic cell death. Apoptosis is usually accompanied by standard morphological features such as cell shrinkage, membrane blebbing, margination of nuclear chromatin and apoptotic body formation unlike necrosis which involves cell swelling and bursting (Zhivotovsky, 2004). Hence the cervical cancer cells were further investigated for various indicators of apoptosis such as DNA fragmentation, plasma membrane integrity loss, mitochondrial membrane depolarisation, etc. Nuclear morphology studies revealed that HeLa cells treated with DIM compounds exhibited clear signs of nuclear fragmentation. Nuclear fragmentation was a major apoptotic indicator evaluated as part of the anticancer activity study of a novel nitrone derivative (Mandal *et al*., 2018). To assess plasma membrane integrity and to eliminate necrosis as the cause of cytotoxicity, AO/EB method was employed (Kasibhatla *et al*., 2006). The studies on DIM-1 and DIM-4 showed plasma membrane leakage and a condensed chromatin indicating apoptotic mode of cell death. Natural DIM (3,3'-diindolylmethane) from cruciferous vegetables has been shown to exert mitochondrial mediated apoptosis in prostate cancer cells, and this apoptotic pathway is usually accompanied by mitochondrial membrane depolarisation (Nachshon-Kedmi *et al*., 2004; Kim *et al*., 2005). Recent anticancer studies by Mandal *et al*., 2018 showed that mitochondrial depolarisation is associated with mitochondrial mediated apoptosis. Hence studies on the mitochondrial health of HeLa cells was performed which revealed that DIM compounds induced mitochondrial membrane depolarisation. The assays using phase contrast and fluorescent imaging provided morphological evidences for cell death in HeLa cells exposed to DIM compounds, hence a few major biochemical markers of apoptosis namely caspase 3, Bax, Bcl2 and c-PARP were investigated with respect to DIM treatment. Caspase 3 activity and Bax expression increases during apoptosis while cleavage by caspase down regulates PARP and Bcl2 (Kirsch *et al*., 1999; Mandal *et al*., 2018). The increase in Bax/Bcl2 ratio itself, can lead to mitochondrial depolarization, release of cytochrome c and subsequent apoptosis induction (Elmore, 2007). The results indicated that DIM-1 and DIM-4

upregulated caspase 3 activity, Bax and c-PARP while downregulating the expression of antiapoptotic Bcl2. The evidence of mitochondrial depolarization along with the change in Bax/Bcl2 ratio observed from western blot points to the activation of intrinsic pathway of apoptosis. Hence the current studies showed that HeLa cells treated with DIM compounds exhibited morphological and biochemical changes associated with apoptosis induction.

Many cytotoxic anticancer compounds currently in use, exhibit anti tumor activity through anti-angiogenesis. Anti-angiogenesis has become the focus of study for controlling cancer recurrence and metastasis. Natural dietary compounds like ursolic acid (terpenoid) and quercetin (flavanoid) are gaining precedence as effective anticancer molecules for their anti-angiogenic and anti-metastatic potential (Kashyap *et al*., 2018). Malignant cells possess the ability to invade and metastasize in addition to uncontrolled proliferation. Scratch wound assay revealed that the migration of HeLa cells was significantly reduced by the DIM compounds. A key enzyme aiding the migration and invasion of cancer cells by ECM degradation are MMP. Elevated levels of MMP-2 and MMP-9 activity in several types of cancer indicate poor prognosis (Roomi *et al*., 2009). Hence the role of DIM compounds were studied with regard to the expression of MMP-2 and MMP-9 and a reduction in the activity of MMP enzymes was observed. This also supports the abrogation of migration in cervical cancer cells. DIM compounds also showed anti angiogenic potential by downregulating the expression of two major pro-angiogenic factors, VEGF and fibronectin. Fibronectin and VEGF are often seen upregulated in several types of tumor cells and promotes vasculature growth near tumors (Goel *et al*., 2014; Wang *et al*., 2017). Docking results showed that DIM compounds showed a better binding affinity towards VEGFR2, which is a receptor closely associated with cancer associated angiogenesis (Smith *et al*., 2010). Recent studies on zerumbone a phytochemical from *Zingiber zerumbet* showed that its anticancer effect was based on its anti angiogenic activity through the inhibition *of VEGF, MMP-9* and *VEGFR* expressions *(Samad et al., 2017).* In conclusion, DIM compounds inhibited key angiogenic markers namely MMP-2, MMP-9, fibronectin, and VEGF in cervical cancer cells.

Cancer is the result of uncontrolled multiplication of abnormal cells that can evade the proliferation checkpoints during cell division. Literature shows that natural DIM induces G1 phase arrest in breast cancer cells accompanied by up-

regulation of p21 expression (Hong *et al*., 2002). Results showed that DIM compounds caused an elevation in the cell number of sub G0/G1 phase along with reduction in the percentage of cells in G1 phase which indicated growth arrest at G1 phase. Recent studies showed that, the anticancer activity of formononetin (isoflavone) against ovarian cancer cells was due to cell cycle arrest at sub G0/G1 phase (Park *et al*., 2018). Studies on the well characterized metabolite 1,8-Cineole showed that upregulation of p53 expression accompanied apoptosis induction and cell cycle arrest (Sampath *et al*., 2018). Hence the possible involvement of p53 in inducing apoptosis and cell cycle arrest in cervical cancer cells were investigated by western blot. A noticeable upregulation in p53 expression consistent with literature was seen in HeLa cells treated with DIM compounds.

Many pathways are frequently found deregulated in cancer cells and such pathways are targets for anticancer drugs. To cite an example, alantolactone is a sesquiterpene which induced apoptosis by NF-κB inhibition and p38 MAPK activation (Liu *et al*., 2018). NF-κB signaling is activated by many carcinogens and is responsible for chemoresistance in many cancer cells. The activity of many anti-cancer drugs, is achieved through the activation of MAPK pathway. MAPK pathways are seen to interact with other pathways, for instance NF-κB is a downstream target of p38 MAPK. And p38 MAPK also has a major role in the activation of Bax translocated to the mitochondria and can interact with p53 to trigger apoptosis or induce cell cycle arrest (Kim *et al*., 2002; Mayr *et al*., 2002). In addition to this, natural DIM was found to be an effective inactivator of NF-κB signaling in breast cancer cells (Rahman *et al*., 2007). So the expression of these major signaling molecules were studied in HeLa cells. DIM compounds increased the phosphorylation of p38MAPK while the downstream target NF-κB was downregulated. But the pro-apoptotic factor p53 was upregulated signifying the ability of the compounds (DIM-1 and DIM-4) to interact with multiple signaling pathways to mitigate cancer growth.

3.5. Conclusion

The novel synthetic compounds DIM-1 and DIM-4 show promising chemotherapeutic activity against human cervical cancer cells. They are selectively cytotoxic towards cancer cells. They attenuate cancer cell growth by inducing apoptosis and cell cycle arrest. They also demonstrate anti-migration and anti-

angiogenic properties. The novel DIM compounds target multiple hallmarks of cancer making them strong anticancer agents. Figure 3.15. represents the possible mechanism of action through which the compounds effect anticancer activity.

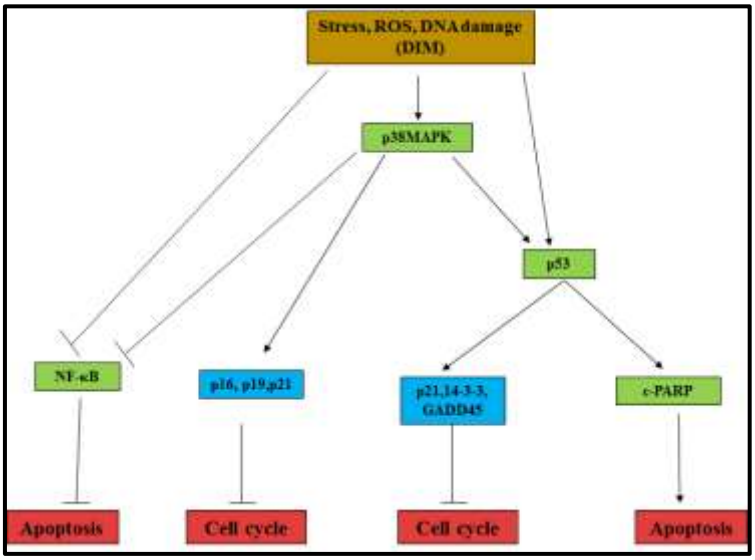

Figure 3.15. Signalling pathways modulated by ortho-biaryl-DIM compounds in HeLa cells.

3.6. References

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Chapter 4

Anticancer activity studies of Biaryl DIM derivatives in human breast cancer cells

4.1. Introduction

Breast cancer is the most frequently diagnosed cancer in women across the world as well as the foremost incident cancer in many regions in the world (Jemal *et al*., 2011; Boyle e*t al*., 2008). According to the estimations of GLOBOCAN in 2010, breast cancer accounts for 1.4 million new cases every year across the globe (Ferlay e*t al*., 2010). Recent estimates by GLOBOCAN in 2018, showed that breast cancer is the second most commonly diagnosed cancer, amounting to almost 11.6% new cases annually (Bray *et al*., 2018).

The survival rate of breast cancer patients has improved considerably with better diagnosis and understanding of the disease. Based on molecular and clinical heterogeneity, breast cancer is divided into five distinct subtypes namely, luminal A, luminal B, HER2 overexpressing, basal-like breast cancer, and normal like breast cancer (Sorlie *et al*., 2003). Receptor status has proved to be a very useful marker, for better diagnosis and treatment of breast cancer (Hon *et al*., 2016). Triple-negative breast cancer (TNBC), which lack estrogen receptor (ER), progesterone receptor (PR), and overexpression of HER2 receptors, can only be treated using toxic chemotherapeutic agents (Kumar *et al*., 2016; Hon *et al*., 2016). TNBC is reported to have a high chance of recurrence and a poor overall survival rate and many studies have shown that chemotherapeutic agents were capable of alleviating these rates (Berry *et al*., 2006; Carey *et al*. 2007).

Anthracycline/taxane based therapy are the currently implemented strategies for breast cancer including TNBC. Studies conducted by Huober *et al*. 2010, showed a high pCR (pathologic complete response) rate which was achieved through anthracycline-taxane based neoadjuvant chemotherapy. Since TNBC has also been associated with BRCA1/BRCA2 mutations, use of DNA crosslinking platinum drugs (cisplatin and carboplatin) and PARP inhibitors have been considered (Huang *et al*., 2017; Metzger-Filho *et al*., 2012). The need for novel targeted therapy to alleviate the poor survival rate in advanced cases of TNBC has led to the

investigation of various molecular targets such as VEGF, PARP, EGFR, PI3K, AKT, and mTOR (Massihnia *et al*., 2016). EGFR, cKIT, cytokeratins 5/6, 14 and 17, p53, p15 and cyclin E are a few of the proteins found overexpressed in TNBC, and hence viewed as possible targets (Nielson *et al*., 2004). TNBC is a highly proliferative cancer that needs constant angiogenesis and hence many angiogenesis inhibitors such as VEGF inhibitors (bevacizumab) and VEGFR inhibitors (sorafenib) have been investigated in clinical trials. (Amos *et al*., 2012; Kumar *et al*., 2016). EGFR inhibitors (cetuximab and erlotinib) and PI3K inhibitors (buparlisib) have been investigated in multiple clinical trials for TNBC (clinicaltrials.gov) (Kumar *et al*., 2016). Loss of PTEN activation in TNBC leads to mTOR activation and hence mTOR inhibitors such as everolimus are under clinical trial in combination with other drugs (clinicaltrials.gov NCT00930930). The molecular targets of TNBC belong to multiple signalling pathways and are currently being investigated in clinical trials.

DIM is the major bioactive metabolite of I3C, from cruciferous vegetables that possesses a wide range of anticancer properties. Thomson *et al*. 2016, describes in detail the specific efficacy of DIM in regulating the development of breast cancer at different stages namely initiation, promotion, progression, and invasion. Hence, DIM exhibits all the attributes needed for the successful attenuation of TNBC. For instance in studies by Bhowmik *et al*. 2013, DIM compounds induced apoptosis in breast cancer cells (including TNBC) by inhibiting the EGFR pathway, while DIM inhibited AKT activation in MDA-MB-231 cells in studies by Nicastro *et al*. 2013. In another study, mTOR and AKT which are signalling moecules that have a major role in TNBC survival and recurrence were inhibited by DIM (Kong *et al*., 2008). Studies by Banerjee *et al*., 2007 and Gong *et al*. 2006, demonstrated that the anticancer activity of DIM in human breast cancer cells involved the release of ROS from mitochondria, cell cycle arrest, and apoptosis. Various modified derivatives of DIM like C-DIM also have been found to exhibit potential activity against multiple cancer cells including TNBC with reduced toxicity. *In vivo* studies involving lung tumors in mice, demonstrated a remarkable regression in markers of metastasis and angiogenesis (MMP-2, MMP-9, EGFR, VEGF) on exposure to C-DIM derivatives (Andey *et al*., 2013). Recent studies on NLC formulations of novel DIM derivatives revealed enhanced anticancer effects against TNBC (Godugu *et al*., 2016). A synthetic DIM derivative phemindole, caused ROS

mediated mitochondrial apoptosis, and ER-stress induced apoptosis in TNBC (Chakraborty *et al.*, 2016). All these studies indicated that DIM and its derivatives are capable of targeting signaling molecules and pathways that are found deregulated in TNBC, and hence are potential candidates for this study.

The current chapter investigated the novel, synthetic, *ortho*-biaryl-DIM derivatives for anticancer activity in TNBC cell line, MDA-MB-231. The compounds were initially screened for cytotoxicity, followed by further studies to understand the mechanism of action of the compounds. The recognised targets of natural DIM and the molecular targets for TNBC treatment were considered for the design of the study.

4.2. Methods

The novel biaryl DIM derivatives were investigated for anticancer activity in TNBC cell line, MDA-MB-231. Initially, the 12 DIM compounds (structures given in chapter 3) were checked for cytotoxicity in breast cancer cells and normal cells H9C2 using MTT assay. Paclitaxel was used as the positive control for comparison. Besides the methods employed in chapter 3, additional methodologies employed to investigate the anticancer activity of DIM compounds in breast cancer cells are mentioned.

- Phosphatidylserine translocation detected by Annexin V staining.
- Caspase 3 and caspase 9 activity assay.
- Western blotting analysis of cleaved-PARP, Bax, Bcl2 and cytochrome c.
- Generation of ROS was investigated using DCFDA staining method.
- Glucose uptake analysis was investigated by flow cytometry.
- Western blot analysis of proteins belonging to PI3K/AKT/mTOR pathway and FAK/RAS/p53 pathway was carried out.

4.3. Results

4.3.1. Cytotoxicity evaluation of DIM compounds in MDA-MB-231 cells

The cytotoxic effects of DIM compounds in TNBC, was studied by MTT assay. The standard of comparison for cytotoxic potential used was GI_{50} or the concentration at which 50% of growth inhibition was achieved. MDA-MB-231 cells were treated with different concentrations (2, 5, 10, 20, and 50 µM) of DIM

compounds for 24 h, and the GI_{50} for each compound was estimated and summed up in Table 4.1. The results indicated that DIM-1, DIM-4 and DIM-8 showed excellent growth inhibition potential with GI_{50} below 10 µM, while the remaining compounds exhibited values above 20 µM. The inhibition of the conjugates was also checked in H9C2 cells, which revealed that DIM-1and DIM-4 were non-toxic at the effective concentration, whereas DIM-8 was toxic making it unfavourable for further investigations. Hence the parent compound DIM-1 and its derivative DIM-4 were selected for detailed studies.

S.No	Compounds	GI_{50} (µM)	
		MDA-MB-231	H9C2
1	**DIM-1**	**9.8±0.219**	**>50**
2	DIM-2	>20	>50
3	DIM-3	>20	>50
4	**DIM-4**	**8.7±0.523**	**>50**
5	DIM-5	>20	>50
6	DIM-6	>20	>50
7	DIM-7	>20	>50
8	**DIM-8**	**8.5±0.727**	**10.00±0.265**
9	DIM-9	>20	>50
10	DIM-10	>20	>50
11	DIM-11	>20	>50
12	DIM-12	>20	>50

Table 4.1. Cytotoxicity evaluation of DIM compounds in MDA-MB-231 cells: *MDA-MB-231 cells were treated with different concentrations of compounds (DIM-1 to DIM-12) for 24 h and the cytotoxicity was evaluated using MTT assay. GI_{50} value of each compound in breast cancer and normal cells is depicted. DIM-1, and DIM-4 showed selective toxicity towards cancer cells with GI_{50} values below 10 µM, while DIM-8 induced toxicity in both cancer and normal cells.Values represented are means, with standard deviations represented as ±.*

The concentration dependent inhibition of MDA-MB-231 cells by DIM-1 (GI_{50} 9.80±0.219 µM) and DIM-4 (GI_{50} 8.70±0.523 µM) is shown in Figure 4.1. It also depicts the nontoxic nature of both compounds in normal H9C2 cell line. Paclitaxel the positive control was toxic towards both normal and cancer cells.

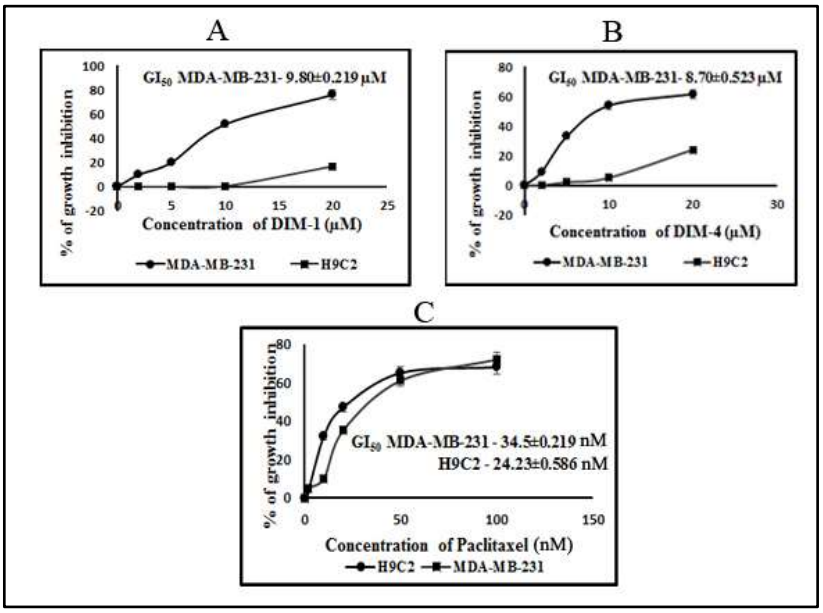

Figure 4.1. Cytotoxicity induced by DIM-1, DIM-4, and paclitaxel in MDA-MB-231 ad H9C2 cells: *The dose response curves of DIM-1, DIM-4, and paclitaxel in MDA-MB-231 and H9C2 cells are represented in this figure. (A) DIM-1 induced a concentration dependent inhibition of breast cancer cells with a GI_{50} value of 9.80±0.219 µM. DIM-1 was non-toxic towards normal cells at the GI_{50} range. (B) DIM-4 induced, growth inhibition in cancer cells (GI_{50} 8.70±0.523 µM), while it was nontoxic towards normal cells. (C) Paclitaxel induced toxicity in normal and cancer cells. Data represented are values± standard deviation (SD) of three independent experiments ($p \leq 0.05$).*

4.3.2. Effects of DIM-1 and DIM-4 in inducing morphological alterations

Apoptosis induces significant alterations in the morphology of cells. The morphological changes induced by DIM-1, DIM-4, and paclitaxel in MDA-MB-231 and H9C2 cells were analysed using phase contrast microscopy. The concentrations for DIM-1 and DIM-4 (5, 10 and 20 µM) and paclitaxel (50 nM) were selected based on the GI_{50} values obtained in the cytotoxicity studies. The control cells showed normal spindle morphology with an intact membrane structure while cells treated with the compounds showed a significantly altered rounded appearance, cell shrinkage, and a marked reduction in the number of cells. At higher concentrations (20 µM) of DIM compounds, signs of membrane blebbing and formation of apoptotic bodies was observed. The morphology of normal H9C2 cells does not show any significant alterations even at higher concentrations. The standard drug paclitaxel induced toxicity in both MDA-MB-231 and H9C2 cells. The results are given in Figure 4.2. The morphological alterations induced by the DIM-1 and DIM-4 in MDA-MB-231 cells suggested apoptosis as the cause for cytotoxicity.

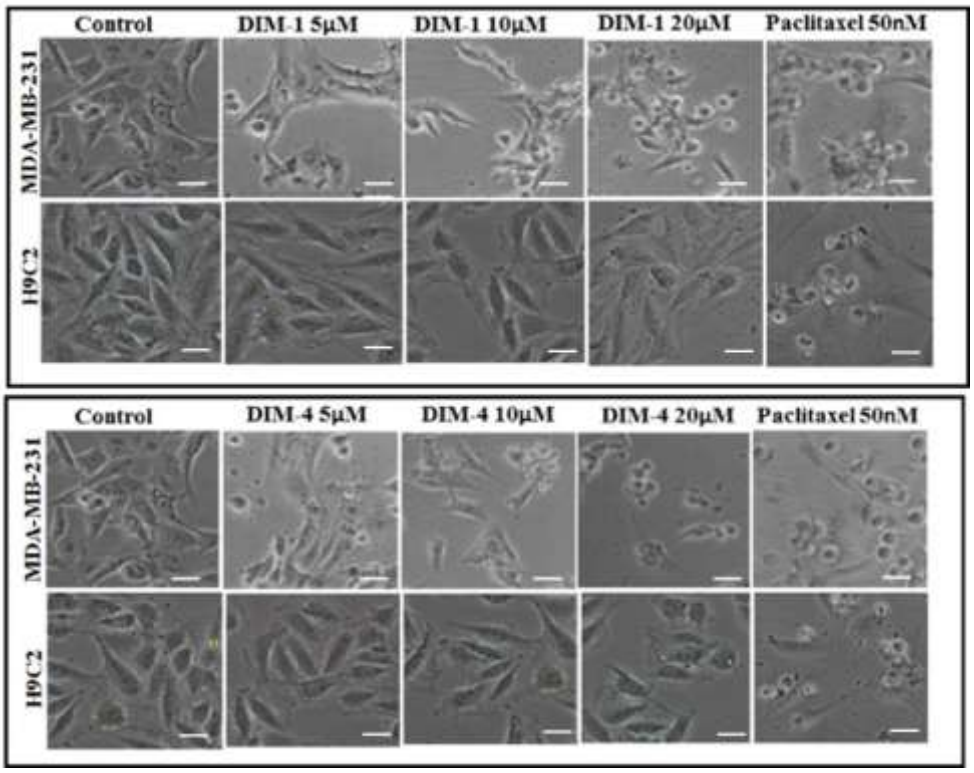

Figure 4.2. Morphology of MDA-MB-231 and H9C2 cells treated with DIM-1 and DIM-4: *Cells were treated with different concentrations (10 µM and 20 µM) DIM compounds for 24 h, and analysed for morphological changes using phase contrast microscopy. (A) DIM-1, and (B) DIM-4, induced significant morphological changes in MDA-MB-231 cells, such as cell shrinkage, blebbing and apoptotic body formation, without affecting the morphology of H9C2 cells. Paclitaxel induced toxic morphological changes in both normal and cancer cells. Scale bar represents a size of 20 µm.*

4.3.3. Effects of DIM-1 and DIM-4 on nuclear fragmentation and plasma membrane integrity

Nuclear fragmentation and membrane leakage are major indicators of apoptotic cell death, and therefore nuclear fragmentation and membrane leakage was studied in MDA-MB-231 cells employing DAPI and AO/EB staining respectively. Breast cancer cells exposed to 10 µM and 20 µM of DIM-1 and DIM-4 showed clear signs of nuclear damage when compared to the intact nucleus of the control cells. Results given in Figure 4.3., indicated that DIM-1 and DIM-4 induced significant nuclear fragmentation in the breast cancer cells, while the control cells showed firm and undamaged nucleus. The positive control paclitaxel also induced significant nuclear fragmentation. Hence the results indicated that DIM-1 and DIM-4 induced nuclear fragmentation in breast cancer cells, which is an indication of apoptosis

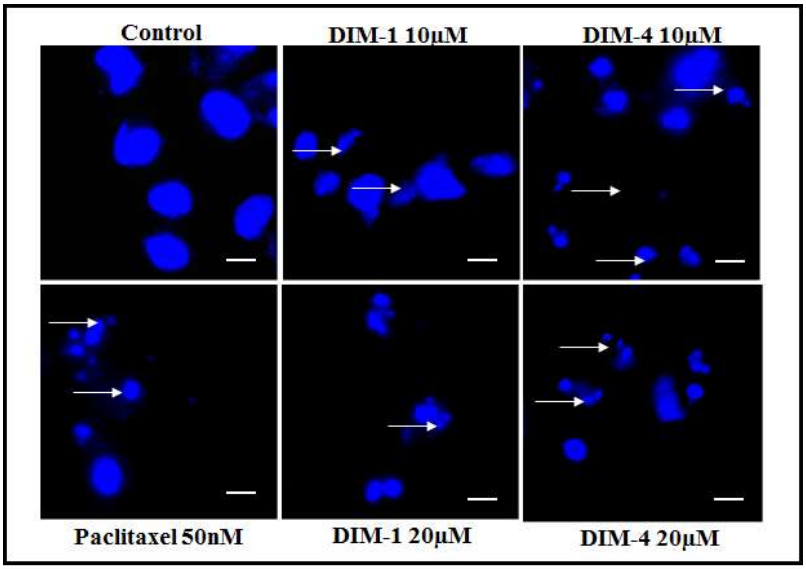

***Figure 4.3. Analysis of DNA fragmentation in MDA-MB-231 cells by DAPI staining**: MDA-MB-231 cells treated with two different concentrations (10 µM and 20 µM) of DIM-1, DIM-4 and paclitaxel (50 nM) showed clear signs of nuclear damage while the control cells exhibited an intact nuclear structure. The arrows indicate nuclear fragmentation and the scale bar represents a size of 10 µm.*

Normal healthy cells have an intact plasma membrane, but during apoptosis the plasma membrane integrity is compromised. AO/EB staining was employed to study the changes in plasma membrane integrity in MDA-MB-231 cells treated with effective concentrations (10 and 20 µM) of DIM-1 and DIM-4. The treated cells showed yellow fluorescence due to co-staining with AO and EB which signified the loss of plasma membrane integrity. The positive control paclitaxel also induced similar effects. Therefore DIM compounds induced loss of plasma membrane integrity in breast cancer cells, which also indicates apoptosis induction.

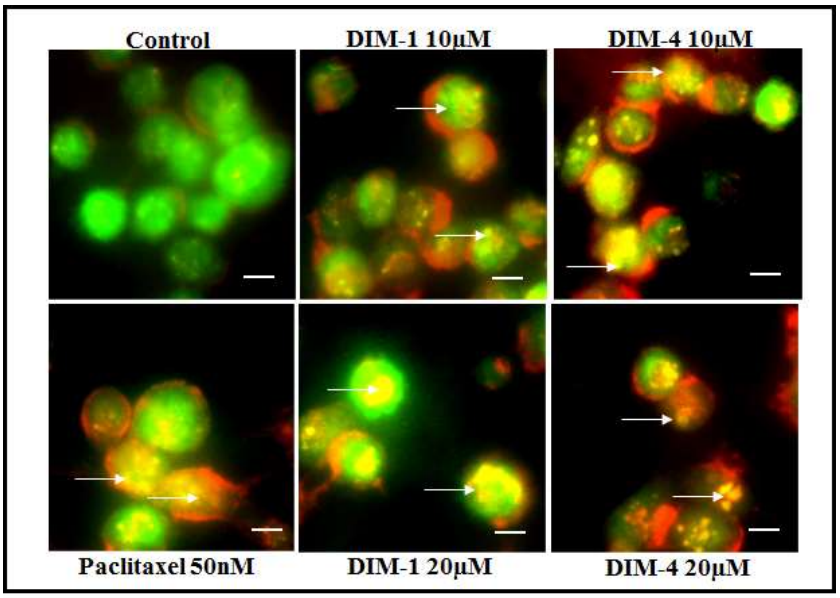

Figure 4.4. Membrane integrity studies in MDA-MB-231 cells by AO/EB staining*: MDA-MB-231 cells exposed to two different concentrations (10 µM and 20 µM) of DIM-1, DIM-4, and paclitaxel(50 nM) exhibited yellow fluorescence (EB co-staining with AO)) which indicates loss of plasma membrane integrity, while the control cells showed only green fluorescence (AO uptake) indicating an intact plasma membrane. The arrows indicate the cells with membrane damage. The scale bar represents a size of 10µm.*

4.3.4. Effects of DIM-1 and DIM-4 on phosphatidyl serine translocation

A reliable indicator of apoptosis is the translocation of anionic phosphatidyl serine (PS) to the outer leaflet of the plasma membrane which is an "eat me" signal to the phagocytes for the removal of post apoptotic debris. Annexin V stain was employed for the detection of PS. Annexin V staining was performed in cells treated with two different concentrations (10 and 20 µM) of DIM-1, DIM-4, and paclitaxel (50 nM) for 24 h. The green fluorescence in control cells is due to the uptake of 6CF while the lack of red fluorescence indicated the absence of PS translocation. But in the cells treated with DIM compounds and paclitaxel, scattered red fluorescence in the outer cell membrane due to Annexin V staining was observed. The results are given in Figure 4.5. Thus DIM compounds induced positive annexin staining in MDA-MB-231 cells indicating apoptosis induction.

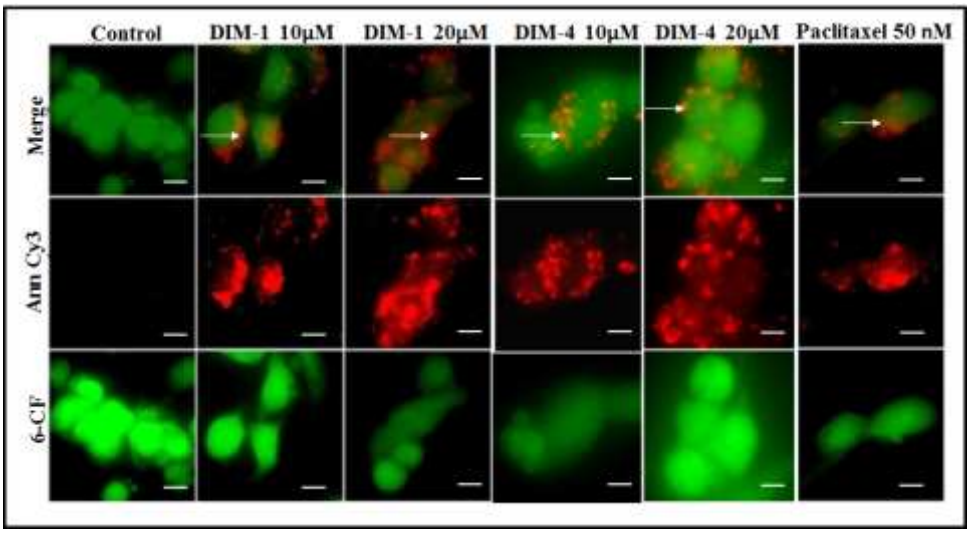

Figure 4.5. Annexin V/CY3 staining of MDA-MB-231 cells treated with DIM-1 and DIM-4: *MDA-MB-231 cells exposed to two different concentrations of DIM -1, DIM-4 and paclitaxel were stained Annexin V/CY3 stain to detect PS translocation associated with apoptosis. The control cells only exhibited green fluorescence due to the uptake of 6-CFDA, but cells treated with DIM-1, DIM-4, and paclitaxel showed scattered red fluorescence on the outer membrane which is an indication of PS translocation along with green fluorescence. The arrows point out positive annexin staining and the scale bar represents a size of 10 µm.*

4.3.5. Effects of DIM-1 and DIM-4 on caspase 3 and caspase 9 activity

Caspase 3 is the terminal effector caspase and caspase 9 is the executioner caspase in the intrinsic apoptotic pathway. Caspase 3 and 9 activities in MDA-MB-231 cells treated with DIM-1, DIM-4 and paclitaxel were determined by fluorescence based assay as given in materials and methods section (Chapter 2). The results are given in Figure 4.6. The results indicated that the treatment of MDA-MB-231 cells with DIM-1 induced an increase in caspase 3 activity by 1.18 (10 µM) and 1.41 (20 µM) fold while DIM-4 increased the activity by 1.3 (10 µM) and 1.45 (20 µM) fold ($p \leq 0.05$). Upon treatment, caspase 9 activity gave a fold increase of 1.54 (10 µM) and 1.82 (20 µM) in DIM-1 treated cells while DIM-4 treated cells exhibited an increase by 1.61(10 µM) and 2.27 (10 µM) fold ($p \leq 0.05$), when compared to the control cells. Therefore DIM-1 and DIM-4 upregulated the caspase 3 and caspase 9 activity in MDA-MB-231 cells, which suggested induction of intrinsic apoptotic cell death.

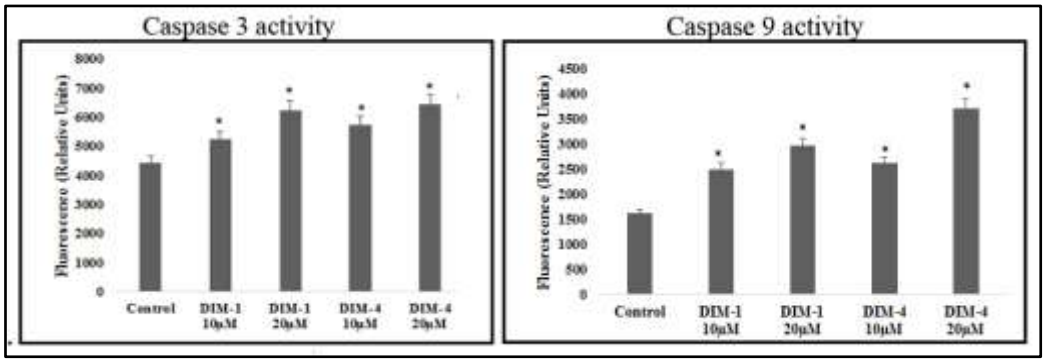

Figure 4.6. Effect of DIM-1 and DIM-4 on caspase 3 and caspase 9 activity in MDA-MB-231 cells: *Caspase 3 and caspase 9 activity in MDA-MB-231 cells treated with DIM-1 and DIM-4 were estimated using a fluorimetric assay. DIM compounds upregulated caspase 3 and caspase 9 activity. Values are means with SD of three independent experiments. * denotes a significant difference from control group ($p \leq 0.05$).*

4.3.6. Effects of DIM-1 and DIM-4 on the expression of apoptosis related proteins

In order to present biochemical substantiation for apoptosis, key proteins involved in the pathway was investigated by western blot analysis. Proapoptotic Bax expression which is essential for apoptosis was found to be significantly upregulated by 2.8 and 3.9 fold DIM-1 and DIM-4 treatment (20 µM) respectively ($p \leq 0.05$). The relative intensity of Bcl2 which is antiapoptotic in nature was found significantly reduced ($p \leq 0.05$) to 0.14 and 0.24 from 1 (control) on treatment with 20 µM of DIM-1 and DIM-4 respectively. Expression of cleaved PARP, a product of caspase 3 cleavage was also substantially upregulated by 2.52 and 2.4 fold by DIM-1 ad DIM-4 respectively ($p \leq 0.05$). Cytoplasmic cytochrome c was upregulated significantly ($p \leq 0.05$) by 1.9 and 1.7 fold by DIM-1 ad DIM-4 respectively. The western blot results are given in Figure 4.7.

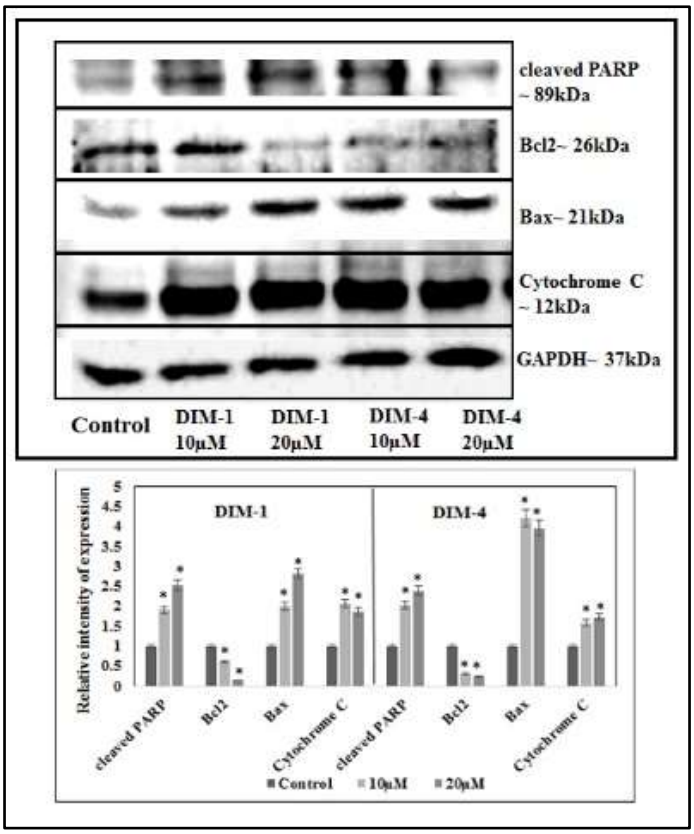

Figure 4.7. Western blot analysis of apoptotic proteins in MDA-MB-231 cells: *Western blot analysis was employed to study the variations in protein expression of c-PARP, Bax, Bcl2, and cytochrome c in MDA-MB-231 cells treated with DIM-1and DIM-4. The expression of c-PARP, Bax and cytochrome c was significantly upregulated and Bcl2 was downregulated in cells treated with the DIM compounds. Values represented are means with SD represented by vertical bars.*indicates a significant difference from control group (p≤ 0.05).*

4.3.7. Effects of DIM-1 and DIM-4 in ROS generation in MDA-MB-231 cells

Mitochondria is the primary target for ROS species, leading to oxidation of MOMP **(**mitochondrial outer membrane permeabilization), therefore the ROS generation status of treated cells were investigated using DCFDA method using fluorescent imaging. Figure 4.8. plainly shows the increase in fluorescence in DIM treated cells compared to the control cells. The fluorescence is a direct indicator of the ROS activity. The graphical representation of the fluorescence depicts an increase of fluorescence in the treated cells by more than two fold when compared to the control cells. To be specific, the DIM-1 treated cells showed a fold increase of ROS activity by 2.1 (10 µM) and 2.7 (20 µM) while DIM-2 treated cells depicted a fold increase of 2.1 (10 µM) and 2.9 (20 µM) ($p \leq 0.05$). Paclitaxel also induced an increase in ROS activity.

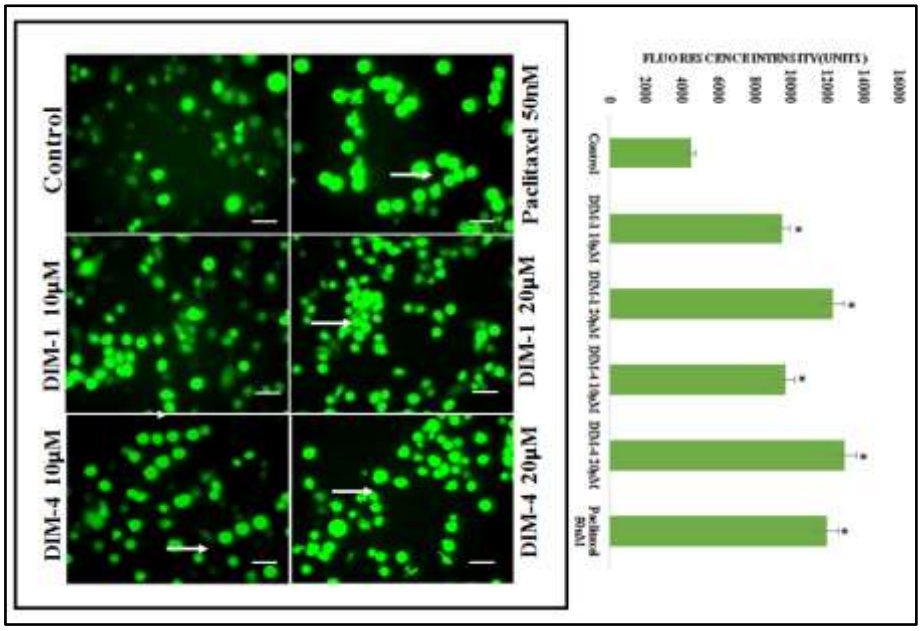

Figure 4.8. DCFDA staining to detect ROS generation in MDA-MB-231 cells: *Cells were treated with two different concentrations of DIM-1, DIM-4 (10 µM and 20 µM), and paclitaxel (50 nM) and stained with DCFDA to investigate ROS activity. The images and intensity graph show the increase in fluorescence in DIM treated cells compared to the control cells. The scale bar represents a size of 50 µm and the arrows indicate the increase in fluorescence compared to the control cells. Values are means, with SD represented by vertical bars. * indicates a significant change from the control cells ($p \leq 0.05$).*

4.3.8. Effects of DIM-1 and DIM-4 on the migration of MDA-MB-231 cells

Cancer has the ability to invade and metastasize to distant location and form secondary tumors and the migration of cancer cells are aided by many factors. The effect of DIM-1 and DIM-4 on the migration of MDA-MB-231 cells, was studied by scratch wound assay. The introduced wound was found to be noticeably reduced on exposure with 10 µM of DIM-1 and DIM-4. The phase contrast images and the graphical representation of percentage of wound closure are given in Figure 4.9. The percentage of wound closure for control cells were 74% and 96% at 24 and 48 h respectively. The wound closure observed at 24 h was drastically reduced to 21% and 17% for DIM-1 and DIM-4 respectively. So DIM-1 and DIM-4 effectively reduced the migration of breast cancer cells, similar to that of paclitaxel.

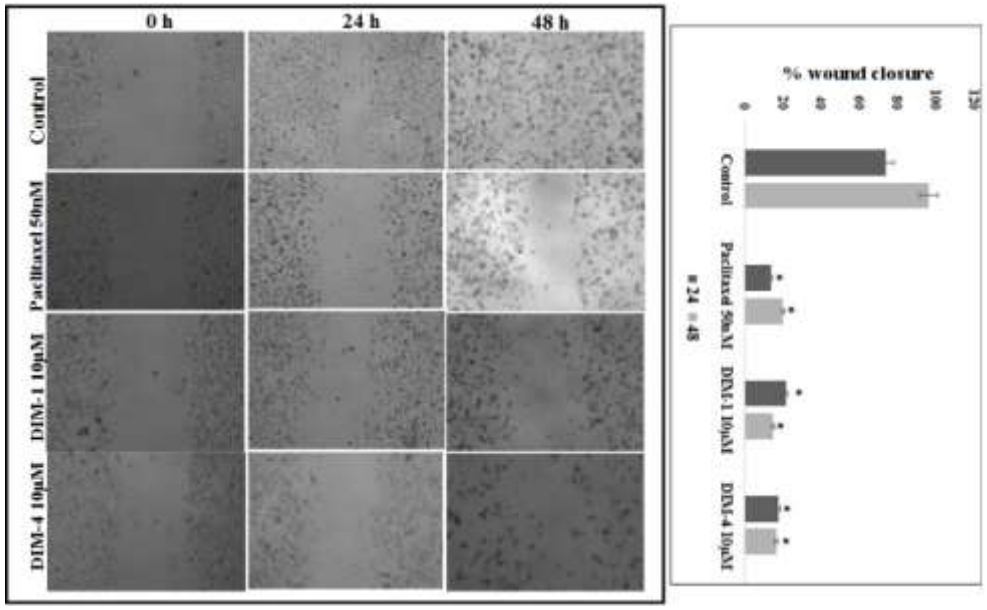

Figure 4.9. Effects of DIM-1 and DIM-4 on migration of MDA-MB-231 cells: *The phase contrast images of cells were taken at 0, 24, and 48 h after treatment with DIM-1, DIM-4, and paclitaxel. The wound introduced in the cell monolayer was found to decrease in control cells due to migration of cells. But the wound closure was significantly reduced in treated cells which signified reduced migration of the cells. The vertical bars represent SD of data means. * indicates significant difference from control group.*

4.3.9. Effects of DIM-1 and DIM-4 on MMP activity and VEGF expression

During angiogenesis, VEGF is the growth stimuli for endothelial cells and MMP helps in cell invasion by the degradation of ECM. The activity of MMP was studied by gelatin zymography, and the results are given in Figure 4.10. MMP-9 activity was significantly reduced by DIM-1 and DIM-4, while MMP-2 activity was suppressed noticeably by DIM-4 but slightly by DIM-1 (10 μM). The relative intensity of expression of MMP-9 was reduced to 0.46 and 0.32 at 20 μM concentration of DIM-1 and DIM-4 respectively, from control expression of 1 ($p \leq 0.05$). Meanwhile MMP-9 expression was reduced to 0.9 (DIM-1) and 0.65 (DIM-4) at 20 μM concentration from control expression which was taken as 1. Thus DIM-1 and DIM-4 significantly downregulated the MMP activity in MDA-MB-231 cells which indicated its anti-migration potential.

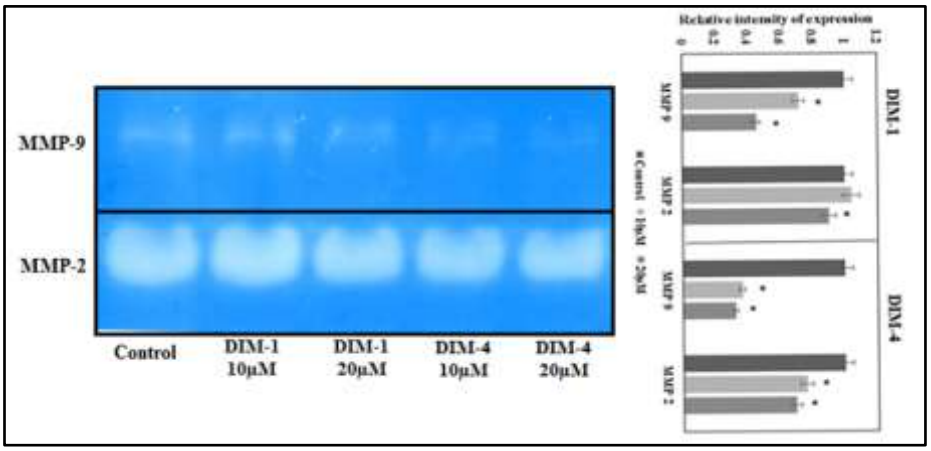

Figure 4.10. Effects of DIM-1 and DIM-4 on MMP activity in MDA-MB-231 cells: *Gelatin zymography revealed that the relative expression of MMP-2 and MMP-9 was downregulated by DIM-1 and DIM-4 in MDA-MB-231 cells. The vertical bars represent SD of means. * indicates significant difference from the control group ($p \leq 0.05$)*

The expression of angiogenic initiator VEGF was studied by western blot and the results are given in Figure 4.11. VEGF expression was significantly reduced in DIM-1 and DIM-4 treated cells compared to control. The relative intensity was reduced to 0.49 and 0.38 at 10 µM concentration by DIM-1 and DIM-4 respectively when compared to the control group expression taken as 1 ($p \leq 0.05$). So the reduction in activity of MMP enzymes and VEGF expression by DIM-1 and DIM-4 indicated that they have anti-angiogenic potential.

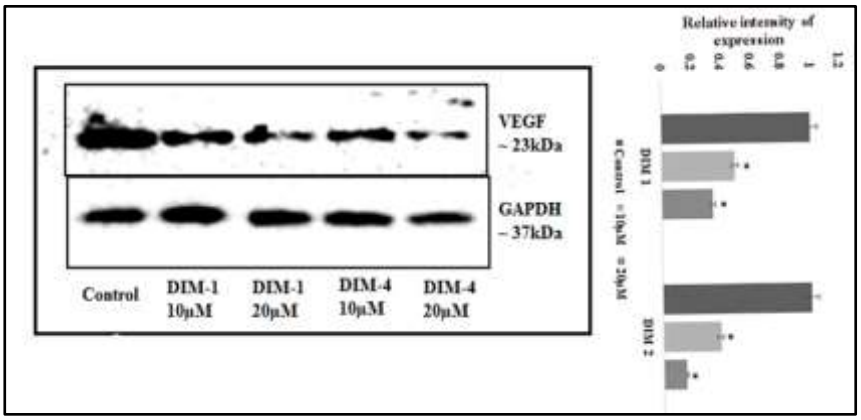

Figure 4.11. Effects of DIM-1 and DIM-4 on VEGF expression in MDA-MB-231 cells: *The expression of VEGF in MDA-MB-231 cells treated with DIM-1 and DIM-4 showed a marked downregulation compared to control. Values are means, with SD denoted by vertical bars. * indicates significant difference from control group ($p \leq 0.05$).*

4.3.10. Effects of DIM-1 and DIM-4 on cell cycle progression

The changes in cell cycle progression of MDA-MB-231 cells on treatment with DIM-1 and DIM-4 was studied flow cytometry after PI staining. The results indicated that cells treated with 10 µM of DIM-1 and DIM-4 did not reveal any significant alteration in the distribution of cells in different phases of the cell cycle. The percentage of cells in different phases were similar to that of the control. The standard drug paclitaxel induced significant variations in sub G0/G1, G1 and S phases indicating cell cycle arrest. Hence DIM-1 and DIM-4 induced cell death in MDA-MB-231 cells independent of cell cycle arrest.

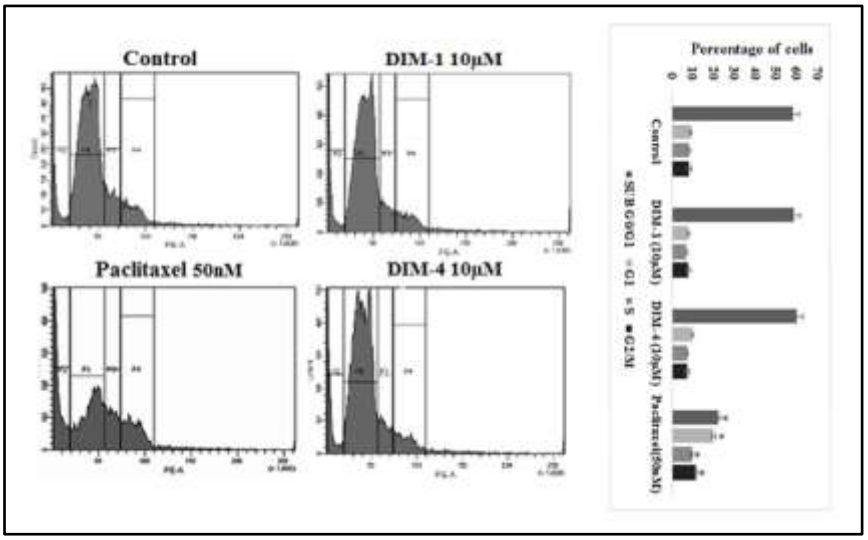

Figure 4.12. Effects of DIM-1 and DIM-4 the on cell cycle progression in MDA-MB-231 cells: *The effect of DIM derivatives on cell cycle progression in MDA-MB-231 cells was investigated by flow cytometry. The distribution of cells remained unchanged in control and treated cells, indicating that DIM compounds had no role in the cell cycle progression of the cells. But paclitaxel treatment, induced variations in all the phases of the cell cycle. Values are means with SD represented by vertical bars. * indicates significant difference from control group (p≤ 0.05).*

4.3.11. Effects of DIM-1 and DIM-4 on glucose uptake

Cancer cells have an altered metabolism with an increased glucose uptake and glycolytic rate and this has been a targeted area in anticancer therapy. The involvement of DIM compounds in glucose uptake was investigated by flow cytometric analysis of glucose uptake using 2-NBDG analog. MDA-MB-231 cells treated with 10 µM of DIM-1 and DIM-4 for 24 h were exposed to 2-NBDG which is a fluorescent glucose analog and the uptake of the dye directly indicates uptake of glucose and hence glycolytic rate. Flow cytometric representation of glucose uptake as histograms, clearly show that the rate of uptake is not affected by DIM

exposure while cells treated with paclitaxel demonstrate a decrease in uptake by almost 4 fold in comparison to untreated cells. The results are shown in Figure 4.13. Thus it was observed that DIM-1 and DIM-4 did not affect the cell metabolism of cancer cells.

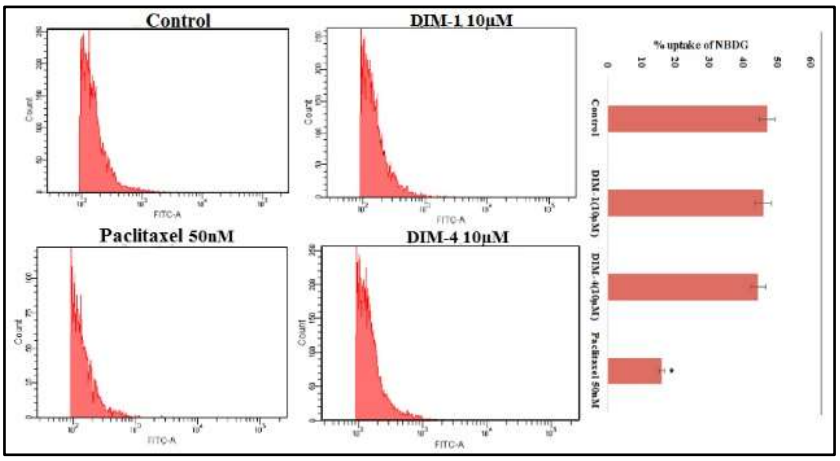

Figure 4.13. Glucose uptake analysis by flow cytometry in MDA-MB-231 cells: *Glucose uptake by cells on treatment with DIM-1. DIM-4, and paclitaxel was investigated using 2-NBDG based flow cytometry. The results indicated that the DIM compounds did not alter the glucose uptake by cells but paclitaxel significantly reduces the uptake. Values are means, with SD represented by vertical bars. * indicates a significant difference from the control cells ($p \leq 0.05$).*

4.3.12. Effects of DIM-1 and DIM-4 on the expression of HIF-1, p53, FAK, pFAK, RAS, and active RAS

In cancer cells the activation of FAK/RAS pathway results in the inhibition of p53 and apoptosis evasion. p53 and HIF-1 α are transcription factors that trigger stress mediated apoptosis. Due to the critical role of these proteins in cancer progression, they are major targets for cancer therapeutics. Western blot analysis was done to analyse the variations in expression of these proteins on treatment with DIM-1 and DIM-4 and the results are shown in Figure 4.14. The expression of p53 increases by 1.4 and 1.33 fold ($p \leq 0.05$) on treatment with 10 µM of DIM-1 and DIM-4 respectively when compared to control. The activated form, pFAK is downregulated by the DIM compounds noticeably, while the expression of inactivated form, FAK remains stable in control and treated cells. The relative intensity of active GTP-RAS bands was found to be significantly reduced to 0.79 (DIM-1) and 0.667 (DIM-4) at a concentration of 20 µM ($p \leq 0.05$) from control (taken as 1). HIF-1α a major transcription factor responsible for stress regulation was also studied and found to be significantly upregulated by 2.4 (DIM-1) and 2.5

(DIM-4) fold at 20 µM ($p \leq 0.05$). GAPDH was used as the standard loading control for comparison. Thus DIM-1 and DIM-4 induced cytotoxicity by upregulating p53 and HIF-1, and by inhibiting the FAK/RAS phosphorylation for its effects.

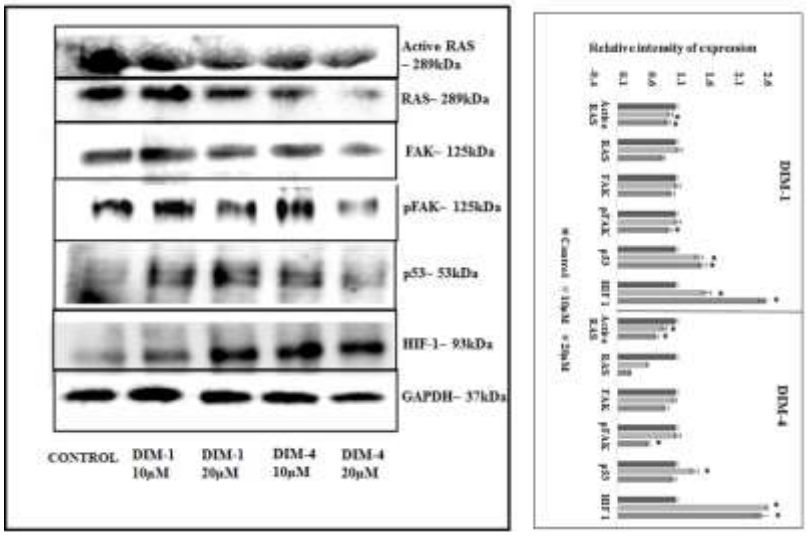

Figure 4.14. Western blot analysis of p53, HIF-1, active RAS, RAS, and FAK proteins in MDA-MB-231 cells*: Western blot analysis of proteins namely RAS, active RAS, FAK, pFAK, p53 and HIF-1α were carried out in MDA-MB-231 cells treated with DIM-1 and DIM-4 for 24 h. The bar graphs representing the relative intensity of protein expression depicts that DIM compounds downregulated active RAS and pFAK while upregulating p53 and HIF-1. Values are means, with SD represented by vertical bars. * indicates a significant difference from the control cells ($p \leq 0.05$).*

4.3.13. Effects of DIM-1 and DIM-4 in the activated proteins of PI3K/AKT/mTOR pathway

The survival pathway which is found to be perpetually activated in many types of cancer, is a major target for anticancer drugs. The expression of major molecules in the signaling pathway, PI3K/AKT/mTOR was analysed by western blotting. MDA-MB-231 cells exposed to DIM-1 and DIM-4 for 24 h was analysed for the expression of phosphorylated (active) and inactive forms of PI3K, AKT and mTOR. The results are given in Figure 4.15. The activated forms of all the investigated proteins were downregulated when treated with DIM derivatives. When the relative intensity of the control taken as 1, pPI3K was reduced to 0.39 (DIM-1) and 0.4 (DIM-4) at 20µM concentration. At the same concentration of the compounds, relative intensity of pAKT was reduced to 0.46 (DIM-1) and 0.38 (DIM-4). DIM-1 and DIM-4 decreased the relative expression of mTOR to 0.33 and 0.34 respectively. This result strongly points out to a concerted inhibition of

multiple components of the survival pathway PI3K/AKT/mTOR by DIM-1 and DIM-4 resulting in apoptosis.

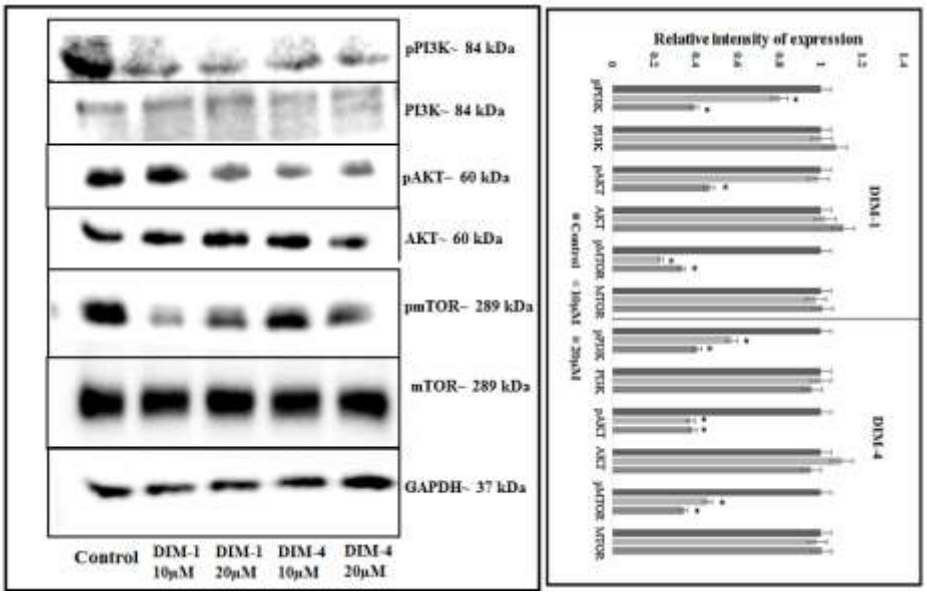

Figure 4.15. Western blot analysis of proteins in the PI3K/AKT/mTOR pathway in MDA-MB-231 cells: *Western blot analysis of proteins pPI3K, PI3K, pAKT, AKT, pMTOR and MTOR in breast cancer cells treated with DIM-1 and DIM-4 for 24 h was conducted and the bar graphs represent the relative intensity of expression of the proteins. The results indicate the suppression of the pathway proteins on treatment with the DIM compounds. Values are means, with standard deviations represented by vertical bars. * indicates a significant difference from the control cells ($p \leq 0.05$).*

4.4. Discussion

Breast cancer, one of the most incident cancer in women, has many subtypes, and the treatment options for the subtypes depend on the hormone receptor status of cancer (Jemal *et al*., 2011; Sorlie *et al*., 2003). TNBC is a subtype of breast cancer, almost 80% of which is comprised of basal-like tumors (Dawood *et al*., 2010). Although it accounts for only 15-20% of breast cancer cases, it is highly invasive, has a high risk of recurrence, and low disease-free survival. Due to the lack of selective targets, chemotherapy remains the mainstay of treatment for this subtype of breast cancer. And many studies have shown that TNBC showed significantly higher pCR rates than the other subtypes of breast cancer, towards neoadjuvant chemotherapy (Liedtke *et al*., 2008).

The current existing treatment options, have moderate efficacy and have unpredicted side effects, demanding the need for new active anticancer agents for the treatment of TNBC. Recent preclinical research adds to the mounting evidence on the beneficial action of natural compounds on multiple cancer pathways

(Kapinova *et al*., 2017). Many currently marketed anti-cancer drugs derived from natural resources such as paclitaxel, camptothecin, and vinblastine, have been successfully used as anticancer agents (Cragg *et al*., 2013). The review by Kapinova *et al*. 2017, also deliberates the use of plant-based functional foods as a treatment for breast cancer. Diet has been identified as a major, variable, risk factor for breast cancer and the role of cruciferous vegetables in cancer chemoprevention has been widely acknowledged. DIM from cruciferous vegetables has found to be effective in all stages of breast cancer development (Thomson *et al*., 2016). Hence this chapter was dedicated to the study of novel ortho-biaryl derivatives of DIM in triple negative breast cancer cell line, MDA-MB-231.

Cytotoxicity is the mode of action of many anticancer agents, and so for the present study the synthetic derivatives of DIM were initially screened for their cytotoxic potential using MTT assay. DIM-1, DIM-4, and DIM-8 induced a concentration dependent cytotoxicity in breast cancer cells with low GI_{50} values below 10 μM indicating a high toxicity potential. The rest of the compounds only showed weak activity with GI_{50} values above 20 μM. The viability study of the DIM compounds in normal H9C2 cells revealed that DIM-8 was the only derivative that was toxic in both normal and cancer cells. Hence, DIM-1 and DIM-4 were chosen for further anticancer activity studies, owing to the low GI_{50} values and non-toxic nature towards normal cells. Paclitaxel was chosen as the positive control for the study, as it is an active agent currently favoured for the clinical treatment of breast cancer (Perez 1998; Miller *et al*., 2007). Similarly IC_{50} values were used for the successful comparison of cytotoxicity of anticancer drugs (docetaxel, paclitaxel, etc.) in multiple cancer cell lines in a study by Florento *et al*. 2012. The morphology of the cells were analysed, to observe the changes associated with the cytotoxicity induced by the DIM compounds. In the present study, morphological response of breast cancer cells to the DIM compounds included a reduction in cell number, cell shrinkage, membrane blebbing, and apoptotic body formation. But in normal cells the alterations if any, were not noticeable. The DIM induced changes in cancer cells resembled the morphological changes accompanying apoptosis induction (Zhivotovsky 2004). A recent review by Nagata 2018, on apoptosis which described the alterations in the morphology of cells undergoing apoptosis also supports the fact that the morphological changes

induced by DIM-1 and DIM-4 indicate apoptosis induction. Therefore the MDA-MB-231 cells treated with DIM compounds were examined for multiple indicators of apoptosis.

Apoptosis ensures efficient removal of damaged or abnormal cells through a programmed multistep process and is usually found deregulated in cancer cells. In summary, apoptotic signals trigger a family of proteases known as caspases which execute the proteolytic cleavage of many functional and structural proteins such as cytoskeletal proteins, repair enzymes, inactivated nucleases, etc. Caspases are responsible for many ultra-structural indicators like nuclear fragmentation, PS translocation, etc. that can be detected using various techniques (He *et al*., 2007). The study of the nuclear morphology of MDA-MB-231 cells treated with DIM compounds revealed the extensive nuclear fragmentation, while the nuclei of the control cells remained undamaged. DAPI was used as a reliable apoptotic assay to examine the changes at DNA level in a study by Sheikh *et al*. 2018. AO/EB staining method revealed that DIM compounds induced a loss of the plasma membrane integrity in breast cancer cells. The yellow-orange, stained nuclei also indicated that the cells exposed to the DIM compounds were in the late stage of apoptosis. This dual staining method was employed by Sheikh *et al*. 2018, to study the apoptosis induction in MDA-MB-231 cells. The removal of apoptotic cell debris by phagocytes are aided by the exposed PS on the surface of the cells. In the current study, the translocation of PS to the outer surface of the cells induced by the DIM compounds was visualised by annexin staining. This gave further proof for the induction of the apoptotic process by the DIM compounds. Annexin staining was similarly used in a study by Al Dhaheri *et al*. 2014, to detect PS translocation and therefore apoptosis. In a study by Kumar *et al*. 2018, mitochondrial-mediated apoptosis by epoxyazadiradione in TNBC was established through caspase 3 and 9 activities, along with the expression of apoptosis-specific proteins such as Bcl2, Bax, PARP, etc. (Kumar *et al*., 2018). Hence in the current study, the potential DIM compounds were investigated and found to induce an upregulation in the activity of caspase 3 and caspase 9, in MDA-MB-231 cells. This signified mitochondrial-mediated apoptosis induction as caspase 9 is the initiator enzyme involved in the intrinsic pathway. Further biochemical proof of apoptosis was obtained by western blot analysis of DIM treated cells, and the data displayed a characteristic upregulation in the expression of proapoptotic Bax and

downregulation of antiapoptotic Bcl2. The expression of c-PARP, the caspase-cleaved repair enzyme and cytosolic cytochrome c was elevated on treatment with DIM-1 and DIM-4. For the activation of caspase 9 in the intrinsic pathway, the mitochondrial release of cytochrome c is indispensable (Cadron *et al*., 2006). The results were in agreement with the mitochondria mediated apoptosis induced by epoxyazadiradione in breast cancer cells which was accompanied by an elevated caspase 3 and 9 activity, increase of Bax/Bcl2 ratio, cleavage of c-PARP, and release of cytochrome c (Kumar *et al*., 2018). Hence the effects induced by DIM compounds in MDA-MB-231 cells suggested the apoptosis to be mitochondrial-mediated.

ROS are signaling molecules that play a major role in cell proliferation and apoptosis (Chen *et al*., 2015). Studies have shown that ROS are capable of directly influencing the mitochondrial potential, causing the release of cytochrome c and thereby activating the intrinsic pathway of apoptosis (Fleury et al., 2002). In a study of the natural compound carnesol in MDA-MB-231 cells, apoptosis was found to be ROS mediated (Al Dhaheri *et al*., 2014). In the present investigation, DIM compounds were found to induce a significant increase in ROS activity in MDA-MB-231 cells. Along with the earlier evidence for the mitochondrial-mediated apoptosis, and the increased ROS activity elicited by DIM compounds, it can be summarised that ROS played a major role in the mitochondrial mediated apoptosis induced by the DIM compounds.

Mortality from TNBC is mainly attributed to the metastasis of the cancer cells. Studies have shown that the expressions of MMP-2, MMP-9, and VEGF are positively associated with the extent of invasion, metastasis, and angiogenesis (Zheng *et al*., 2006). MMP's play a major role in cancer cell invasion and formation of new blood vessels in angiogenesis, while VEGF is a marker for tumor angiogenesis. In an *in vivo* study involving tumor-bearing mice, the cancer metastasis was repressed through the inhibition of MMP and VEGF (Huang *et al*., 2015). In the present study, the scratch wound assay revealed a significant inhibition in the migration of breast cancer cells treated with DIM compounds, compared to the control cells. Gelatin zymography demonstrated that DIM compounds significantly downregulated MMP-2 and MMP-9 activity in breast cancer cells. Western blot analysis revealed a marked downregulation in the expression of angiogenic marker VEGF in breast cancer cells treated with DIM

compounds signifying its anti-angiogenic effect. In conclusion, the DIM compounds slowed down the migration of the breast cancer cells, suppressed the activity of ECM degrading enzymes and down regulated VEGF expression, all data indicating the ability of DIM compounds in effectively attenuating the migration and angiogenesis in MDA-MB-231 cells.

In the earlier chapter, studies on the effect of DIM compounds on cell cycle progression revealed the induction of cell cycle arrest in HeLa cells, and the literature also proclaimed natural DIM's ability to suppress cancer proliferation through cell cycle arrest (Hong et al., 2002). Therefore in the current chapter, the role of DIM compounds in the cell cycle progression of MDA-MB-231 cells was investigated, and the flow cytometry histograms exhibited no indications of cell cycle arrest. Deregulation of glucose metabolism to preferentially use aerobic glycolysis is a well-known hallmark of cancer and TNBC has been associated with upregulated expression of GLUT's (Warburg et al., 1926; Hussein et al., 2007). A recent study on Metformin revealed that it targeted glucose metabolism and was particularly potent against TNBC (Wahdan-Alaswad et al., 2018). In the current study, the glucose uptake was studied by flow cytometry, and the results indicated that DIM compounds did not affect the glucose uptake in MDA-MB-231 cells. Hence it can be concluded that the anticancer activity of DIM compounds was not accomplished through cell cycle arrest or alteration of glucose metabolism.

FAK signaling is a major target for cancer therapeutics, as it is a key regulator in integrin and growth factor-mediated signalling, and is often found to be overexpressed in metastatic cancer. It regulates multiple downstream effectors to promote migration and metastasis (Lee *et al*., 2015). FAK interacts with p53 negatively, leading to its ubiquitination which is aided by mdm2 (Cance *et al*., 2008). And p53 is a potential therapeutic target as it is usually found deregulated in TNBC and has a significant role in the execution of apoptosis and cell cycle arrest (Turner *et al*., 2013). RAS is a signalling molecule usually activated by growth factor receptors and is implicated in cancer initiation and development. The role of RAS in promoting cancer cell migration is attributed to its complex interaction with integrin-mediated FAK signalling (Antonyak et al., 2009). In the present study DIM compounds were found to downregulate the expression of activated RAS and FAK along with a relative increase in the expression of p53 in agreement with the above literature. Both signalling molecules (RAS and FAK) that promote cancer

proliferation and metastasis were downregulated by the DIM compounds signifying the interruption of either an integrin mediated signalling or growth factor mediated signalling (Lee *et al*., 2015; Antonyak et al., 2009). The suppression of FAK or increased ROS activity (stress signal) might be responsible for the stabilization of p53. HIF-1α is usually associated with angiogenesis and poor cancer prognosis, but it is also a stress response regulator and stabilises p53 during hypoxic conditions (An *et al*., 1998). In agreement with the findings of An *et al*. the potential DIM compounds induced the upregulation of HIF-1α in TNBC. Hence with the above findings it can be concluded that DIM-1 and DIM-4 inhibits the integrin-FAK signalling accompanied by the concerted upregulation of p53 expression.

PI3K/AKT/mTOR is a pathway usually found dysregulated in multiple types of cancer including breast cancer and is responsible for the proliferation, survival, and metastasis of cancer cells (Saini *et al*., 2013). The pathway maybe activated by mutation of upstream molecules, activation of growth factor receptors, or the loss of PTEN function. Western blot analysis revealed that DIM compounds suppressed the expression of PI3K, AKT, and mTOR. This result was in agreement with a recent study where flavonoids induced apoptosis and autophagy in breast cancer cells by the downregulation of PI3K/AKT/mTOR signalling (Zhang *et al*., 2018). Another example is the anticancer activity of shikonin, a natural naphthoquinone achieved through the inhibition of PI3K/AKT/mTOR pathway (Ni *et al*., 2018). The current data along with the literature points out that the anticancer activity shown by DIM-1 and DIM-4 is associated with the suppression of PI3K, AKT, and mTOR pathway.

In conclusion, DIM compounds induced apoptosis in TNBC by influencing multiple pathways and processes including ROS generation, p53 stabilisation, and survival pathway inhibition.

4.5. Conclusion

The novel ortho-biaryl derivatives showed promising anticancer activity in the TNBC cell line, MDA-MB-231 through the induction of cell cycle-independent mitochondrial-mediated apoptosis accompanied by upregulated ROS activity, p53 stabilisation, suppression of integrin-FAK signalling and suppression of PI3K/AKT/mTOR pathway. The hypothesized mechanism of action of DIM compounds in metastatic breast cancer cells is summarised in Figure 4.16.

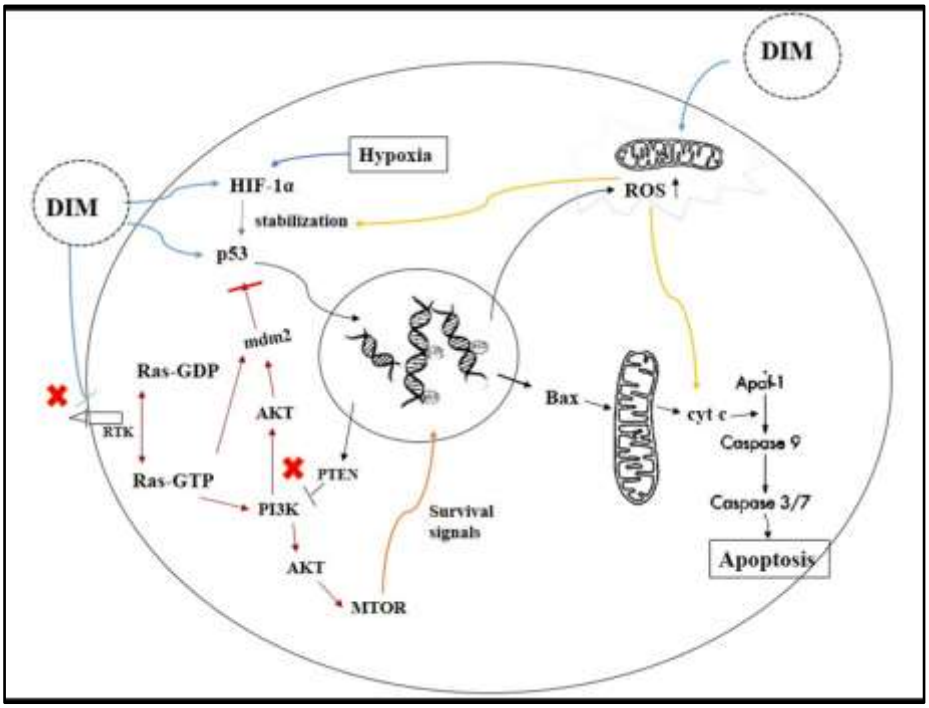

Figure 4.16. Mechanism of action of DIM-1 and DIM-4 in MDA-MB-231 cells: *The effect of DIM compounds on different pathways and signalling molecules are summarised in this figure. The cross-communication between various pathways was surmised from literature.*

4.6. References

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Chapter 5

Comparative anticancer studies of DIM-1 and DIM-4 in breast cancer cells under 2D and 3D culture conditions

5.1. Introduction

Tumor microenvironment plays a crucial role in the development and progression of cancer. Tumor cells activate the adjacent stromal cells, resulting in the formation of the cellular component of tumor microenvironment such as cancer-associated fibroblasts (CAFs), tumor infiltrating mesenchymal stem cells (MSCs), tumor infiltrating lymphocytes (TILs), etc. (Friedl *et al*., 2011). These cells further change the architecture of the matrix by secreting cytokines and growth factors and depositing extracellular matrix (ECM) components, which in turn induces changes in the tumor biology, enhancing invasion and metastasis (Egeblad *et al*., 2010).

Cell culture is one of the basic techniques used to study cancer cell biology in life sciences. The traditional cell culture method employs a two-dimensional (2D) monolayer culture of cells, which does not completely represent the architecture and microenvironments of *in-vivo* tumors. The 2D cultured cells exhibit key differences in morphology, proliferation, communication, signal transduction, differentiation, etc. from *in vivo* tumor cells (Wiegelt *et al*., 2014). Hence 2D culture system lacks cell-cell and cell-matrix interactions that are present in native tumors. The need for better culture systems that accurately represents the tumor characteristics led to the development of three-dimensional (3D) culture methods that closely mimic the native environment of tumors (Abbott *et al*., 2003).

3D culture has been used in cancer biology as an intermediate model between *in vitro* cell culture and *in vivo* models. Many studies have revealed that 3D-cultured cells acquire morphological and cellular characteristics closely similar to *in vivo* tumors (Ma *et al*., 2012). Studies by Ma *et al*. also revealed that spheroids could be used as a screening tool for nanoparticles. Another study by Holliday *et al*. 2009, involved the 3D co-culture of different breast cancer cell types which revealed features similar to *in situ* breast ductal carcinoma. The

comparison of gene and protein expression profiles between 2D cells and multicellular tumor spheroid culture by Kumar *et al*. 2008, revealed that many metabolic, stress regulators, structural, signal transduction, and transport proteins were overexpressed in the 3D system. Moreover, cell adhesion and junction proteins that maintain the cell communication of *in vivo* tumors were found to be upregulated in spheroids compared to 2D cultured cells (Kang *et al*., 2007). These studies state some of the merits of using 3D culture methods for anticancer studies. 3D culture also alleviates practical and ethical issues associated with tumors grown *in vivo* in experimental animals.

Multiple models for the 3D culture of tumor cells have been developed. Ishiguro *et al*. 2017, stated four representative methods for 3D culture of cancer cells namely, organotypic multicellular spheroids, tumor-derived organoids, tumor-derived spheroids and multicellular tumor spheroid culture (MCTS). MCTS system has many advantages over the other 3D culture methods such as clonality of cells, ease of maintenance, and reproducibility making it an appropriate tool for drug testing (Friedrich *et al*., 2009). MCTS are interestingly derived from cancer cell lines and, mimic the metabolic and proliferative properties of *in vivo* tumors. Multiple research has shown that the drug sensitivity/ resistance behaviour of cancer cells cultured in a 3D system more accurately mimics the *in vivo* tumor (Zanoni *et al*., 2016).

This chapter was designed to investigate the anticancer activity of DIM-1 and DIM-4 in MDA-MB-231 cells grown in a 3D culture system. The study focused on translating the anticancer effects of DIM compounds obtained in monolayer culture in MDA-MB-231 cells into a 3D system. The 3D culture being a close representation of an *in vivo* model. Comparison of DIM activity between the two systems was based on multiple parameters including cytotoxicity, expression of apoptotic proteins, activity of MMP enzymes, and expression of pathway proteins.

5.2. Methods

This study involves the investigation of MDA-MB-231 cells cultured in 3D conditions. The 3D culture condition was achieved through poly-HEMA coating of culture plates. The results obtained for active compounds DIM-1 and DIM-4 in 2D conditions were compared to the effect of the compounds in 3D cultured cells. The

parameters subjected to comparative analysis were cytotoxicity, apoptosis, migration, angiogenesis, integrin-FAK pathway proteins, and PI3K/AKT/mTOR pathway proteins. Immunofluorescence method was employed to determine the effect of the DIM compounds on EGFR activation. Finally the toxicological studies of the DIM compounds *in vivo* was carried out by Acute Zebrafish Emryo Toxicity Test (ZFET).

5.3. Results

5.3.1. Establishment of 3D culture of MDA-MB-231 cells in poly-HEMA coated plates

To culture MDA-MB-231 cells in a 3D condition, the cell culture plates were prepared by coating with poly-HEMA. The coated 96 well plates were then seeded with 1 x 10^5 cells/well, and after 24 h incubation, observed for morphological changes using phase contrast microscopy. The cells were found to be in close contact with each other on all sides and held together in an aggregated spheroid. The morphology of the spheroid, resembled a tumor-like morphology, with cells growing on top of each other. The structure of the aggregate was compact, motile, and held together by ECM like scaffold. Figure 5.1. depicts the comparison of the morphology of MDA-MB-231 cells grown in 2D and 3D conditions. In 2D cultured cells, the cell-cell contact was limited, while in aggregates the cells were compactly arranged indicating good communication.

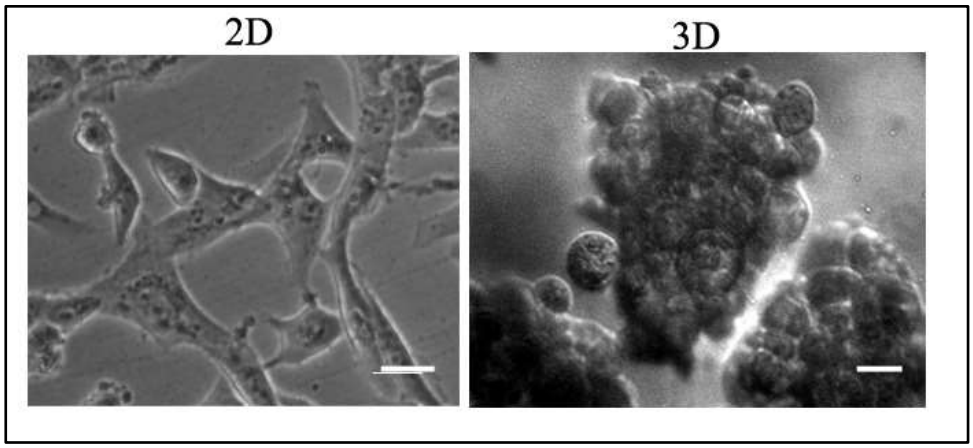


Figure 5.1. Morphology of MDA-MB-231 cells grown in 2D and 3D conditions: *MDA-MB-231 cells were seeded on normal and poly-HEMA coated 96 well culture plates and incubated for 24 h. The morphology of the cells were observed using phase contrast microscope and the cells grown in coated plates showed a tumor like morphology with formation of aggregated spheroids. The scale bar represents a size of 30 µm.*

5.3.2. Cytotoxicity analysis of MDA-MB-231 cells grown in 2D and 3D conditions

MDA-MB-231 cells were seeded in uncoated and poly-HEMA coated 96 well plates, and left for attachment for 24 h. The cells were then treated with multiple concentrations of DIM-1 and DIM-4 for 24 h and the percentage of growth inhibition was estimated by MTT assay. A concentration-dependent inhibition was observed in cells cultured in 2D as well as 3D conditions, but interestingly in 3D culture inhibition was attained at higher concentrations (Figure 5.2.). The GI_{50} value obtained for DIM-1 in attached cells was 9.8±0.219 μM, while in 3D cultured cells 50% growth inhibition was attained at twice the concentration (24±0.724 μM). This indicated that the cells in 3D conditions resisted the compound effect owing to better intercellular interactions. The same was observed for DIM-4, where GI_{50} value was higher in 3D condition (19.20±0.375 μM) compared to that of 2D condition (8.7±0.523 μM). Hence it can be stated that DIM-1 and DIM-4 successfully induced cytotoxicity in 3D cultured cells.

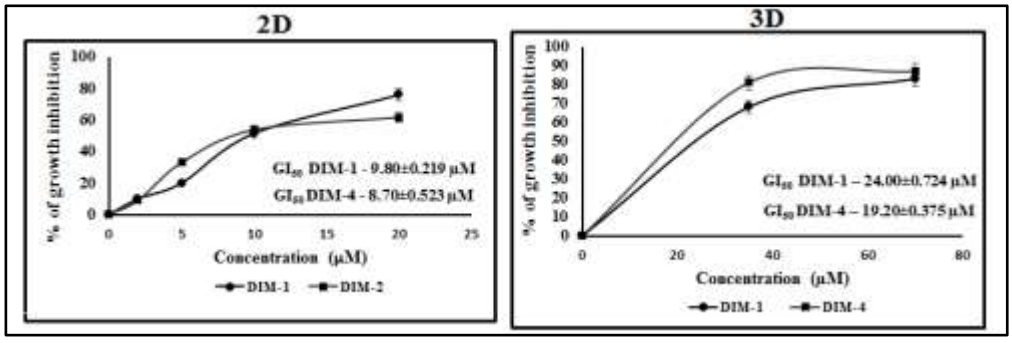


Figure 5.2. Cytotoxicity studies in MDAMB-231 cells cultured in 2D and 3D conditions: *Cells were seeded into normal and poly-HEMA coated 96 well plates and left for 24 h. The cells were then exposed to DIM-1 and DIM-4 compounds for 24 h, followed by which MTT assay was performed. Both compounds induced cytotoxicity in 3D cultured cells at a higher concentration compared to attached cells. Data represented are values ± standard deviation (SD) of three independent experiments.*

5.3.3. Comparison of the morphology of MDA-MB-231 cells on treatment with DIM-1 and DIM-4 in 2D and 3D conditions

MDA-MB-231 cells were seeded in normal, and poly-HEMA coated 96 well plates and incubated for 24 h. This was followed by treatment with multiple concentrations of DIM-1 and DIM-4 for 24 h. The analysis of the morphology through phase contrast microscopy revealed that in attached cells the morphology of the cells showed indications of cytotoxic damage such as cell shrinkage,

membrane blebbing and apoptotic body formation (Figure 5.3.). In 3D cultured cells, we found a disruption in the aggregate morphology and a reduction in the number of cells forming the aggregates, at a concentration of 10 µM for both DIM-1 and DIM-4. At a higher concentration of the DIM compounds (20 µM), the morphology of the cells further deteriorated with apoptotic body formation. And the spheroid morphology was found damaged with presence of detached single cells. DIM-1 and DIM-4 at 50 µM, reduced the cell aggregates to scattered groups of cells. The positive control paclitaxel, induced morphological indications of death in both 2D and 3D conditions. Therefore it can be said that DIM compounds induced morphological signs of cell death in a 3D system along with disruption in the spheroid morphology.

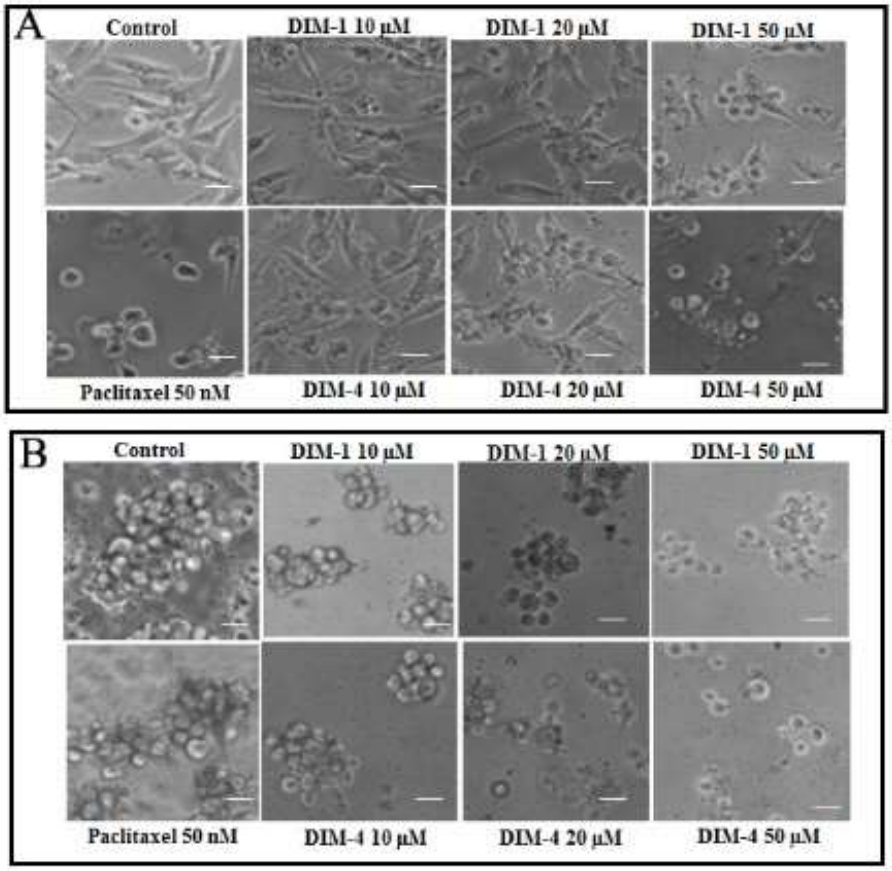


Figure 5.3. Morphology of MDAMB-231 cells treated with DIM-1 and DIM-4 in 2D and 3D conditions: *Cells were seeded in poly-HEMA coated and uncoated plates and treated with different concentrations of DIM-1 and DIM-4 for 24 h. In both 2D and 3D cultures morphology was significantly altered. In 3D cultured cells, DIM compounds affected the aggregate morphology by reducing the number of cells forming the aggregate and detaching the cells from the spheroid. At higher concentrations, the cells are found in small scattered clusters. The scale bar indicates a size of 20 µm.*

5.3.4. Anoikis induction in MDA-MB-231 cells by DIM-1 and DIM-4 by calcein staining

Anchorage independent growth is a key feature of metastatic cancer cells, and anoikis is programmed cell death induced in cells after detachment. To further validate the behaviour of MDA-MB-231 cells under DIM-1 and DIM-4 treatment, anoikis assay was performed. Cells grown in 2D and 3D conditions when treated with the DIM compounds for 24 h induced a significant decrease in calcein fluorescence. The fluorescence which is a direct indicator of viability, was documented by microscopy and microplate reader. Figure 5.4. shows the fluorescent images of cancer cells in 2D and 3D conditions treated with the DIM compounds along with the relative intensity of fluorescence. The fluorescence intensity of control cells in 3D was found to be lower than that of 2D system, indicating a reduction in proliferation rate. In adherent culture, the fluorescence intensity (viability) of DIM-1 and DIM-4 treated cells dropped to almost half of the control fluorescence at 10 µM concentration ($p \leq 0.05$). Meanwhile, in the 3D system the fluorescence intensity was reduced by half, only at an increased DIM concentration of 20 µM. To be more specific, the relative intensity of fluorescence for control cells in 3D culture was 54951, which on treatment with 20 µM of DIM-1 and DIM-4 decreased significantly to 31357 and 28023 respectively ($p \leq 0.05$). And the cell aggregates appear to lose the compactness when exposed to DIM compounds. Paclitaxel which was used as the positive control, also induced cell death in both 2D and 3D conditions. This data provides additional proof that DIM compounds were able to induce apoptosis in metastatic breast cancer cells cultured in 3D conditions which are usually resistant to anoikis. And that the proliferation in 3D cells was found to be lower in untreated cells along with a reduced sensitivity towards DIM compounds.

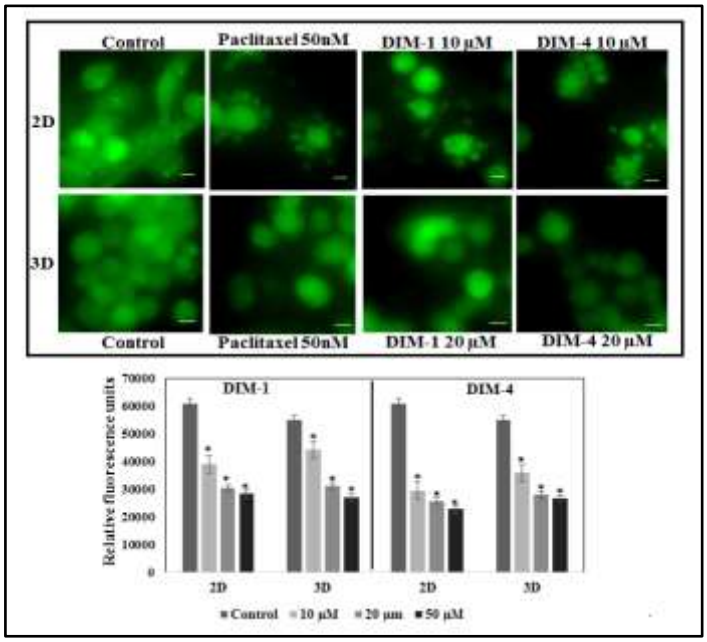


Figure 5.4. Anoikis assay using calcein dye in MDAMB-231 cells treated with DIM-1 and DIM-4: *Cells seeded in 2D and 3D conditions after treatment with DIM-1 and DIM-4 was stained with calcein dye. The results indicate a change in spheroid morphology on treatment along with a reduction in relative fluorescence which indicates decrease in viability or induction of anoikis. The scale bar represents a size of 10 µm. Values represented are means with SD represented by vertical bars.*indicates significant difference from control group ($p \leq 0.05$).*

5.3.5. Effects of DIM-1 and DIM-4 on caspase 3 and 9 activity in MDA-MB-231 cells cultured in 2D and 3D conditions

MDA-MB-231 cells were cultured in 2D and 3D condition and treated with DIM-1 and DIM-4 for 24 h. The caspase activity was estimated with assay kits, and the relative fluorescence was measured using a microplate reader (Figure 5.5.). The fluorescence intensity is a direct indicator of enzyme activity. The cytotoxicity comparison of the two systems revealed that a comparable growth inhibition in 3D culture was only achieved at double the concentration used in 2D culture (10 µM and 20 µM). Hence for further studies increased concentrations were used in the 3D system (20 µM and 40 µM). The caspase 3 activity in attached cells were significantly increased by 2.54 and 2.3 fold at 20 µM concentration of DIM-1 and DIM-4, respectively when compared to the control cells ($p \leq 0.05$). Though caspase 3 activity in 3D system, was also significantly upregulated by the compounds, the fold increase was much reduced compared to 2D cultured cells. To be specific, the fold increase in caspase 3 activity was 1.24 and 1.81 at 40 µM concentration of DIM-1 and DIM-4 respectively. Caspase 9 activity in 2D cultured cells was found to be upregulated notably by 1.8 and 2.27 fold by DIM-1 and DIM-4 (20 µM

concentration) when compared to the control cells. And in cells grown in 3D conditions, 40 µM of DIM-1 and DIM-4 increased caspase 9 activity by 1.39 and 1.63 fold ($p \leq 0.05$). The caspase 9 activity was significantly upregulated in 3D condition but the fold increase was reduced when compared to that of attached cells. Hence DIM compounds effectively increased caspase 3 and caspase 9 activity in breast cancer cells cultured in both 2D and 3D conditions.

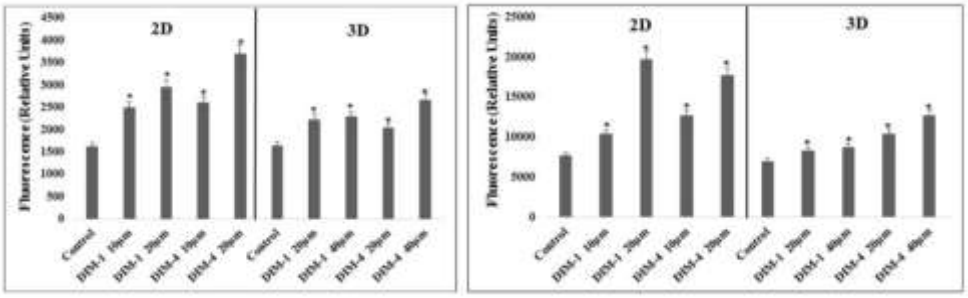


Figure 5.5. Caspase 3 and 9 activity in MDA-MB-231 cells treated with DIM-1 and DIM-4 cultured in 2D and 3D conditions: *Cells were grown in adherent and non-adherent conditions and subjected to treatment with DIM compounds. The caspase 3 and 9 activity was upregulated in both 2D and 3D cultured cells by the DIM compounds. In 3D conditions the caspase induction was reduced compared to 2D cells. Values are means with standard deviation of three independent experiments represented by vertical bars. * indicates significant difference from control group ($p \leq 0.05$).*

5.3.6. Effects of DIM-1 and DIM-4 on the expression of apoptotic proteins in 2D and 3D conditions

MDA-MB-231 cells were seeded into 6 well plates and grown under 2D and 3D conditions, and then treated with effective concentrations of DIM-1 and DIM-4 in each system for 24 h. The total cell lysate was extracted and subjected to western blot analysis. The results indicated that the expression of each investigated apoptotic protein from 2D and 3D conditions on DIM treatment, indicated apoptosis induction. DIM compounds induced upregulation of pro-apoptotic proteins Bax and Bad, while anti-apoptotic protein Bcl2 was downregulated. Meanwhile, caspase-cleaved protein c-PARP and cytosolic cytochrome c were found to be upregulated by the DIM compounds. Figure 5.6. shows the western blot data of apoptotic proteins and their relative intensity of expression. According to the densitometric analysis, the relative intensity of expression of Bax in 3D cultured cells was upregulated by more than two fold by the DIM compounds at 40 µM concentration. At the same concentration, Bad expression was increased in 2D culture by 1.53 and 1.84 fold by DIM-1 and DIM-4 respectively ($p \leq 0.05$). Bcl2

was found to be significantly downregulated from control expression (taken as 1) to 0.089 (DIM-1) and 0.212 (DIM-4) at 20 µM concentration of compounds ($p \leq 0.05$). In the 3D system, the expression of c-PARP and cytochrome c was also upregulated by many folds compared to the control on treatment with DIM-1 and DIM-4 ($p \leq 0.05$). The western blot results indicate that the DIM compounds regulated the investigated apoptotic proteins in both 2D and 3D cultured cells in a pro-apoptotic manner.

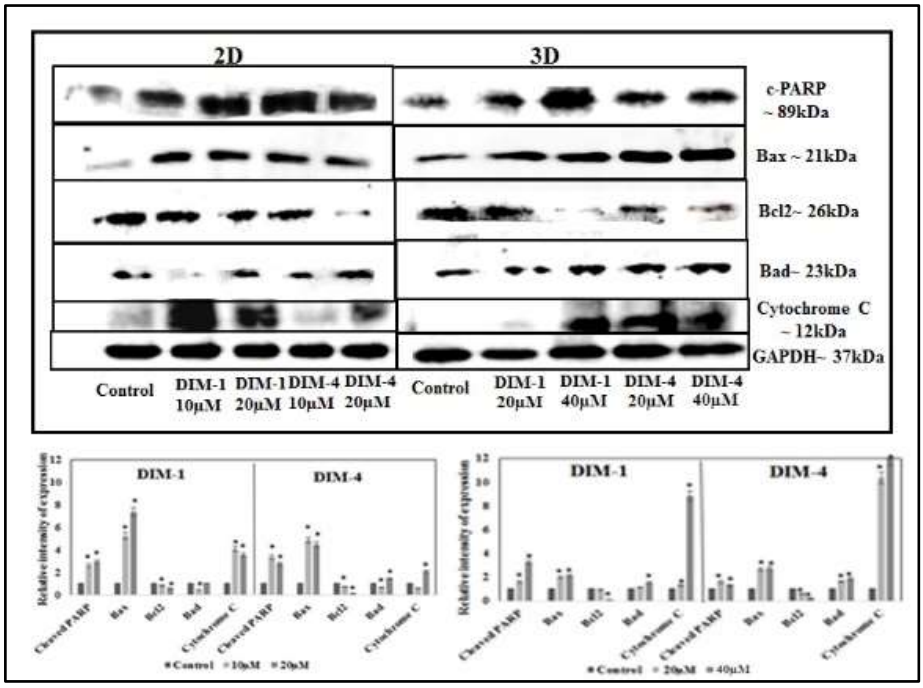


Figure 5.6. Western blot analysis of apoptotic proteins in MDA-MB-231 cells treated with DIM-1 and DIM-4 in 2D and 3D conditions: *Cells grown in 2D and 3D conditions were exposed to 24 h treatment of DIM compounds were subjected to western blot analysis. The results indicated that DIM compounds altered the expression of apoptotic proteins: Bax, Bad, Bcl2, c-PARP, and cytochrome c in cells cultured in 3D conditions indicating apoptosis induction. The relative intensity of expression is also shown. GAPDH was used as the standard protein. Values represented are means with SD represented by vertical bars.*indicates significant difference from control group (p≤ 0.05).*

5.3.7. Effects of DIM-1 and DIM-4 on ECM proteins and MMP: 2D and 3D comparison

Numerous studies have shown that various ECM proteins are aberrantly expressed in cancer and MMP enzymes have a major role in promoting invasion and angiogenesis. MDA-MB-231 cells cultured in 2D system was treated with 10 µM and 20 µM of DIM-1 and DIM-4, while cells cultured in 3D system were treated with 20 µM and 40 µM of the compounds for 24 h. The activity of MMP-2

and MMP-9 enzymes from the cells in the two different culture systems are presented in Figure 5.7. The relative intensity analysis revealed that in the 2D system, DIM compounds at 20 µM concentration reduced relative intensity of MMP-9 to ~ 0.2 and MMP-2 to ~ 0.4 from control (taken as 1). But in the cells grown in 3D culture the DIM compounds (40 µM) reduced MMP-9 expression to ~ 0.7 from control which was taken as 1. MMP-2 expression was significantly reduced to 0.632 and 0.322 by DIM-1 and DIM-4 respectively ($p \leq 0.05$). The results indicated that the MMP activity was significantly inhibited in 2D cultured cells, but the downregulation of enzyme activity in spheroid system was not as pronounced as in the adherent cells. But it can be stated that DIM compounds inhibited the MMP enzyme activity in both 2D and 3D system, signifying the ability of the compounds to inhibit migration in anoikis resistant cells.

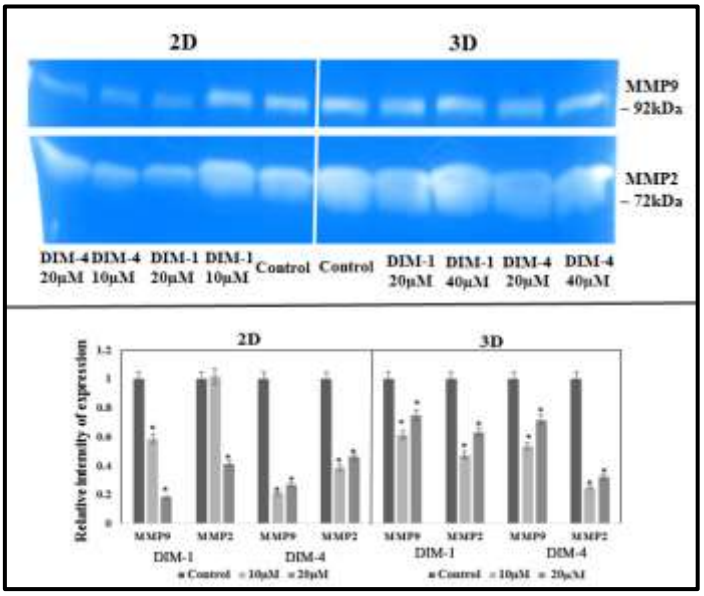


Figure 5.7. Gelatin zymography to determine the effects of DIM-1 and DIM-4 on the activity of MMP-2 and MMP-9 enzymes: *MDA-MB-231 cells were cultured in both 2D and 3D systems and the medium was analysed for MMP enzyme activity employing gelatin zymography. The results revealed a significant inhibition of MMP-2 and MMP-9 activity by the DIM compounds in the 2D system. DIM compounds were also inhibited the enzyme activity in 3D cultured cells, but the inhibition was much reduced. Represented values are means with SD represented by vertical bars.* indicates significant difference from control group ($p \leq 0.05$).*

The expressions of MMP-2, MMP-9 and fibronectin were determined using ELISA (Figure 5.8). The expression of MMP enzymes obtained from ELISA also supports the results from gelatin zymography as MMP acitivity was downregulated by the DIM compounds at 20 µM and 40 µM concentration, in 2D and 3D culture systems respectively. Fibronectin, an ECM protein found to promote tumorigenesis

at various stages, was studied using ELISA technique in cells cultivated in both 2D and 3D culture. Interestingly the expression of fibronectin in control cells from 3D culture was found to be higher than that in the control cells from 2D system. The expression of fibronectin was found to be downregulated by DIM-1 and DIM-4 in attached as well as unattached cells, providing further evidence for the anticancer potential of the compounds.

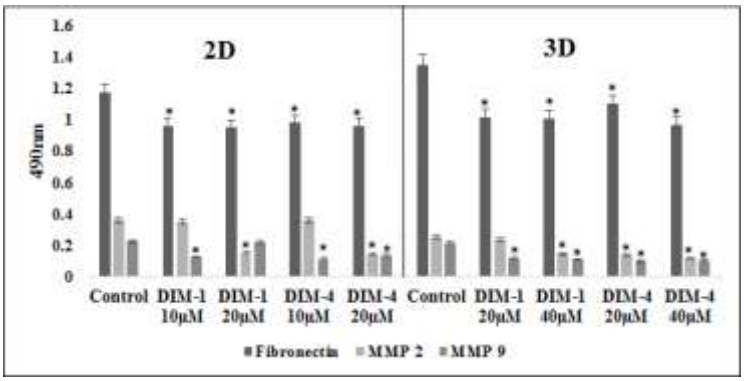


Figure 5.8. Effects of DIM-1 and DIM-4 on the activity of MMP-2, MMP-9 and fibronectin in 2D and 3D system by ELISA: *MDA-MB-231 cells were analysed for the activity of MMP-2, MMP-9 and fibronectin by employing ELISA. The expression of all three proteins were downregulated by the DIM compounds in cells cultured in 2D and 3D system. Values represented are means with SD represented by vertical bars.* indicates significant difference from control group ($p \leq 0.05$).*

5.3.8. Effects of DIM-1 and DIM-4 on VEGF expression in 2D and 3D cultured cells

The anti angiogenic potential of DIM compounds was assessed in cells grown in 2D and 3D culture by western blot analysis. The expression of VEGF was found to be significantly downregulated by the DIM compounds in both systems. The western blot data of the protein is depicted in Figure 5.9. The relative intensity of expression was downregulated by DIM-1 and DIM-4 at 20 μM concentration, in 2D system from control (taken as 1) to 0.83 and 0.083 respectively. At the same time in cells from 3D culture, the expression was decreased to 0.64 and 0.10 by DIM-1 and DIM-4 (40 μM) respectively ($p \leq 0.05$). The VEGF inhibition was more pronounced in cells treated with DIM-4. The results thus point out the significant anti-angiogenic potential exhibited by DIM compounds in cells cultured in 2D and 3D condition.

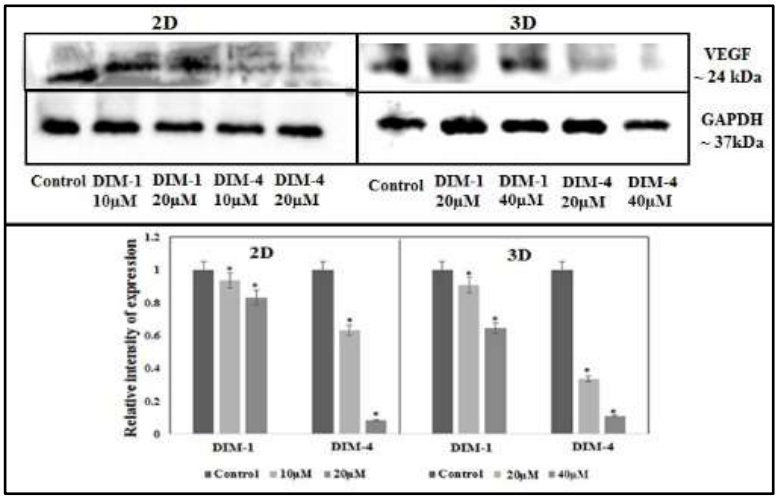


Figure 5.9. Comparison of VEGF expression in cells treated with DIM-1 and DIM-4 in 2D and 3D system: *MDA-MB-231 cells cultured in adherent and non-adherent culture were investigated for VEGF expression using western blot analysis. The study revealed an inhibition in the expression of the pro angiogenic protein by DIM compounds. Represented values are means with SD represented by vertical bars.* indicates significant difference from control group (p≤ 0.05).*

5.3.9. Effects of DIM-1 and DIM-4 on the expression of p53 and integrin-FAK pathway proteins in 2D and 3D comparison

MDA-MB-231 cells were cultured in both 2D and 3D conditions and subjected to treatment with DIM-1 and DIM-4 for 24 h. The changes in the expression of integrin, FAK, and p53 proteins effected by DIM compounds were determined by western blot analysis in both culture systems. The western blot data depicted in Figure 5.10. indicated that the DIM compounds downregulated the expression of integrin and pFAK, while upregulating p53 expression in MDA-MB-231 cells irrespective of the culture conditions. Importantly the stabilisation of p53 is significant in 3D cultured cells, indicating that DIM compounds are effective in spheroids along with monolayer culture. In cancer cells cultured in the 3D system, the expression of integrin, was reduced from control (taken as 1) to 0.44 and 0.48 by DIM-1 and DIM-4 (40 μM) respectively. And the intensity of pFAK was downregulated to 0.43 and 0.23, by DIM-1 and DIM-4 at 40 μM concentration, correspondingly ($p \leq 0.05$). The expression of p53 at the same time was upregulated by 7.6 and 9.6 fold by DIM-1 and DIM-4 respectively at 40 μM concentration ($p \leq 0.05$). The data indicated the suppression of integrin/FAK pathway and the stabilisation of p53. The results showed that DIM-1 and DIM-4 were able to elicit the response in cells cultured in 2D and 3D conditions.

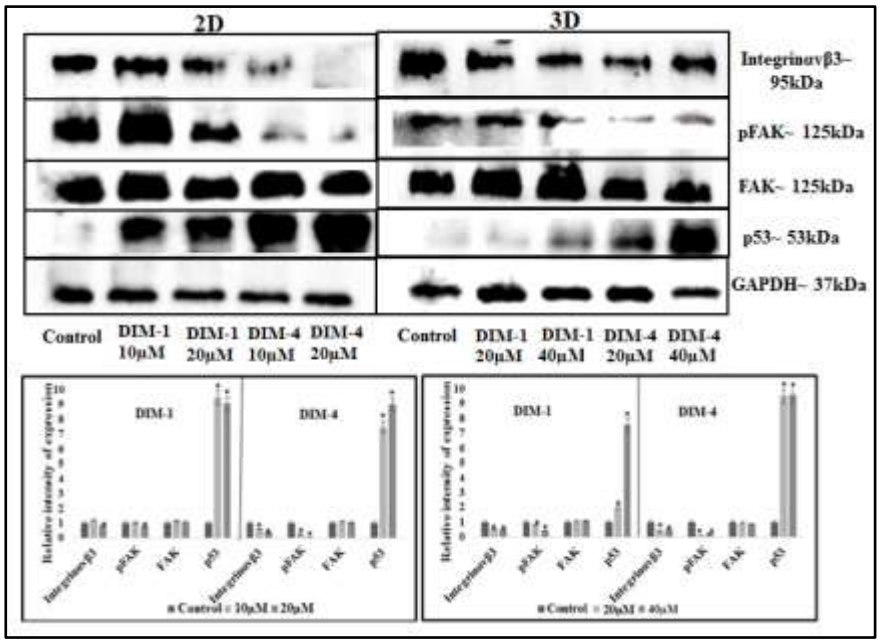


Figure 5.10. Western blot analysis of p53 and integrin/FAK pathway proteins on treatment with DIM-1 and DIM-4: *MDA-MB-231 cells cultured in 2D and 3D culture were investigated for alterations in expression of proteins namely integrin, pFAK, and p53 on treatment with DIM-1 and DIM-4. The study revealed downregulated expression of both integrin and pFAK by DIM compounds in cancer cells grown in 2D and 3D system. But the expression of p53 was found upregulated. Represented values are means with SD represented by vertical bars.* indicates significant difference from control group (p≤ 0.05).*

5.3.10. Effects of DIM-1 and DIM-4 on PI3K/AKT/mTOR pathway proteins in 2D and 3D condition

The expressions of phosphorylated (active) and inactive forms of PI3K, AKT, and mTOR proteins were analysed using western blot, after treating the cells with DIM-1 and DIM-4 in 2D and 3D conditions. As already determined in chapter 4, DIM compounds suppressed the activation of PI3K/AKT/mTOR pathway in adherent cells. The comparison of the results in 2D and 3D system is presented in Figure 5.11. The results indicated that DIM compounds suppressed the active/phosphorylated forms of the studied proteins in 3D cultured cells as well. To elaborate, at a concentration of 40 μM, DIM-1 and DIM-4 reduced the expression of pPI3K from control (taken as 1) to 0.54 and 0.37 respectively, while pAKT expression was reduced to 0.59 and 0.044. DIM-1 and DIM-4 (40 μM) also suppressed the expression of pMTOR from 1 (control) to 0.46 and 0.025 respectively ($p \leq 0.05$). The results indicated that the DIM compounds-mediated suppression of survival pathway proteins in 2D conditions were effectively translated into the 3D system.

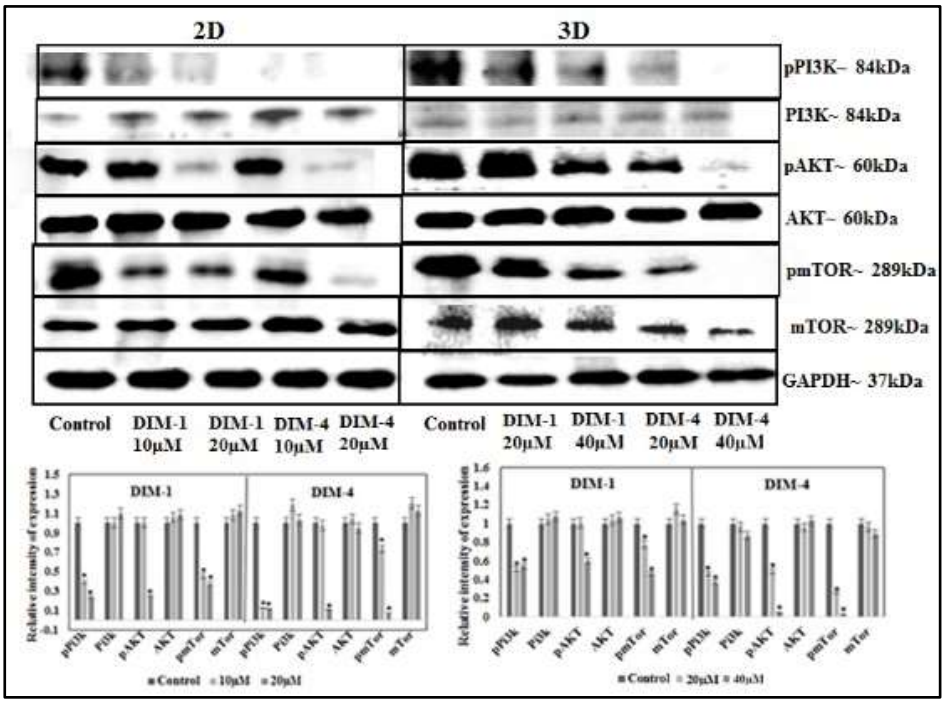


Figure 5.11. Western blot analysis of proteins in the PI3K/AKT/mTOR pathway on treatment with DIM-1 and DIM-4: *MDA-MB-231 cells grown in 2D and 3D conditions were investigated for alterations in the expression of proteins in the survival pathway namely, PI3K, AKT, and mTOR using western blot analysis. The study revealed that DIM compounds downregulated the expression of all the investigated proteins in cells grown in 2D and 3D system. Represented values are means with SD represented by vertical bars.* indicates significant difference from control group (p≤ 0.05).*

5.3.11. Docking analysis of DIM-1 and DIM-4 with receptor proteins

The possibility of interaction between DIM compounds and receptors was investigated using Autodock 4.2 software, which enabled the study of virtual binding between the molecules. The binding probability of the DIM compounds with many receptors were carried out and the interactions with lowest free binding energy are represented in Figure 5.12. namely, folate receptor, tyrosine kinase (EGFR), transforming growth factor receptor (TGF) and G protein-coupled receptor (GPCR). The best conformations of DIM compounds and receptors are also shown in the figure. The free binding energy signifying the affinity of interaction between receptors and DIM-1 and DIM-4 is represented in Table 5.1. On the basis of binding affinity, DIM compounds had the best binding affinity with tyrosine kinase domain of EGFR. To be specific, the least free binding energy for DIM-1 and DIM-4 was obtained with EGFR, -9.91 and -9.37 respectively. Hence it can be said that among the multiple receptors investigated, DIM compounds showed increased binding probability with EGFR.

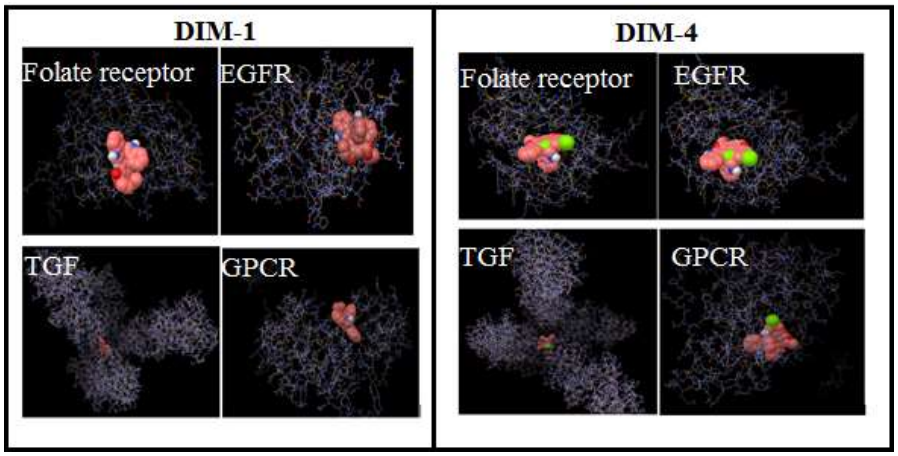


Figure 5.12. Docking analysis of DIM-1 and DIM-4 to various receptors: *The binding affinity of DIM -1 and DIM -4 with folate, EGFR, TGF and GPCR was studied using Autodock 4.2 .Ligand – protein docking was employed and the best binding conformations obtained with each receptor is shown.*

RECEPTORS	**BINDING ENERGY**	
	DIM-1	**DIM-4**
Folate	-7.78	-9.5
Tyrosine kinase	-9.91	-9.37
TGF	-7.9	-8.17
GPCR	-7.74	-9.5

Table 5.1. Binding energy of DIM-1 and DIM-4 with various receptors: *The free binding energy between DIM compounds and receptors were obtained from the docking software Autodock 4.2. The results indicate that Dim compounds showed better binding affinity with tyrosine kinase receptor.*

5.3.12. Inhibition of EGF induced proliferation by DIM-1 and DIM-4 in MDA-MB-231 cells

The autodock results along with the suppression of PI3K/AKT pathway indicated the involvement of EGFR mediated signaling in the anticancer activity of DIM compounds. Hence a proliferation study was designed employing EGF, which is one of the ligands of EGFR. MDA-MB-231 cells were seeded in normal and non-adherent plates, then the cells were subjected to serum starvation. This was followed by incubation of the cells in EGF supplemented medium for 1 h, followed by treatment with DIM compounds for 24 h. The proliferation of the cells were then observed through phase contrast microscopy (Figure 5.13.). From morphology of the cells it was evident that EGF increased proliferation in MDA-MB-231 cells in 2D and 3D culture. And it can also be stated that in both culture systems, DIM compounds were able to induce cytotoxicity in normal as well as EGF

supplemented cells indicating that EGF induced signalling was interrupted by the DIM compounds.

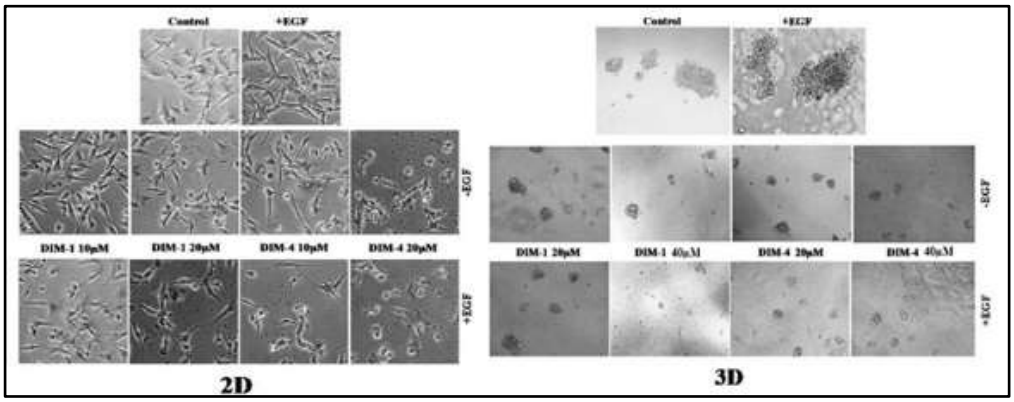


Figure 5.13. Phase contrast images of EGF induced proliferation study in 2D and 3D culture: *MDA-MB-231 cells were cultured under 2D and 3D conditions and subjected to serum starvation. This was followed by proliferation induction by EGF exposure for 1h. Then the cells were treated with DIM compounds for 24 h. The morphology of the cells were analysed by phase contrast microscopy. The images represent cells grown in 2D and 3D conditions, each system subjected to EGF induced and absent growth and the effect of DIM compounds in each system. Analysis indicate that DIM compounds inhibited EGF induced proliferation in cells grown in 2D and 3D conditions. The scale bar represents a size of 50 μm.*

The changes in proliferation was also quantified employing calcein staining, where the fluorescence was measured in situ in adherent and non-adherent cells. The relative intensity of fluorescence is shown in Figure 5.14. Based on fluorescence intensity, EGF treated cells showed an increased proliferation rate compared to deprived cells. In attached cells, EGF increased the proliferation of control cells almost by two fold. But DIM compounds reduced the proliferation of EGF supplemented cells by almost half of the control cells. In cells cultured in 3D conditions the reduction in proliferation was significant in EGF supplemented cells but not as pronounced as in the attached cells. The proliferation induced by EGF in control cells was more than twice that of un-induced cells in 3D system as well. The results indicate that EGF increased the proliferation rate of the cancer cells in 2D and 3D conditions, but DIM compounds were able to inhibit the proliferation induced by EGF in both the culture systems. Therefore it can be stated that DIM is able to suppress the signalling triggered by EGF.

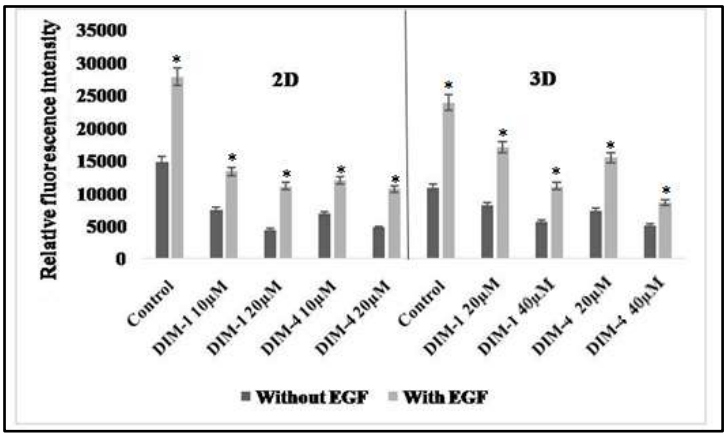


Figure 5.14. EGF induced proliferation study by calcein staining in 2D and 3D culture on treatment with DIM-1 and DIM-4: *MDA-MB-231 cells cultured under 2D and 3D conditions were subjected to proliferation induction by EGF exposure for 1h. Then the cells were treated with DIM compounds for 24 h. The proliferation rate was measured by calcein staining, which was measured. EGF induced proliferation in cancer cells, but this was decreased by the DIM compounds in both adherent and non-adherent culture. Represented values are means with SD represented by vertical bars.* indicates significant difference from control group ($p \leq 0.05$).*

5.3.13. Immunofluorescence studies on EGFR expression in MDA-MB-231 cells on treatment with DIM-1and DIM-4

The docking studies as well as the EGF induced proliferation studies indicated that DIM-1 and DIM-4 were capable of blocking the EGF mediated signalling for induction of cytotoxicity. The expression analysis of EGFR in cells treated with DIM-1 and DIM-4 was investigated next by immunofluorescence studies. On treatment with DIM-1 and DIM-4, MDA-MB-231 cells were labelled with fluorescent EGFR and pEGFR antibody, and analysed for variation in expression of the proteins. Figure 5.15. displays the confocal images of the FITC and DAPI labelled breast cancer cells. Interestingly in the control cells, EGFR fluorescence was seen localised inside the nucleus, while pEGFR was seen concentrated on the cell membrane. In EGF exposed cells the EGFR and pEGFR fluorescence was seen to be upregulated in the membrane as well as inside the cells. And on treatment with DIM-1 and DIM4 expression of EGFR was decreased in the outer membrane and inside the cells. pEGFR expression which was seen concentrated in the outer membrane in control cells was found downregulated on treatment with the DIM compounds. The results thus indicated that DIM-1 and DIM-4 interact with EGFR negatively leading to reduction in the activation of the receptors.

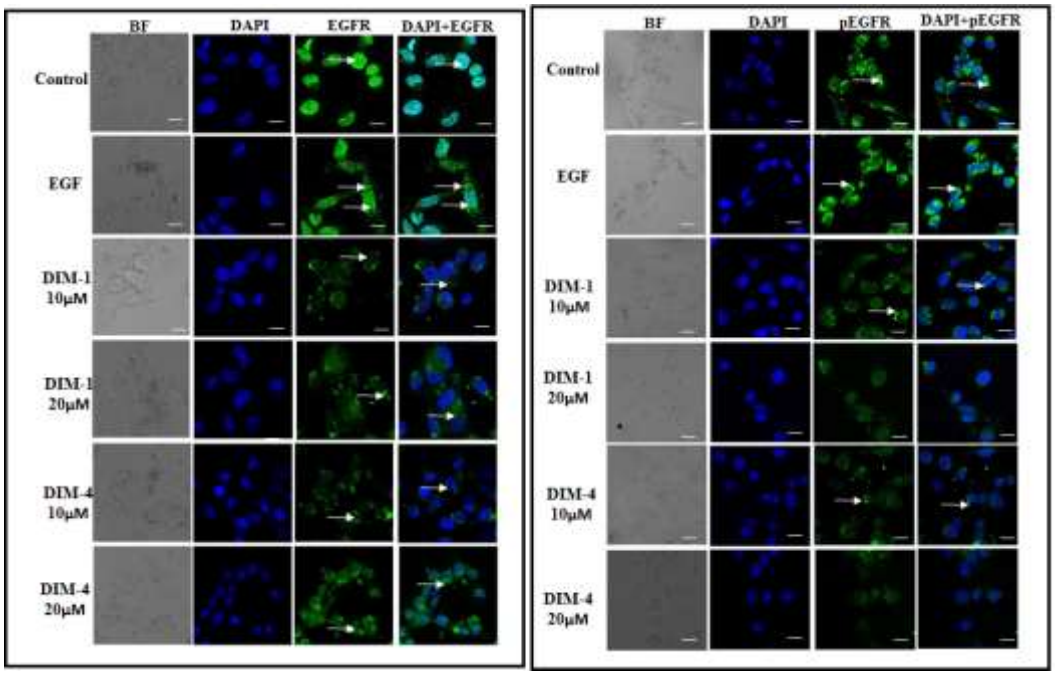
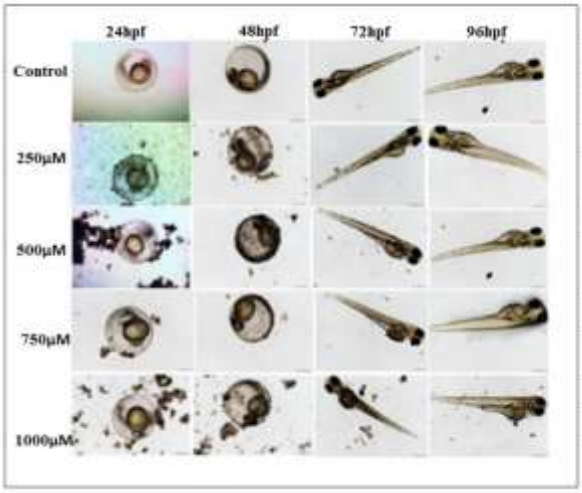


Figure 5.15. Effects of DIM-1 and DIM-4 on EGFR expression and activation: *MDA-MB-231 cells were analysed for EGFR and pEGFR expression by immunofluorescent staining. The images indicate that DIM compounds reduce the EGFR expression and pEGFR activation in the MDA-MB-231 cells. Bright field is represented as BF. Arrows indicate EGFR or pEGFR expression. The scale bar represents a size of 10 μm.*

5.3.14. Toxicological studies of DIM-1 and DIM-4 in zebrafish model system

The acute/lethal toxicity of the DIM compounds were tested in the embryonic stages of zebrafish. This is a standard test used to assess the toxicity of chemicals in a living system. If a chemical has toxic effects, it is manifested in the embryos as coagulation of embryos, lack of somites formation, lack of tail detachment and heartbeat. The freshly fertilized embryos were exposed to different concentrations of DIM-1 and DIM-4 for 24, 48, 72, and 96 hpf and examined for any lethal effects on the embryonic structure. Each set of experiments were carried out in replicates and the cumulative mortality % was calculated. The experiment revealed that DIM-1 and DIM-4 did not induce any morphological defects or lethality in the embryos. Figure 5.16. represents the microscopic images of embryo exposed to different concentrations of DIM-1 for varying time periods. Figure 5.17. represents the effect of DIM-4 on zebrafish embryo at different exposure period. Each experimental setup consisted of 30 embryos for each time interval and concentration and percentage of mortality was calculated. And results show that in every experimental setup the mortality % obtained was zero, signifying that both DIM-1 and DIM-4 had no impact on the zebrafish embryo either at higher

concentration or at increased exposure. This test gives sufficient proof regarding the non-toxicity of the DIM compounds in *in vivo* system, supporting its claim as selective anticancer compounds.



Duration	Replicates	Control	250 µM	500 µM	750 µM	1000 µM
24-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
48-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 1/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
72-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
96-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
Cumulative Mortality %		0%	0%	0%	0%	0%

Figure 5.16. Toxicological study of DIM-1 in the embryonic stages of zebrafish: *Freshly fertilized zebrafish embryo were exposed to varying concentrations of DIM-1 and observed at regular intervals for any lethal effects. The test revealed that DIM-1 did not induce any lethality/mortality in zebrafish embryo even at a higher concentration (1000 µM) or at maximum exposure period (96 hpf). The experiment was done in triplicates*

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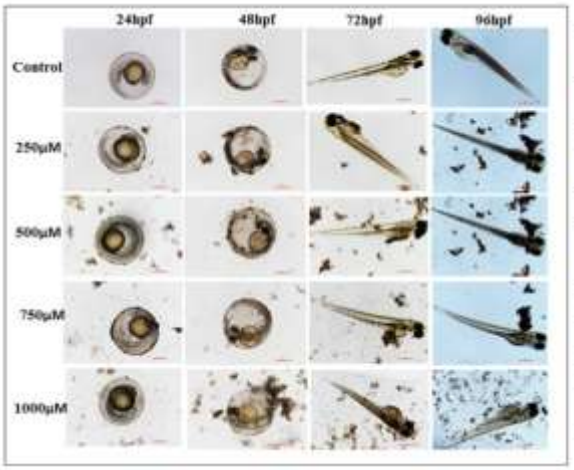

Duration	Replicates	Control	250 µM	500 µM	750 µM	1000 µM
24-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
48-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 1/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
72-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
96-hpf observation	R1 R2 R3	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10	0/10 0/10 0/10
Mortality %		0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%	0/30; 0%
Cumulative Mortality %		0%	0%	0%	0%	0%

Figure 5.17. Toxicological study of DIM-4 in the embryonic stages of zebrafish: *Newly fertilized zebrafish embryo were exposed to multiple concentrations of DIM-4 for different time periods and analysed for lethality. The microscopy images revealed that DIM-4 did not induce any noticeable defects in the embryonic stages at each time interval. The cumulative mortality % revealed that DIM-4 was non-toxic to zebrafish embryo till 96hpf even at higher concentrations. All experiments were carried out in triplicates.*

5.4. Discussion

Although the use of cell culture for drug screening and studies pose many advantages such as being economical, time-saving, high throughput, and minimizing the use of animals, they fundamentally lack the pharmacological

advantages of *in vivo* assays. Studies have also found that the conversion of tissue cells into *in vitro* cells, result in many metabolic alterations inherently changing the biochemistry of the cells (Cassim *et al*., 2017). Also, the literature indicates that cell culture activity may not be a good indicator of *in vivo* activity. The failure of successful translation of novel drugs from lab to patients has led to an impeded growth of the anticancer drug discovery research (Eastman *et al*., 2017).

Metastasis is the main reason for cancer-associated mortality. As a barrier towards metastasis, when normal lose contact with ECM or adjacent cells, detachment-induced apoptosis known as anoikis is initiated. Cancer cells acquire malignant potential by developing mechanisms to resist anoikis and thereby survive detachment and metastasis (Simpson *et al*., 2008). Many EMT-promoting proteins are also linked with anoikis resistance.

In vitro 3D cell culture techniques provide a solution for both these problems, as they afford better *in vivo* mimicking conditions, and provide anchorage independent studies for anoikis resistance (Abbott *et al*., 2003; Martian *et al*., 2017; Kang *et al*., 2007). For the present study, poly-HEMA was the chosen matrix used to induce an anchorage independent growth of MDA-MB-231 cells. MDA-MB-231 is a metastatic cell line which exhibits anoikis resistance. A recent study even reported the expression profile of these cells to determine the proteins responsible for anoikis resistance. And the breast cancer cells were forced to grow in culture dishes coated with the anti-adhesive polymer, poly-HEMA. The anticancer activity of DIM compounds obtained in 2D culture was compared to the anticancer effect of the compounds in cells grown in 3D conditions.

Poly-HEMA coated culture vessels were employed to establish a floating 3D culture of the MDA-MB-231 cells. The morphological analysis of the cells in 3D culture indicated the existence of cells as spheroids or aggregates. In the spheroids, the cells were found to be in close contact with each other and were connected by matrix like substance. In contrast, breast cancer cells in monolayer culture were more extended and spindle-shaped in morphology. A study by Ivers *et al*. 2014, reported that MDA-MB-231 cells exist in 3D culture, either as single motile cells or cell aggregates. Lawlor *et al*. 2002, conducted studies on multicellular spheroids of Erwin tumor cells, and found that the spheroids shared many similarities with the primary tumors in multiple aspects. According to Lee *et al*. 2015, a spheroid is formed due to the initial attraction of dispersed cells to form loose aggregates due

to their ECM fibers with RGD motif which binds to integrin resulting in the upregulation and accumulation of cadherin. The cells become more compact due to cadherin-cadherin interaction. And E-cadherin was found to be responsible for the tight packing in MDA-MB-231 cells (Ivascu *et al*., 2007).

The effect of DIM-1 and DIM-4 on MDA-MB-231 cells in 3D culture was investigated using MTT viability assay. One interesting fact regarding 3D culture was that the proliferation rate of cells was much reduced compared to 2D culture. A recent comparative study conducted by Souza *et al*. 2018, also reported the lowered rate of proliferation in 3D cultured cells. The main observation regarding the present study was that the concentration required to induce half maximal cell death in cells cultured in 3D culture was almost twice that of the attached cells, *i.e.,* the activity of the DIM compounds was reduced to half in the 3D cultured cells. Studies by Souza *et al.* reported increased chemoresistance of spheroids towards paclitaxel and docetaxel compared to its 2D counterpart. Another study by Dubois *et al*. 2017, compared the cytotoxic response of TNBC cell lines in 3D culture in the presence of increasing concentrations of *drugs like* cisplatin, docetaxel, and epirubicin, which revealed that spheroids were less sensitive towards chemotherapeutic agents than cells cultured in 2D system. These studies supported the requirement for increased concentration of DIM compounds for its cytotoxic action in the 3D system. To further strengthen the present result, anoikis assay was carried out, which showed a reduction in the proliferation rate of cells in the 3D system, and the reduced sensitivity of the cells towards DIM compounds requiring a higher dose for obtaining half-maximal inhibition. But the anoikis induction was evident in cells cultured in 3D condition.

The next parameter that was subjected to comparison was apoptosis induction. The current study revealed that DIM compounds triggered caspase 3 and caspase 9 activity in both 2D and 3D cells. But the increase in caspase enzymes was much pronounced in adherent cells. The increase in caspase activity in the 3D system was much reduced. A 2D vs. 3D comparative study by Imamura *et al*. 2015, revealed that caspase 3 activity was much lower in BT-474 cells cultured in the 3D system than in the 2D system, suggesting that the 3D environment was anti-apoptotic. The same study compared the caspase activity of primary cultured cells, in both systems, again finding lowered caspase activity in the 3D cultured cells. The apoptotic resistance of 3D spheroids was reported by Ikram *et al*. 2017, in

lymphoma cells, where anti-apoptotic proteins were upregulated (Bcl2), and pro-apoptotic proteins (Bax) were suppressed. To establish the apoptosis induction in the 3D system in the current study, western blot analysis of major apoptotic proteins were carried out which revealed that the DIM compounds were able to regulate the investigated proteins (Bax, Bcl2, Bad, c-PARP and cytochrome c) in a pro-apoptotic manner in the 3D cultured cells. This indicated that DIM compounds were able to induce apoptosis despite the resistant behaviour of the cells in a 3D environment.

Tumor microenvironment plays a key role in migration and metastasis. Hence the effect of DIM compounds on key tumorigenic ECM components, namely MMP-2, MMP-9, VEGF, and fibronectin were investigated in 3D culture. According to the studies by Ikram *et al*. on lymphoma cells, 3D spheroids showed an upregulated expression of VEGF and MMPs. In the present study, gelatin zymography revealed elevated expression of MMP-2 and MMP-9 enzymes in control cells in the 3D system. It also showed that in the 3D culture, the DIM-induced suppression of MMP enzymes was comparatively lower than the 2D system. But VEGF inhibition was significant in the 3D cultured cells indicating the strong angiogenic potential of the DIM compounds. According to Hazlehurst *et al*. 2003, fibronectin production is a prominent reason for the CAM associated drug resistance apart from spheroid formation. And many studies have been reported on the upregulation of fibronectin in spheroids and in the present study a marked downregulation of fibronectin was induced in both 2D and 3D system by the DIM compounds.

Integrins mediate cell survival by cell-ECM interactions and regulates major survival pathways such as PI3K/AKT and MAPK through FAK activation. In cancer cells, anoikis resistance is attributed to the deregulation of integrin-mediated cell survival, and anoikis signaling (Guan *et al*., 2010). FAK activation is considered necessary for anchorage-independent existence of tumor cells and its metastasis (van Nimwegen *et al*., 2005). A study by Walker *et al*. 2016, stated that in 3D culture of breast cancer cells, FAK activation resulted in the suppression of apoptosis. And in the current study, the expression of both integrin and FAK were found to be suppressed by DIM compounds in the 3D system, interrupting the integrin-mediated survival signalling and thereby triggering anoikis induction in the floating cells. DIM compounds also suppressed the expression of PI3K, AKT,

and mTOR proteins in the 3D system, which also complemented the earlier result of FAK inhibition, as FAK is an upstream regulator of PI3K mediated survival pathway. A recent study by Xu *et al*. 2017, provides a similar result where the inhibition of integrin/FAK signalling and downstream PI3K/AKT signalling resulted in growth arrest of malignant ovarian carcinoma. PI3K inhibitors reduced cell proliferation in 2D and 3D systems of lung cancer cells (Bonelli *et al*., 2015). Another spheroid-based study stated that the inhibition of proteins like Bcl2, EGFR, or IGF1R overcame the resistance to PI3K/mTOR inhibition (Muranen *et al*., 2012).

In the current study, autodock analysis of DIM compounds with various cell surface receptors usually overexpressed in cancer indicated a favourable affinity towards EGFR tyrosine kinase receptors. EGFR is usually found overexpressed in many cancer types including TNBC (Wee *et al*., 2017). And in addition, DIM compounds induced the downregulation of the survival pathway proteins, PI3K, AKT, and mTOR which are downstream molecules in the EGFR signal transduction pathway. All these results suggested the involvement of EGFR mediated inhibition of PI3K signalling. Many EGFR inhibitors and tyrosine kinase inhibitors have been approved by FDA as anticancer agents. Hence in the current study MDA-MB-231 cells were subjected to EGF induced proliferation assay in 2D and 3D system. The principle of the assay was that when EGFR binds to its cognate ligand EGF, it activates receptor associated proteins and triggers downstream effectors resulting in cell proliferation. And the results showed that EGF increased the proliferation in the control cells by activating the EGFR pathway and DIM-1 and DIM-4 were able to inhibit EGF induced proliferation. This indicated a disruption of EGF induced signalling by the DIM compounds. The involvement of EGFR in DIM activity was investigated by immunofluorescence studies. MDA-MB-231 cells treated with the DIM compounds were labelled with EGFR and pEGFR antibodies, which revealed the alteration in the expression of this receptor. In control cells, EGFR was found localised in the nucleus which indicated increased transcription and therefore cell survival. Inside the nucleus, EGFR functions as a co-transcription factor for several genes responsible for cell proliferation and angiogenesis. Nuclear localized EGFR is highly associated with poor prognosis in cancer, and chemoresistance (Brand *et al*., 2013). EGF exposure increased the expression and activation of surface EGFR indicating increased

proliferation. And treatment with the DIM compounds resulted in the downregulation of EGFR expression and its activation in the breast cancer cells. The results indicated that on treatment with the DIM compounds the activation of EGFR was significantly reduced. This suggested that the compounds negatively interacted with the receptor, resulting in its inactivation, which could explain the suppression of the PI3K/AKT/mTOR signalling and the subsequent apoptosis induction. Various studies in literature show that natural DIM interacts negatively with EGFR attenuating cancer cell growth. Studies by Kandala *et al*. 2012, presents an example for this where DIM suppresses ovarian carcinoma by blocking EGFR activation. Another example for the inhibition of EGFR/AKT axis by DIM is by studies by Loganathan *et al.* 2012.

The toxicological analysis of the DIM compounds using ZFET showed that DIM-1 and DIM-4 did not induce any toxic or lethal reactions in the acute embryo test indicating the safety of the compounds in a living system without undesirable toxic actions.

5.5. Conclusion

The anticancer activity of DIM compounds were compared in 2D and 3D cell culture and the results indicated successful translation of the anticancer effects of DIM compounds into a 3D system which is a representative model of an *in vivo* environment. The DIM compounds induced cytotoxic activity accompanied by caspase activation, regulation of apoptotic proteins, anti angiogenic effects, inhibition of integrin/FAK signalling, p53 stabilisation, and inhibition of PI3K/AKT/mTOR pathway. The inhibition of PI3K/AKT pathway was accompanied by a decrease in the activation of EGFR. The anticancer activity of DIM compounds could be EGFR mediated (Figure 1.18.). DIM compounds were found to be completely non-toxic in *in vivo* zebrafish model.

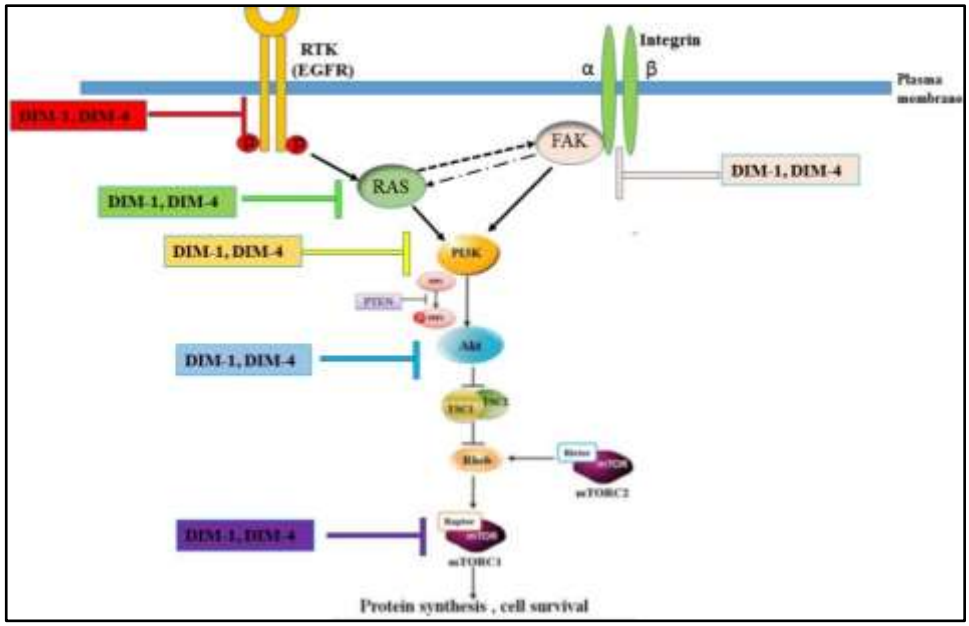

Figure 5.18. Suppression of EGFR/PI3K/AKT/mTOR pathway by DIM-1 and DIM-4 in MDA-MB-231 cells.

5.6. References

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Chapter 6

Summary and conclusion

Drug discovery in cancer biology focuses on discovery or synthesis of therapeutic strategies that present minimal side-effects and increased survival rate. Chemotherapeutic drugs have been found to be the most appropriate and favoured choice to mitigate the multifactorial disease. Given that many currently available drugs are derived from natural resources, the quest for novel anticancer drugs of natural origin is still rampant. The current study was centred on this rationale.

DIM is a natural compound found in cruciferous vegetables that has exhibited anticancer activity against various hallmarks of cancer through multiple targets. The current study investigated the *in vitro* anticancer activity of novel derivatives of DIM conjugate of biaryls in cervical and breast cancer cells. The DIM compounds which were synthesized by a one-step condensation reaction was a novel combination of two potential chemotherapeutic compounds, DIM and biaryls. Anticancer activity studies on these compounds have not been reported.

Chapter 1 discusses the literature associated with the study and **chapter 2** explains in detail the materials and methods required to accomplish the investigation.

Chapter 3 discusses the anticancer potential exhibited by potential DIM compounds (DIM-1 and DIM-4) in cervical cancer cells. The preliminary cytotoxicity screening of the parent compound DIM-biaryl derivative (DIM-1) and its 11 derivatives revealed that DIM-1 and DIM-4 induced potential cytotoxicity with low GI_{50} value of 11.00±0.707 µM and 8.33±0.416 µM respectively in cervical cancer cells while being nontoxic towards normal H9C2 cells. DIM-1 and DIM-4 were chosen for detailed analysis. The apoptosis induced by DIM compounds was accompanied by nuclear fragmentation, plasma membrane integrity loss, mitochondrial membrane depolarization, increased caspase 3 activity, and regulation of apoptosis-related proteins (Bax, Bcl2, c-PARP). DIM-1 and DIM-4 also inhibited the migration, angiogenesis, and cell cycle progression (G1 phase arrest) in cervical cancer cells. The study of cancer-associated proteins

revealed that the two potential DIM compounds also induced pro-apoptotic changes in the expression of p38MAPK, p53, and NFκB proteins. Thus it can be concluded that DIM-1 and DIM-4 induce apoptosis and cell cycle arrest in cervical cancer cells by regulating multiple cancer-associated pathways.

Chapter 4 investigated the anticancer effects of the novel DIM compounds in metastatic breast cancer cells. The cytotoxicity screening of the 12 novel derivatives showed that DIM-1 and DIM-4 showed good activity with GI_{50} values of 9.8 ±0.219 µM and 8.7±0.523 µM, respectively, while being non-toxic towards normal cells. The compounds induced apoptosis in breast cancer cells, which was accompanied by nuclear fragmentation, loss in membrane integrity, increased phosphatidylserine translocation, caspase 3 activity, and expression of proapoptotic Bax and decreased expression of antiapoptotic Bcl-2. The upregulation of caspase 9 activity, cytosolic cytochrome c release, and increased ROS generation suggested that the apoptosis was mitochondrial mediated. The compounds inhibited cell migration, the activity of MMP-2, MMP-9 and VEGF expression, but did not affect cell cycle progression or glucose uptake. This indicated that apoptosis induced by the compounds was not through cell cycle arrest or by regulating cell energetics. Upregulation of p53 and inhibition of FAK phosphorylation were also observed. Also, the phosphorylations of key signaling molecules in the PI3K-AKT-mTOR survival pathway were inhibited by these compounds. In conclusion, the studies indicated that DIM-1 and DIM-4 induced p53 mediated mitochondrial apoptotic cell death in breast cancer cells by downregulating the survival pathway PI3K-AKT-mTOR.

Chapter 5 compared the effects of DIM-1 and DIM-4 in MDA-MB-231 cells cultured in 2D and 3D conditions. This chapter aimed at studying the effects of the compounds into a more *in vivo* mimicking system to ensure its anticancer effect in a living system. Cytotoxicity studies revealed that cells cultured in the 3D environment required higher concentrations of DIM-1 and DIM-4 to induce a cytotoxicity comparable to the 2D system. The compounds upregulated caspase activity, and apoptosis-related proteins in both 2D and 3D system in a comparable manner. The migration and angiogenic markers were also downregulated in 2D and 3D conditions similarly. The integrin/FAK/p53 pathway and PI3K/AKT/mTOR pathway were downregulated in both culture systems in a similar manner. The suppression of the pathways suggested the involvement of upstream receptors.

Autodock analysis of the potential compounds with various receptors revealed an increased affinity with EGFR. DIM-1 and DIM-4 inhibited proliferation of EGF activated cells in both 2D and 3D culture conditions also indicating a receptor-mediated inhibition. Immunofluorescence studies have revealed an increased nuclear localisation of receptors in control cells which signifies increased protein translation. On treatment, the receptor expression was found to be significantly downregulated, while the active form of the receptor, phoshoEGFR (Y845) also was found to be significantly reduced, which is indicative of receptor inactivation.

The chapter also reports the non-toxicity shown by DIM-1 and DIM-4 in a living system (zebrafish embryo) established through Fish Embryo Acute Toxicity test.

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- "Epoxy azadiradione isolated from the *Azadirachta indica* induced mitochondrial mediated apoptosis in HeLa cells", in First International Conference on Neutraceuticals and Chronic Diseases, Cochin, Ernakulam, Kerala. September 2016. (Oral presentation).
- "Signaling pathways involved in p53 mediated apoptosis induced by a synthetic di (indolyl) methane derivative in metastatic breast cancer cells", in International Seminar on Recent Biochemical Approaches in Therapeutics, University of Kerala, Trivandrum, Kerala. January 2018, (Oral presentation).
- "Detachment induced cell death (Anoikis) promoted by a purified Lectin from *Morus Alba* leaves on human breast cancer cells", in National Seminar on Recent Approaches in Biochemical Research, University of Kerala, Trivandrum, Kerala. October 2014, in Trivandrum (Poster presentation).