SCREENING AND BIOLOGICAL CHARACTERIZATION OF A NATURAL AND SYNTHETIC ANTICANCER MOLECULE

A THESIS

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **"SCREENING AND BIOLOGICAL CHARACTERIZATION OF A NATURAL AND SYNTHETIC ANTICANCER MOLECULE"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2006 to June 2011 under the supervision of Dr. Partha Roy, Associate Professor, Department of Biotechnology and Dr. Kaushik Ghosh, Assistant Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(AJANTA CHAKRABORTY)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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The Ph.D. Viva-Voce Examination of **AJANTA CHAKRABORTY**, Research Scholar, has been held on 10/10/11

Signature of Supervisors

Signature of External Examiner

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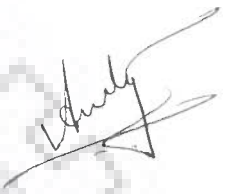

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List of Publications

1. **Chakraborty A**, Kumar P, Ghosh K, Roy P. Evaluation of a Schiff base copper complex compound as potent anticancer molecule with multiple targets of action. *Eur. J. Pharmacol.* 2010, 647(1-3):1-12.

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List of Abbreviations

μM	Micro molar
3 αHP	3α-dihydroprogesterone
5-DK	5-deoxykaempferol
5αHP	5α-dihydroprogesterone
7,3′,4′-THIF	7,3′,4′-trihydroxyisoflavone
ABCs	ATP-binding cassette transporters
ACAT	Acyl-CoA:cholesterol *O*-acyl transferase
AcOH	Acetic acid
AFI	Activation function element 1
AJCC	American Joint Committee on Cancer
ALP	Alkaline phosphatase
AMACR	α- methyl-acyl CoA racemose
AMPK	AMP activated protein kinase
AO	Acridine orange
Atg	Autophagic protein
ATP	Adenosne tri-phosphate
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
BH3	Bcl-2 homology domain
BSA	Bovine serum albumen
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
Cav-1	Caveolin 1
cc	Cubic centimeter
CDK	Cyclin-dependent kinase
CDKIs	CDK inhibitors
ChIP	Chromatin immunoprecipitation
CIS	Carcinoma in situ

Cisplatin	Cis-diamminedichloroplatinum (II)
COMT	Catechol-O-methyltransferase
CSF-1R	Colony-stimulating factor receptor
D8D7I	3β-hydroxylsterol-Δ^8-Δ^7-isomerase
dH_2O	deionised water
DHCR-7	3β-hydroxylsterol-Δ^7-reductase
dl	Decilitre
DMBA	Dimethylbenz(a)anthracene
DMSO	Dimethyl sulphoxide
DNA	De-oxy ribonucleic acid
DRs	Death receptors
DT	Doubling time
DTT	Dithiothreitol
EB	Ethidium bromide
ECM	Extracellular matrix
EDTA	Ethylene di-amine tetra acetic acid
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ER	Estrogen receptors
ERE	Estrogen responsive elements
ERK	Extracellular-signal-regulated kinases
EtOAc	Ethyl acetate
g	Gram
GC-MS	Gas chromatography - mass spectrometry
GSH	Glutathione reduced
GSSG	Glutathione oxidized
H_2O_2	Hydrogen peroxide
HAT	Histone acetyl transferaseFIGO

HER-2	Human epidermal growth factor receptor -2
HOCl	Hypochlorus acid
HPLC	High performance liquid chromatography
HRT	Hormone Replacement Therapy
IC_{50}	Half inhibition concentration
IF	Inhibitor function region
IGF	Insulin like growth factors
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin like growth factor binding protein
IL	Interleukin
FIGO	International Federation of Gynecology and Obstretics
JNK	c-Jun N-terminal protein kinases
kg	Kilogram
KLK4	Kallikrein 4
LC3	Light chain proteins of microtubulin
LD_{50}	Half lethal dose
LOH	Loss-of-heterozygosity
MAPK	Mitogen associated protein kinases
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDR	Multidrug resistance
MDR1/P-gp	MDR1/P-glycoprotein
Mg	Miligram
MGMT	O6-methylguanine DNA methyltransferase
mm	Milimeter
MMPs	Matrix metalloproteinase
MNU	N-Methyl-N-Nitroso Urea

mRNA	messenger RNA
MRP1	Multidrug resistance associated protein
mTOR	Mammalian target of Rapamycin
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADH	Nicotinamide dinucleotide
NADPH	Nicotinamide dinucleotide phosphate
NBT	Nitroblue tetrazolium
NF-κB	Nuclear factor of κB
NIH	National Institute of Health
NIPER	National Institute of Pharmaceutical Education and Research
nM	Nano molar
nm	Nano meter
NO	Nitric oxide
$O2^-$	Superoxide anion
OH	Hydroxyl radical
ORO	Oil red o
PARP	Poly-(ADP-ribose) polymerase
PBPE	N-pyrrolidino-4-(phenylmethyphenoxyl)-ethanamine,HCl
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptor
PI	Phosphatidyl Inositol
PMSF	Phenyl methyl sulphonyl fluoride
pNP	Para nitrophenol
pNPP	Paranitrophenol phosphate
PR	Progesterone receptors
PRE	Promoter
PTEN	Phosphatase and tensin homolog tumor suppressor gene
PTP	Protein tyrosin phosphatase

PVDF	Polyvinylidene fluoride
RARβ	Retinoic acid receptor β
RIP	Rho Interacting Protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
S.E.M.	Standard error of mean
SAPK	Stress-activated protein kinases
SDF-1	Stromal cell derived factor
SDS	Sodium dodecyl sulphate
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SGR	Specific growth rate
SH3	Src homologue domain
SIM	Selected ion monitoring mode
SRC1	Steroid receptor co-activator 1
SREBP1c	Sterol regulatory element-binding protein 1c
STAT	Signal transducer and activator of transcription
TDLU	Terminal Ductal Lobular Unit
TGF β	Tumor growth factor β
TIMP	Tissue inhibitor of matrix metalloproteinase
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
TNM	Tumor Necrosis Metastasis
TRAIL R1	Tumor necrosis factor related apoptosis-inducing ligand receptor 1
TSC	Tuberous Sclerosis Complex
UICC	International Union Against Cancer

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World health organization

Abstract

The present thesis entitled "**Screening And Biological Characterization Of A Natural And Synthetic Anticancer Molecule**" deals with anticancer properties and the mechanism of action of a novel Schiff base copper complex and pterostilbene in breast cancer treatments.

Breast cancer is the commonest malignancy in women. Since the past two decades, rates from breast cancer have increased at an alarming rate. Cancer is a silent disease which develops through years of undiagnosed symptoms. The use of hormone replacement therapy (HRT) for the cure and management of several diseases related to menopause and age has caused advert effect and aided the development of breast cancers (Campagnoli et al., 2009). Moreover, already available chemotherapeutic drugs which are commonly used have not given a cure to the disease and have a lot of side effects. Hence is the requirement for the search of new molecules with better efficacy and low toxicity.

The **objectives** of present study are to initially screen both novel synthetic compounds and plant extracts to find an effective compound from each category then understanding each of their molecular mechanisms as to elucidate how they can be inhibitory to breast cancer cell proliferation. The first stage biochemical and microscopic analysis led to the study the molecular pathways mainly by reverse transcriptase polymerase chain reaction and western blot analysis. Interactions with major proteins are also confirmed by few transactivation studies. Not only the efficacy of the compounds is checked in *in vitro* cell based systems, but their effect was validated in the breast cancer tumor models developed with MNU (Thomson and Adlakha, 1991).

At the beginning, **Chapter 1** introduces briefly the present scenario of the chemotherapeutic drugs available to the patients. It also deals with the key factors present in cancer cells that need to be targeted. These cancer cell characteristics can serve as major points to guide the screening of novel molecule for their respective efficacy. Finally detailed goal to be attained in the study is specified here.

Followed by this, **Chapter 2** presents (i) a detailed review of general genetic abnormalities related to cancer and the recent molecular targets; (ii) the characteristic anomalies of breast cancer; (iii) a vivid description of the till date discoveries for designing of synthetic chemicals which guides the selection of the Schiff base copper complexes among others; and

(iv) recent research detailing benefits of phytochemicals and their characteristics to target multiple pathways in cancer therapy; and finally (v) the hypothetic idea behind the present thesis to manipulate the cancer cells towards death. Each of these hypotheses is explored in subsequent chapters of the thesis.

In **Chapter 3**, the basic principles of anticancer screening assays adapted to preliminary shortlist molecules that bear cytotoxic properties are specified. Whether cytotoxicity is related to apoptotic death or any other form of cell death is then studied. Cancer cells are highly proliferating cells where the mitotic index is very high with chromosomal anomalies (Kops et al., 2005). Although a cell can die in a number of processes, the main form of cell death that is targeted in cancer cells is a sequestration of a sequence of events, rightly called as programmed cell death or apoptosis (Kerr et al., 1972). In this concern, this chapter contains the overall principles of all the experimental assays that were performed to identify a molecule to be anticancer compound. This is explained as stage I, the biochemical parameters like MTT screening, chromatin body formation, DNA fragmentation patterns, activation of caspases are studied initially to select a novel drug; and in stage II, the principles of the biological mechanisms are studied at transcriptional and translational expression levels of various genes to understand how a molecule acts intracellularly, mainly the assays which show the basic determination of the underlying pathways.

Investigating the above features in **Chapter 4** the anti-cancer properties of a series of copper centered synthetic molecules and crude extracts of medicinal plants are investigated. Copper is a biologically relevant metal due its association with various biomolecules related to essential physiological activities. Anticancer compounds having a copper as a metal center is hypothesized to be less toxic and more potent. The series, designated as a family of Schiff base copper complexes, is screened and the best compound [Cu(Pyimpy)Cl_2] where Pyimpy is a tridentate ligand containing two pyridine and one imine nitrogen donor is selected. [Cu(Pyimpy)Cl_2] represented here as CuP1, is found to be cytotoxic at the lowest concentration in the series. It is found to show DNA binding in vitro and caused apoptosis in MCF 7 cells as observed by acridine orange staining assay. In the next part similar cytotoxicity screening assays are performed to select the best of the plant extract tested. The methanolic extracts are only considered for the screening procedures. The basic idea for the screening was to check the effect of polyphenolic fractions in these crude extracts. The results are also validated for apoptosis

characteristic by acridine orange staining. The phytochemical screening results led to the selection of the methanolic extract of the heartwood of *Pterocarpus marsupium* as most effective against MCF-7 breast cancer cell lines.

Next, in **Chapter 5**, the biological characteristics of CuP1 are further tested. The effect of the synthetic CuP1 on DNA content of the MCF-7 is validated by comet assay and DNA fragmentation assay. Not only this, the study also focuses on whether the molecule activates caspases, which is a positive marker of apoptosis. Finally the copper Schiff base compound is tested on rat breast tumor models and the characteristic pathways which lead to the reduction in breast tumor volume is investigated.

After the initial screening and selection of the methanolic extract of *Pterocarpus marsupium* in chapter 4, the present chapter deals with the purification and characterization of the lead compound in the extract for anticancer properties. Maurya et al., (1984) showed that the major content in the heartwood *Pterocarpus* sp. is mainly stilbene. The pterostilbene, a dimethyl ester derivative of resveratrol is thus isolated in **Chapter 6**. Thereafter, the isolated pure compound is tested for a similar efficacy in the breast cancer cell lines as was obtained with the extract. Finally the pathways leading to apoptosis are checked biochemically and then at the transcriptional and translational level. Finally the efficacy of the molecule is tested on the rat mammary tumor model. It is worth mentioning here that the involvement of reactive oxygen species is found as a basic mechanism to activate apoptosis by pterostilbene. This effect is somewhat different than the general trend of activation of phytochemicals. Hence the involvement of ROS as an effector agent in the action of pterostilbene is further taken as a matter of study in the following chapter.

Because of the fact that mode of action of pterostilbene is found to be mediated by the induction of mitochondrial oxidative stress **Chapter 7** initially observes the effect of pterostilbene in presence of ROS scavengers. The results show that the molecule not only apoptosis, low and sub-acute dose of pterostilbene ceases the mitotic and metastatic potential in MCF-7 cell lines although they were still live. This study shows that at low dose and long term treatment pterostilbene leads to a gradual accumulation of neutral lipids in MCF-7 cells and characterize them to differentiate into more of epithelial like morphology. Simultaneously, it shows that there is an induction of autophagic characteristics which are adapted by the breast cancer cells as an alternative form of cell death due to long term exposure to the stilbene

molecule. Thus this study shows for the first time, that pterostilbene increases in the expression of adipogenic differentiation marker c/EBPα and intracellular accumulation of the oxysterols and induces the phenomenon of autophagy in breast cancer cells. Studies on tamoxifen and its metabolite supports the present findings very strongly (Medina et al., 2009; Payre et al., 2008).

In the next section (**Chapter 8**), the effect of pterostilbene is studied on the progesterone receptor positive breast cancer cell lines. This study is in continuation to the observed fact in chapter 6 that pterostilbene is also effective in the progesterone receptor (PR)-positive cell lines. Progesterone receptor is expressed in breast cancers along with estrogen receptor (Hoskins et al., 2009). This study focuses on the inhibition of the PR mediated pathways by pterostilbene in T47D cells. Pterostilbene inhibits the PR transcription and expression. It also inhibits the progesterone up-regulated breast cancer markers Kallikrein 4 which known to involve in cancer cell metastasis. The results in this chapter also describes that pterostilbene inhibits the localization of PR to the nucleus as seen in the PR-GFP transfection studies and the immunoblot analysis. Finally the PR antagonism of pterostilbene is checked at the in vivo animal models.

Finally, **Chapter 9** summarizes the complete work in brief and the future prospects that is still to venture. The scientific findings dealt with in this thesis may be of use to the future researchers working in this area. And finally the list of bibliographies which was consulted in course of the present work is presented in **Chapter 10**.

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Introduction

Cancer is a disease that is responsible for the death of almost 70% of human population within 5 years of detection. Cancer research is an extremely broad topic and mere focus on the basic biological mechanisms underlying the control of tumor development and then on clinical aspects, is a long journey for therapeutic drug search. Research for the development of new strategic therapies against this disease is still on. This has led to the search for novel drugs and understanding of their mode of actions.

Breast cancer is the commonest malignancy in women. Since the past two decades, deaths from breast cancer have increased with the use of hormone replacement therapy (HRT), which utilizes a combination of estrogen and progesterone and is one of major therapeutic choice for the cure and management of several diseases related to menopause and age (Campagnoli et al., 2009). Apart from genetic causes such as mutations in tumor suppressor genes (BRCA, p53) (Brugarolas and Jacks, 1997), the environmental mutations (DNA damage due to carcinogen exposure from tobacco) (Hecht, 2002) are also responsible for the increase in breast cancers in women at an alarming rate (Jemal et al., 2007). The anti-estrogen molecules (eg. tamoxifen) are commonly used for controlling this disease but the use of such anti-hormone drugs generally results in drug-resistance with simultaneous induction of more aggressive forms of breast cancers (Fisher et al., 1996; Peto, 1996). Moreover, there is a simultaneous activation of several cell proliferation pathways at the tumor milieu. Therefore, mere targeting of hormone-receptor functions cannot be the only therapy for controlling these highly growing cells.

One of the interesting features of breast is that it is an organ where the apoptotic pathway is naturally active. A balance between cell proliferation and apoptosis controls the normal development of breast (Reed, 1999; Tamm et al., 2001). The breast cells proliferate and differentiate during, puberty: first stage of incomplete development, and pregnancy: the final stage of complete development (Kumar et al., 2000). Following lactation there is a massive restructuring and apoptosis leading to involution and a return to a primary breast structure (Kumar et al., 2000). The three main female hormones, estrogen, progesterone and prolactin tightly control all these cycle of events.

Targeting breast cancer is a major challenge in cancer therapy. The drug should arrest the hormone mediated cell proliferation and induce a potent apoptosis in the cancer cells specifically, leaving the normal cells at their own functions. In order to achieve this goal an initial understanding of the following are needed in a cancer cell : (i) the presence of serum growth factors in an individual; (ii) growth factors at the tumor microenvironment; (iii) the receptors at the cancer cell surface; (iv) the amount and nature of intracellular proteins that cause cell division; (v) the amount of tumor suppressor and apoptotic proteins present in a tumor cell; (vi) the genetic mutations and finally; (vii) the metabolic status of a cancer cell. All these will provide clues to design a drug to target only the cancer cells in particular. Among these, the relative amount of the mitotic and apoptotic protein collaborators have led to the identification of biomarkers from various microarray analyses of the tumor and normal tissues (Finak et al., 2008). Targeting the exact network of these proteins, to finally check mitosis is a herculean task for the cancer biotechnologists.

In spite of all these complexities, some specific drug targets have been already identified in cancer cells. Some of these are summarized below: (i) The Akt pathway: Akt phosphorylation and dephosphorylation status mainly mediates the intracellular growth-promoting cascade (Brognard et al., 2007). This protein not only activates other quiescent mitogenic proteins but also supplies the cancer cells with proper energy status for its functional significance (Elstrom et al., 2004). The inhibitors of this pathway are gaining importance in the molecular therapy. (ii) Cyclin-CDK pathway: The cyclins and cyclin dependent kinases (CDK) regulate cell cycle. The gain-of-function of cdks and the loss-of-function of inhibitor kinase is a second approach which a drug targets (Gojo et al., 2002). (iii) Apoptotic and autophagic pathway: The activation of cell death machineries by apoptosis and autophagy control excessive cell proliferation. Some drugs cause self-destruction of cells by these pathways (Maiuri et al., 2007). (iv) DNA damage pathway: The cancer cells harbor a large number of mutations in the DNA. The DNA repair enzymes in cancer cells are more prone to skip these mutations. A possibility to cause a general high up-regulation of DNA damage that is irreparable by the repair enzymes is an approach to kill these obnoxious body cells (Gurung et al., 2010). (v) Oxidative stress: The redox potential of the cancer cells differs from that of the normal cells. The antioxidant enzymes cannot efficiently maintain the normal redox state of a transformed cell. Increase in overload of the mitochondrial membrane potential is also a major point to target cancer (Henry-Mowatt et al., 2004). (vi) Tumor suppressor protein pathways: Activation of tumor suppressors (like p53,

PTEN etc.) from inactive state to free and active form can lead to the control of cell proliferation pathways (Beltran et al., 2008). (vii) Cancer cell metabolism: Inhibition of cancer cell metabolism by the inhibition of glucose uptake pathways in cancer cells is an important strategy to induce nutrient stress to the cancer cells (Thompson et al., 2005). (viii) Immune system: Activation of the immune system of the individual to target cancer. New discoveries of certain drugs that can stimulate the immune cells of the body to clear off the cancer cells are very promising (Dunn et al., 2002; Steinman and Banchereau, 2007).

The major focus of this work is to study the anticancer properties of some novel molecules and thereby correlating their effects in prevention and management of breast cancer. In recent years, there has been an explosion of life-saving treatment advances against breast cancer, bringing new hopes and excitements. However, hormonal (anti-estrogen) therapy, chemotherapy, perhaps radiation, and/or surgery are the only clinical options for the patients. None of these has proved to provide a definite cure to the disease. This is one among the hundreds of new findings to figure an ideal drug for the treatment of cancer. Overall, the study attempted to achieve the following objectives.

1. Screening of a group of synthetic molecules for their anticancer properties.
2. Testing the efficacy of the most potent synthetic molecule using in vitro and in vivo approaches.
3. Screening of a group of plant extracts for their anticancer properties.
4. Purification of a lead molecule from the most potential plant extract.
5. Understanding the mode of action of the purified phytochemical in vitro and in vivo in breast cancer in relation to activation of cell death machinery and the redox status of the cells.
6. Understanding the signal transduction cascade of the most efficient anticancer molecule leading to growth arrest in breast cancer and its cross talk with steroid receptors

CHAPTER 2

REVIEW OF LITERATURE

2 Review of literature

"Beneath the complexity and idiopathy of every cancer lie a limited number of 'mission critical' events that have propelled the tumor cell and its progeny into uncontrolled expansion and invasion. One of these is deregulated cell proliferation, which, together with the obligate compensatory suppression of apoptosis needed to support it, provides a minimal 'platform' necessary to support further neoplastic progression. Adroit targeting of these critical events should have potent and specific therapeutic consequences. The mantra from the cancer research community has been that cancer is not a single disease for which there will be a single cure, and the task of developing therapies suitable for treatment of the full gamut of cancers is depicted as Herculean and almost impossible". - Gerard I. Evan & Karen H. Vousden

2.1 Cancer in General

Cancer is a group of diseases in which genetically altered abnormal cells divide without control and can invade nearby tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems (NIH dictionary of Cancer terms). Cancer cells communicate with the cellular environment through delivery of surface proteins, release of soluble factors (growth factors and cytokines), and sophisticated nanovehicles (exosomes) for establishment of invasive tumor growth. This communication occurs in part through constitutive exocytosis, regulated exocytosis, or release of intraluminal vesicles (Hendrix et al., 2010). The altered mitogenic signaling pathways in cancer cells facilitate sustained cellular proliferation, angiogenesis, insensitivity to cell death, invasion and migration, escape from immunosurveillance and senescence. The development of tumor is a slow and long-term process. A concerning statistics of this slow killer has been reported by the National Cancer Institute, USA, that new cancer cases and deaths for 2010 is approximately 1,529,560 and 569,490 respectively. These figures are a matter of concern and require proper understanding of the mechanism of cancer.

2.2 Development of a cancer cell is ideally rare

Awareness of the evolutionary nature of cancer offers a number of important insights into the malignant process. First, and perhaps most striking, is the rarity of the cancer cell. An estimated mutation rate (as given by Oller et al., 1989) is 1 in 2×10^7 per gene per cell division and the fact that there are some 10^{14} target cells in the average human. An abundant repertoire of genes regulate all aspects of cell expansion and it is remarkable that cancers should theoretically arise in only once in three lifetimes of an individual (Evan and Vousden, 2001). This is even

more striking when one considers that oncogenic mutations, by their nature, foster clonal expansion of the affected cell. Therefore propagating the initial mutation and thereby increasing the number of target cells available for (and hence the probability of) further oncogenic mutation will be still higher. The rarity of cancer highlights the efficacy of potent anti-tumorigenic mechanisms presiding over somatic cells. Cancers prevail only when all these master mechanisms have failed (Evan and Littlewood, 1998) (Fig 2.1).

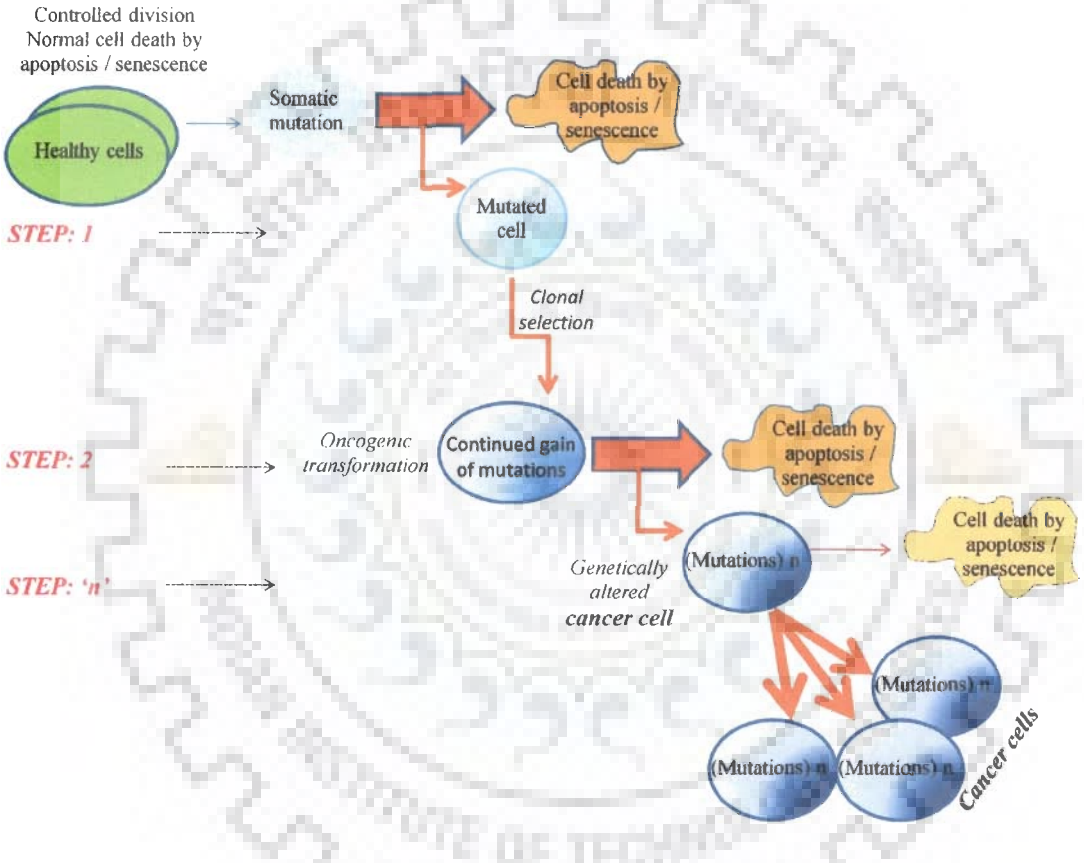


Fig. 2.1 *Schematic representation of the origin of cancer cells after accumulation of mutations over a long period of time*

Therefore, the lesions that power the relentless proliferation of tumor cells, and the compensatory mutations that arise to ensure their survival are the major factors that take over the normal cell division machinery over a long time, may be over more than many years. The challenge before the research community is to identify and understand the molecular anatomy of such pivotal steps in tumor progression and to develop therapies that directly attack these points of convergence.

2.3 Why do cancer cells no longer obey normal regulation?

Cells become genetically and physiologically modified because of repeated mutations in the oncogenic alteration (Fig. 2.1). Regulation of cell growth is inexorably linked to regulation of metabolism, and the unique ways that cancer cells deregulate cell growth and metabolism distinguish them from normal cells. The phosphatidylinositol (PI) 3-kinase pathway functions in growth factor receptor signal transduction to activate the cell growth and proliferative responses to nutrients and growth factors. Here is a role of growth hormones mediating these pathways. Insulin like growth factors (IGF), steroids, epidermal growth factors that are present in the tumor microenvironment (stroma), are among the major stimuli to trigger the protein kinase pathway for cell proliferation. The result is activation of Akt (protein kinase B) (Fig. 2.2). Akt inhibits the tuberous sclerosis complex (TSC) and thereby activates mTOR (Hemmings, 1997). mTOR in turn activates pathways that promote translation, cell cycle progression, nutrient uptake and glycolysis. The ultimate result is cell division, protection from cell death and rapid but inefficient ATP production by glycolysis. Activation of the PI 3-kinase pathway is an exquisite formula for driving proliferation of tumor cells, as a result of which constitutive activation of this pathway is so common in tumors (Downward, 2004). PI3K is a kinase that plays central role in signaling pathways important to cell survival, proliferation, motility, and tissue neovascularization. PI3K is upregulated in many cancers. (Cantley, 2002; Hidalgo and Rowinsky, 2000). Cell-surface receptors induce the production of second messengers such as phosphatidylinositol 4,5-bisphosphate 3 and phosphatidylinositol 3,4,5- trisphosphate, which convey signals to the cytoplasm from the cell surface. Phosphatidylinositol 4,5-bisphosphate 3 signals activate the kinase 3-phosphoinositide-dependent protein kinase-1 (PDK), which in turn activates the kinase, Akt. Akt activation leads to phosphorylation of certain proteins that lead to cell survival (Cantley, 2002). For example, phosphorylation of IκB by Akt leads to activation of NFκB that promotes survival. Phosphorylation of Bad by Akt leads to its inactivation and blocking of the apoptotic signals. In addition, phosphorylation of caspase 9 blocks the induction of apoptosis. Akt also phosphorylates the protein Forkhead-related transcription factor 1. Several proteins important in human cancers can be dysregulated in the PI3K pathway. Epidermal growth factor stimulation activates Akt via PI3K, (Lin et al., 1999) as does HER-2/neu activation (Wen et al., 2000). Loss of phosphatase and tensin homolog tumor suppressor gene (PTEN) also augments the activity of this pathway (Cantley and Neel, 1999). Abnormalities in

cyclin D, p53, pRB, p16, and p27 can increase the PI3K activity in many cancer cells. These control cell cycle progressions from G1 to S phase, and their inhibition leads to growth arrest (Hidalgo and Rowinsky, 2000). Constitutive activation of the PI 3-kinase pathway is achieved in multiple ways, such as activation of PI 3-kinase itself, inactivation of its inhibitor (the tumor suppressor PTEN), or activation of Akt and its downstream signals. A common feature of human solid tumors is their reliance on

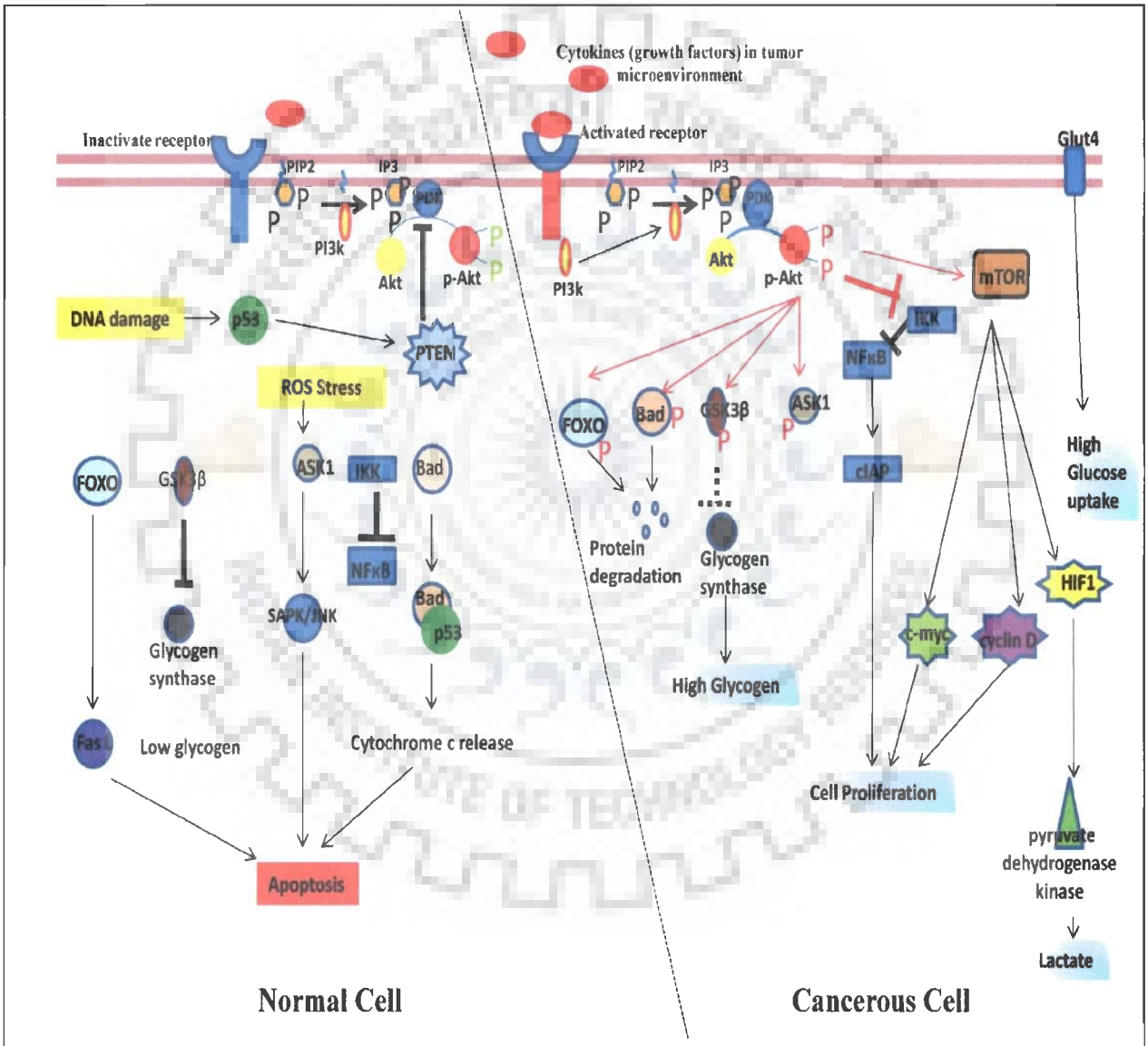


Fig. 2.2: Akt-PI 3-kinase pathway in normal and cancer cells

glycolysis even under aerobic conditions – the Warburg effect (Warburg, 1956). Glycolysis is triggered by oncogene activation, including activation of ras, Akt and myc, and by hypoxia in the tumor microenvironment through HIF-1α induction. Akt is also thought to be involved in

pathways that control the availability of nutrients acting through AMP activated protein kinase (AMPK), which controls glucose and lipid metabolism by sensing changes in nutrient and extracellular energy levels (Engelman and Cantley, 2010). Glycolysis might also be triggered by the accumulation of damaged mitochondria that have an impaired capacity for ATP generation through oxidative phosphorylation (Dang et al., 1997; Elstrom et al., 2004; Gatenby and Gillies, 2004; Semenza, 2003). This suggests that the Akt-mediated oncogenic pathway could be regulated by nutrients. Therefore it is suggested that increased activation of PI3K-AKT-mTOR pathway contributes to immortalization of cells. In this regard, the Warburg effect could be considered as a positive modifier of cancer and can be targeted specifically only to cancer cells. All of these events commonly occur in human tumors. Whether or not the dependence on glycolysis directly contributes to tumor growth is unclear. Glycolysis is a less efficient means for ATP generation compared with oxidative phosphorylation. Thus, the more a tumor cell is dependent on glycolysis, the more crucial is its requirement for nutrients such as glucose. To compensate for inefficient energy production, many of the mechanisms that promote glycolytic metabolism also facilitate uptake of nutrients from extracellular sources (Thompson et al., 2005). Another source of nutrients is catabolism achieved by activation of the autophagy pathway (Jin and White, 2007). Thus, it is found that cancer cells adapt to the nutrient deficiency by easily switching over to low energy glycolysis through PI 3-kinase pathway and can tolerate the hypoxic conditions generated thereby. Normal cells are found to die (autophagy / apoptosis) in case of nutrient stress or hypoxic condition. The inherent adaptation to energy stress along with the presence of extracellular growth factors in the tumor microenvironment relieves the tumor cells from the general growth control that is active in normal cells. Moreover, the cancer cells have active ubiquitin proteasome system which degrades the short lived proteins and cause cell survival differentially (Jana et al., 2001).

2.4 General Breast Cancer

The main theme of the present work was to target cancer in female breast with the help of synthetic molecules and dietary polyphenols. Cancers may occur anywhere in the body and are named according to the organ where it originates. The breast is usually made up of fatty, connective and lymphatic tissue which constitute the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). In breast cancer the cells usually forms a lump or mass called tumor. Most of these masses are benign and do not grow or spread uncontrollably. Some breast cancers are in situ or benign and do not spread. Cancers can be ductal carcinoma or

lobular carcinoma. These can be cured if they do not reach the next stage of metastasis. According to the survey by American Cancer society (2009-2010) most of the breast tumors are invasive and infiltrating. They start as lobular or ductal carcinoma and spread to other parts of the body like lymph nodes, lungs, liver, brain and bone via blood stream (Price et al., 1997). This phenomenon is called metastasis. Breast cancer occurs in both men and women, although male breast cancer is rare (Guinee et al., 1993). Breast cancer is the leading cause of cancer death in women between the ages of 15 and 54, and the second cause of cancer death in women with age group of 55 to 74 (Jemal et al., 2010). Seventy-one percent of black women diagnosed with breast cancer experience a five-year survival rate, while eighty-six percent of white women experience five-year survival. In a survey of the cancer death statistics in USA the cancer death rates between 1991 and 2006 decreased by 12.3%, with decreases in breast and colorectal cancer rates accounting for 60% of the total decrease (Jemal et al., 2010).

2.5 Structure of normal breast and tumor breast

Breast is a specific target where the steroid hormones estrogen and progesterone act and cause the development of the both normal mammary gland and breast cancer (Lapidus et al. 1998; Russo and Russo 1998). The development of mammary gland occurs throughout the female life and can be divided into several stages that differ in morphology, function, and hormonal responsiveness (Ronnov-Jessen et al., 1996; Russo and Russo 1998). Since birth to puberty the chances that occur in the mammary gland is common in both male and female. From the onset of puberty, the female mammary gland responds to the production of the ovarian steroid hormone, estrogen and the epithelium branches into numerous ducts with terminal endbuds or alveoli which are collectively referred to as terminal ductal lobular unit (TDLU) (Fig.2.3). Initially TLDU is composed of 6 to 11 ductules per lobule (Lobule type 1) which progresses to lobule type 2 with higher number of ductular structures per lobule in the post pubertal virgin gland with the onset of menstrual cycle (Russo and Russo 1998). During pregnancy the elevated levels of gonadal steroid hormones (estrogen and progesterone) and the pituitary hormones (prolactin, oxytocin and endorphine) stimulate both lobule type 1 and lobule type 2 to progress to lobule type 3 (Fig 2.3). Lobule 3 is characterized by epithelial expansion of the existing pubertal alveoli to 80 small lobules per alveoli. These changes prime the mammary gland for the milk secretion from the alveoli and called the secreting mammary gland lobule type 4 (Fig 2.3). After parturition, the lactating mammary gland becomes insensitive to estrogen dependent regulation of growth. When the breast-feeding is stopped the post-weaning involution

phase starts and the breast again becomes responsive to estrogen. Finally, with the cessation of lactation, the alveoli collapse and the mammary gland regresses apoptotically to its resting pre-pregnancy state revering lobule type 3 to type 2 with more extensive framework of branching than lobule type 1. Thus female breast experiences recurrent cycles of regulated growth and differentiation, apoptosis and regression with the constant responsiveness and unresponsiveness to various hormonal status (Ronnov-Jessen et al., 1996, Russo and Russo 1998) (Fig 2.3).

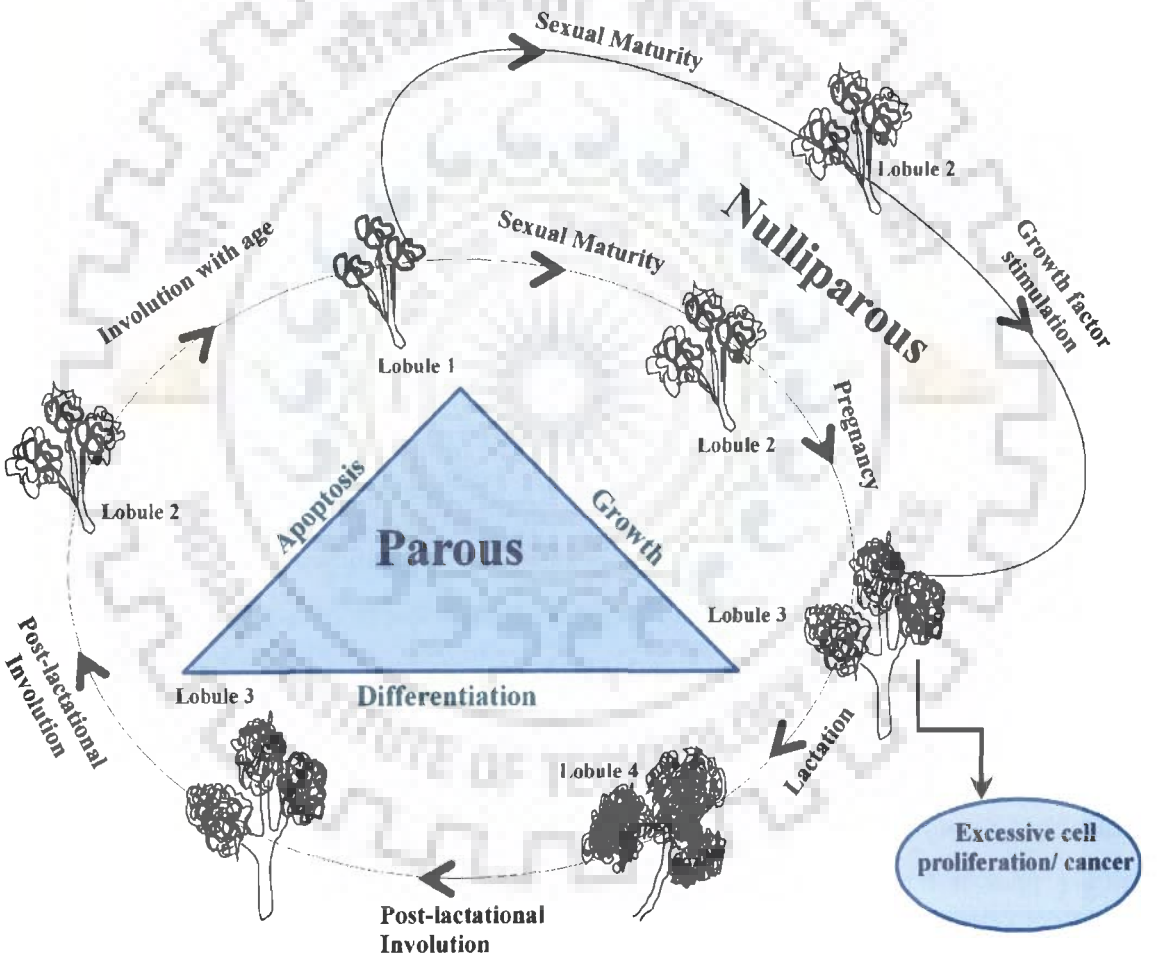


Fig. 2.3: *Schematic diagram showing the changes in mammary gland throughout the female lifetime*

While the development of cancer in breast, few cells become genetically modified and try to form a solid mass due to uncontrolled growth, loss of contact inhibition and apoptosis. They gradually evades the host immune surveillance processes and start gaining invasive

properties (El-Ashry and Lippman, 1994; Thompson, 1994). As these cells acquire these cellular functions, they eventually become the monoclonal malignant focus. In the basic mammary tissue architecture, these may occur in the glandular epithelium with cellular features of malignancy although with no infiltration. These structures are called carcinoma in situ (CIS). The carcinoma can be restricted to lobules (lobular carcinoma in situ) and ducts (ductal carcinoma in situ). Both these lesions arise in terminal duct lobular unit (Beckmann et al., 1997). These structures can again take various shapes and are named accordingly as comedo, cribriform, papillary, micropapillary, signet cell etc. When the cancer cells become invasive and spread to other parts of the body, they are referred to as invasive carcinomas. The majority of invasive carcinomas are referred to as ductal (85–95%). Within this group there are some tumors with characteristic features and an individual name (Histological typing of breast tumors, WHO, 1981). These encompass about 15–20% of all breast cancers, for example, tubular, medullary, mucinous, papillary, adenoid cystic, metaplastic, apocrine, squamous, secretory, lipid-rich, and cystic hypersecretory (Holland and Hendicks, 1994; Ernster et al., 1996).

According to the severity of the primary site of cancer and whether or not it has spread to different organs, National Cancer Institute staged tumors into different categories according to the Cancer Staging Factsheet. The TNM system is one of the most widely used staging systems. The TNM system is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of distant metastasis (M). A number is added to each letter to indicate the size or extent of the primary tumor and the extent of cancer spread. This system has been accepted by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). PDQ®, NCI's comprehensive cancer information database, also uses the TNM system. Primary Tumor is represented by T, primary tumor that cannot be evaluated (TX), no evidence of primary tumor (T0). Tis, is carcinoma in situ (CIS) abnormal cells are present but have not spread to neighboring tissue; although not cancer, CIS may become cancer and is sometimes called pre-invasive cancer. T1, T2, T3, T4 denotes size and/or extent of the primary tumor. (N) represents regional Lymph Nodes tumor. Regional lymph nodes that cannot be evaluated (NX), no regional lymph node involvement (N0), and N1, N2, N3 denotes involvement of regional lymph nodes (number of lymph nodes and/or extent of spread). Distant Metastasis is represented by M. MX denotes distant metastasis cannot be evaluated, no distant metastasis (M0), M1 is distant metastasis is present. TNM combinations correspond to one of five stages: Stage 0 is carcinoma in situ. Stage I, Stage II, and Stage III:

higher numbers indicate more extensive disease *i.e.* larger tumor size and/or spread of the cancer beyond the organ in which it first developed to nearby lymph nodes and/or organs adjacent to the location of the primary tumor. Stage IV means the cancer has spread to another organ(s) (NCI, USA, Factsheet about cancer staging, as reviewed on 09/22/2010).

The Indian Minister of State for Health S. Gandhiselvan said that by 2020, it is expected that breast cancer will overtake cervical cancer at the current rate of increase in cancer cases (August 17, 2010, IANS). This is a figure of concern among the Indian oncologists. Risks for breast cancer include a family history, atypical hyperplasia, delaying pregnancy until after age 30 or never becoming pregnant, early menstruation (before age 12), late menopause (after age 55), current use or use in the last ten years of oral contraceptives, and daily consumption of alcohol. Breast cancer typically produces no symptoms when the tumor is small and most treatable. When breast cancer has grown to a size that can be felt, the most common physical sign is only a painless mass. Sometimes breast cancer becomes metastatic and spreads to underarm lymph nodes and cause a lump or swelling, even before the original breast tumor is large enough to be felt. Less common signs and symptoms include breast pain or heaviness; persistent changes to the breast, such as swelling, thickening, or redness of the breast's skin; and nipple abnormalities such as spontaneous discharge, erosion, inversion, or tenderness (Breast Cancer Facts & Figures, American Cancer Society, 2009-2010).

2.6 The tumor micro-environment

Tumor tissues modify the local environment according to their requirements. The tumor microenvironment was lately recognized as the product of a developing crosstalk between different cells types. For instance, in epithelial tumors these cells include the invasive carcinoma and its stromal elements. Critical stromal elements include cancer-associated fibroblasts, which provide an essential communication network via secretion of growth factors and chemokines, inducing an altered extracellular matrix thus providing additional oncogenic signals enhancing cancer-cell proliferation and invasion (Kalluri and Zeisberg, 2006). The tumor microenvironment has an additional inflammatory response (Leek and Harris, 2002). Active contribution of tumor-associated stromal cells and macrophages to cancer progression has also been recognized (Hanahan and Weinberg, 2000; Liotta and Kohn, 2001). Stromal elements consists of the extracellular matrix (ECM) as well as fibroblasts of various phenotypes, and a scaffold composed of immune and inflammatory cells, blood and lymph vessels, and nerves.

However, it is required to answer how exactly do cancer cells acquire these traits. Recent scientific evidence points towards cancer cells using activated transcription factors from developmental programs, thus gaining pleiotrophic abilities. This means that similar gene expressions that had occurred during the developmental processes are again stimulated in the adult cells. The microenvironment is of critical importance for success in this process (Weinberg, 2007). Major autocrine and paracrine factors in the tumor micro-environment are matrix metalloproteinase (MMPs) (Gingras et al., 2003), vascular endothelial growth factor (VEGF) (Ferrara et al., 2004; Holash et al., 2002; Hurwitz et al., 2004), vascular endothelial growth factor receptor (VEGFR) (Manley et al., 2004; Morgan et al., 2003; Wedge et al., 2002), nuclear factor of κB (NF-κB) (Bartlett et al., 2004; Sleijfer et al., 2004), tumor necrosis factor (TNF) (Bartlett et al., 2004; Sleijfer et al., 2004), interleukin (IL) (Bartlett et al., 2004; Sleijfer et al., 2004), platelet-derived growth factor receptor (PDGFR) (Druker, 2004; Pietras et al., 2003; Mendel et al., 2003), tumor growth factor β (TGF β) (Kojima et al., 2010), colony-stimulating factor receptor (CSF-1R) (Pietras and Hanahan, 2005), stromal cell derived factor (SDF-1) (Kojima et al., 2010) etc. The tumor microenvironment not only provides the niche for the growth of these specialist tumor cells but also helps them escape the immune surveillance.

2.7 Tumor micro-environment of breast cancer

As already described, breast cancer is one of the representatives of solid tumor which progresses very slowly in middle-aged women. The tumor stroma itself is not a uniform entity, and is comprised of several elements, including chiefly fibroblasts, neovessels (endothelial cells), immune cells, and blood cells (Fearon and Vogelstein, 1992). Just two decades ago, only few researchers would have imagined that somatic genetic and genomic alterations could also occur in stromal cells apart from the known mutations in tumor cells. One such pioneer who dared to "think outside the box", Fattaneh Tavassoli and his group, showed, for the first time, that genetic events, specifically loss-of-heterozygosity (LOH) at microsatellite markers could occur in breast cancer stroma (Moinfar et al., 2000). The stroma specific LOH occurred at markers on 11q21-q23, 3p14.2, 16q23-q24, and 17q24 (Moinfar et al., 2000). The frequency of stromal LOH in the invasive carcinomas was higher than those in the ductal carcinoma *in situ*. This suggests that genetic alterations in the tumor stroma accumulate to contribute to tumorigenesis (Moinfar et al., 2000). Genomic and chromosomal alterations have been found in a broad variety of solid tumors and nonmalignant conditions. LOH, microsatellite instability, consistent cytogenetic abnormalities, and/or telomere attrition in stroma have been independently

described by different investigators for a broad variety of solid tumors and nonmalignant conditions such as head and neck squamous cell carcinomas, colorectal adenomas and carcinomas, barrett esophagus and esophageal adenocarcinomas, esophageal squamous cell carcinomas, and carcinomas of the cervix, ovary, bladder, and prostate, as well as inflammatory bowel disease (Man et al., 2001; Bian et al., 2007; Paterson et al., 2003; Ishiguro et al.. 2006; Matsumoto et al., 1989; Matsumoto et al., 2003; Yagishita et al., 2008; Hu et al., 2006a). A total genome LOH scan using almost 400 microsatellite markers on epithelium and stroma from 134 sporadic invasive breast carcinomas revealed that the frequencies of LOH in the epithelium were higher than those in the corresponding markers in stroma, but the number of markers affected by LOH in the stroma was significantly higher than that in the epithelium (Fukino et al., 2004). Thus, one hypothesis stemming from these observations and interpretation would be that genetic and genomic alterations within tumor stroma would affect clinical outcome.

2.8 The necessity of targeting breast stroma

Cancer is a very slow disease and is detected only when enough number of tumor cells has accumulated at the site of origin and cause some distraction to the physiological conditions of the normal body cells. In invasive breast carcinoma the cells move mostly to the lungs and lymph nodes although the migration to other organs is also quite common. Loss of stromal caveolin 1 (Cav-1) is a novel biomarker for cancer-associated fibroblasts that predicts poor clinical outcome in breast cancer and DCIS patients. When the mammary epithelial cancer cells are co-cultured with fibroblasts, the cancer cells have the ability to drive Cav-1 downregulation in adjacent normal fibroblasts, thereby promoting the cancer associated fibroblast phenotype. Co-culture of immortalized human fibroblasts with MCF7 breast cancer cells leads to Cav-1 downregulation in fibroblasts (Martinez-Outschoorn et al., 2010). In order to investigate metastatic solid tumor–metastatic microenvironment interactions, a similar *in vitro* three-dimensional co-culture system was constituted with prostate cancer epithelium and bone stroma, mimicking metastatic prostate cancer to bone (Sung et al., 2008). Led by Leland Chung, these investigators showed that permanent consistent cytogenetic and epigenetic alterations occurred in the bone stroma when co-cultured with prostate cancer epithelium (Sung et al., 2008). Importantly, they also showed that these stromal-specific alterations were secondary to reactive oxygen species. These prostate cancer–influenced bone stromal cells were highly inductive of human prostate cancer growth in mice, and were shown to express high levels of extracellular matrix and chemokine genes, such as those encoding versican, *BDNF, CCL5, CXCL5,* and

CXCL16 (Sung et al., 2008), all of which have been found to be elevated in human metastatic prostate cancer as well. Phytochemicals (like genistein) has a general tendency to get oxidized in the hypoxic environment (D'Alessandro et al., 2003) and can thereby be responsible for protecting the tumor stomal inflammatory response. It is therefore important to target the cells of the tumor microenvironment. A single tumor cell may express more than one survival pathways. So targeting merely one pathway may be effective in in vitro culture but it is rather not very successful in the in vivo tumor model. The next generation search for the anticancer drug therapy is the development of new molecules that affects multiple pathways simultaneously.

2.9 Factors associated with breast cancer

Breast is a specific target where the steroid hormones mainly estrogen and progesterone acts and causes the development of the both normal mammary gland and breast cancer (Lapidus et al., 1998). It is often linked with over- expression of estrogen and/or progesterone receptors (ER and/PR). Almost 75% of breast cancers are ER positive and out of that majority (about 65%) are also progesterone receptor-positive (Hoskins et al., 2009). Early menarche was more consistently associated with ER/PR-positive breast cancers (Althius et al., 2004). Apart from the hormonal regulation, breast cancer is marked by the over-expression of human epidermal growth factor receptor type 2 (HER2) (Ross and Fletcher, 1998) or insulin-like growth factor-1 receptor (IGF-1R) (Canzian et al., 2006; Cheng et al., 2006; Setiawan et al., 2006). At an early stage there is a development of genetic polymorphisms in the breast tissue.

2.9.1 Estrogen receptor

The estrogen receptor (ER) is a steroid receptor which acts as a transcription factor for a number of genes and is over expressed in breast cancer. Apart from acting at the genetic level the receptor also interactions with other cellular proteins to mediate their functions. This class of receptor regulates the expression of genes involved in cell proliferation and/or differentiation (Dontu et al., 2004; Platet et al., 2004). Binding of an estrogen (or an antiestrogen) causes a conformational change in the receptor leading to their dimerization, strong association with DNA and recruitment of co-activators (or co-repressors) as well as other transcription factors (Leclercq, 2002). The ligand activated hormone-receptor complex is responsible for the interaction with promoters of cyclin D1, c-myc, progesterone receptor, FOXA1 and many other transcription factors (Fig. 2.4) (Carroll et al., 2005; Dubik and Shiu, 1992; Petz and Nardulli,

2000; Sabbah et al., 1999). The gene expression profiling on microarrays have identified potential ER target genes in human breast cancer cells. Estrogen responsive elements (ERE) have been identified within the 1kb 5'-proximal region of the estrogen-regulated genes TFF-1 (pS2), EBAG9, and Cathepsin D (Augereau et al., 1994; Berry et al., 1989; Ikeda et al., 2000). It also interacts with other transcription factors like p53 and protects its degradation. Apart from these functions the receptor binds to a number of co-activators CBP/p300, SRC, TIF-2 or co-repressors like NCoR, SMRT, DAX to finally tune its transcription (Kurebayashi et al., 2000). Three related coactivators named as p160s, (SRC-1, TIF2, and AIB1) interact with CBP and activate the activation function (AF-2) domain of ER-α. SRC-1 and CBP exhibit autonomous histone acetyl transferase (HAT) activity. Both p160s and CBP bind to a potent HAT. It has been believed that HAT activity of these coactivators may enable them to remodel chromatin and allow access to the transcriptional machinery (Kurebayashi et al., 2000). On the other hand different ER corepressors regulate steroid receptor activity through a variety of mechanisms, including formation of multiprotein complexes that are able to affect chromatin remodeling, histone deacetylation, or basal transcription. Other mechanisms include competition with coactivators, interference with DNA binding and homodimerization, alteration of receptor stability, sequestration in the cytoplasm, and effecting RNA processing. Most ER co-repressors can control the receptor's activity through more than one mechanism, and it is possible that the synergy between different pathways cooperates to fully inhibit ER transcriptional activity, and create an integrated response to a variety of different cellular signaling pathways (Dobrzycka et al., 2003). Apart from the over-expression of estrogen receptor in breast cancer, ER polymorphisms in intron 1 have no known functional effect on gene expression (Nedelcheva et al., 1999) although intronic polymorphisms may affect the post-transcriptional processing of gene products and receptor binding affinity. Once the steroid receptor mediated transcriptional machinery is activated, the cell proliferation molecules like cyclin D1 and c-myc in turn switch-on other growth promoting factors that help the cancer cells to divide and finally transform. Long exposure to estrogen leads to the development of breast cancer. Estrogens are oxidized in the breast first to catechols and then to form two ortho-quinones ($E_{1/2}$-3,4-Q) that reacts with DNA to form depurinating adducts leading to mutations (Singh et al., 2009). These DNA adducts serve as an important etiology of breast cancers (Gaikwad et al., 2008).

2.9.2 Progesterone receptor

Out of a majority of hormone responsive breast cancers, about 65% are also progesterone receptor-positive (PR) (Hoskins et al., 2009). Early menarche is more consistently associated with ER/PR-positive breast cancers. Progesterone receptor is another major steroid hormone receptor that functions as a ligand mediated transcription factor. Apart from the transcriptional regulation, PR binds to a number of cytosolic proteins and regulates their actions. Progesterone-PR complexes have been reported to activate PI3K/Akt/NFκB pathway to initiate cell cycle (Saitoh et al., 2005), Wnt/β-catenin survival signals (Cloke et al., 2008) and decreases the expression of BIM, a tumor suppressor (Labied et al., 2006). Such an effect is due to the conventional N-terminal DNA binding domain or the SH3 domain interaction but occurs through the interaction of phosphorylation sites of PR with cyclin D1 (McGowan et al., 2007). Both these genomic and non-genomic actions of progesterone have been shown even in breast and endometrial tissues (Ballare et al., 2006). PR binds to non-liganded ER and modifies its function as a proliferative transcription factor (Ballare et al., 2003). This fact could be complementary to the role of PR as an antiproliferative protein at least in ER responsive cancers but this is not always the case in breast cancers. In breast cancers the PR adds to the proliferation caused by ER. PR exists in two isoforms PRA (~94kDa) and PRB (~116kDa) which are slightly distinct in function, structure and intracellular distribution. Basically, PR-A is truncated form of PR-B lacking the first 164 N-terminal amino acids. Two distinct promoters within the single copy gene for PR have been shown to independently regulate the expression of PR isoforms (Kastner et al., 1990; Pieber et al., 2001). Further, it has also been reported that the PR isoforms in breast and endometriod cancers are differentially expressed. Other than FIGO grade I tumors (in which the tumors are graded according to the histological nuclear chromatin structure by International Federation of Gynecology and Obstretics- FIGO) other tumors expressing PR do not bear 1:1 ratio of PRA:PRB which aids to its proliferative effects (Arnett-Mansfield et al., 2001). This may be due to the functional antagonism of the effecter genes to PR. A major difference exists in the differential sub cellular localization of PR, which results in its associations with MAPK for its extra nuclear proliferative effects (Qiu and Lange, 2003). PR-B is present both in the nucleus and cytoplasm whereas PR-A is strictly nuclear (Boonyaratanakornkit et al., 2007) and PR-A and PR-B, respectively has 2 and 3 MAPK induced phosphorylation sites. Thus PR not only acts on the genes bearing progesterone response element, but it also interacts with other genes bearing estrogen response element or c-

src, c-myc, ERK1/2, MEK or ras binding sites (Boonyaratanakornkit et al., 2008) which are prominently responsible for cell proliferation (Fig 2.4). Further, PR isoforms also has differences in the transcriptional activities (Narayanan et al., 2005b; Pierson-Mullany and Lange, 2004; Shen et al., 2001; Takimoto et al., 1996), turnover rates (Takimoto et al., 1996; Narayanan et al., 2005b), protein complex formation and target gene specificity (Qiu et al., 2003; Takimoto et al., 1996). Hence PR is found to exhibit both proliferative and anti-proliferative activities. The expression of pure homodimers of PR-A and PR-B has shown that they act as repressors and activators of transcriptions, respectively (Mohammed et al., 1994). Further, the DNA binding domain contains the sites for phosphorylation, sumoylation, ubiquitination and acetylation (Weigel, 1996; Lange et al., 1998; Abdel-Hafiz et al., 2002). In case of PR-B, 14 residues can remain phosphorylated while 8 residues in case of PR-A of which few sites are basally phosphorylated and few gets activated upon ligand binding (Lange, 2004). Protein kinases like casein kinase, mitogen-activated protein kinase, cyclin dependent kinase can also cause PR phosphorylation upon activation by growth factors (Weigel et al., 1995; Shen et al., 2001). Moreover, progesterone can be linked to single nucleotide polymorphism (SNP) of PR since it has been reported that valine to leucine polymorphism of PR (V660L) increases the risk of breast cancer (Pijnenborg et al., 2005; Pooley et al., 2006). Further an inhibitor function region (IF) has also been characterized at 292 amino acids upstream of activation function element 1 (AFI) which is known to inhibit the function of both activation function elements (AFI and AFII). Since PR-B contains an additional activation function element (AFIII) which is not inhibited by IF, that could explain the functional difference between these receptor isoforms (Hovland et al., 1998). The activity of this receptor is also guided by the cellular status of co-activators and co-repressors similar to that of ER. Apart from this, the hormone, progesterone, is metabolized in the target tissue like breast to form 5α-dihydroprogesterone (5αP) and 3α-dihydroprogesterone (3 αHP) by 5 α-reductase and 3 α-hydroxysteroid oxidoreductase enzymes, respectively (McGowan et al., 2007). But these two metabolites are completely antagonistic in nature. While 5αP is tumorigenic the other metabolite i.e. 3αHP is an apoptotic molecule. Wiebe *et al.* (2009) recently showed that the proliferation of breast cancer cells, both estrogen responsive and unresponsive, can be suppressed by blocking the former and/or increasing the latter. The differential presence of progesterone metabolites have been reported among various tumorous and normal breast tissues irrespective of age, subtypes, grades of carcinoma, estrogen or progesterone positive and/or negative (McGowan et al., 2007). Till date no study has been

proposed using either these metabolites or their analogues as drugs. These may act as the key modulators for the downstream signaling of effecter cascade to cause cell proliferation or apoptosis.

2.9.3 Human epidermal growth factor receptor-2

The breast cancers are also marked by a high expression of human epidermal growth factor receptor -2 (HER-2) (Ross and Fletcher, 1998). The HER-2/neu oncogene encodes a transmembrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor. HER-2/neu has been widely studied in breast cancer. HER-2/neu gene amplification has been associated with the development of breast cancer in animal models (Slamon and Clark, 1988). The HER-2/neu protein is a component of a four-member family of closely related growth factor receptors, including EGFR or HER-1 (erb-B1); HER-2 (erb-B2); HER-3 (erb-B3) and HER-4 (erb-B4) (Lupu et al., 1995).

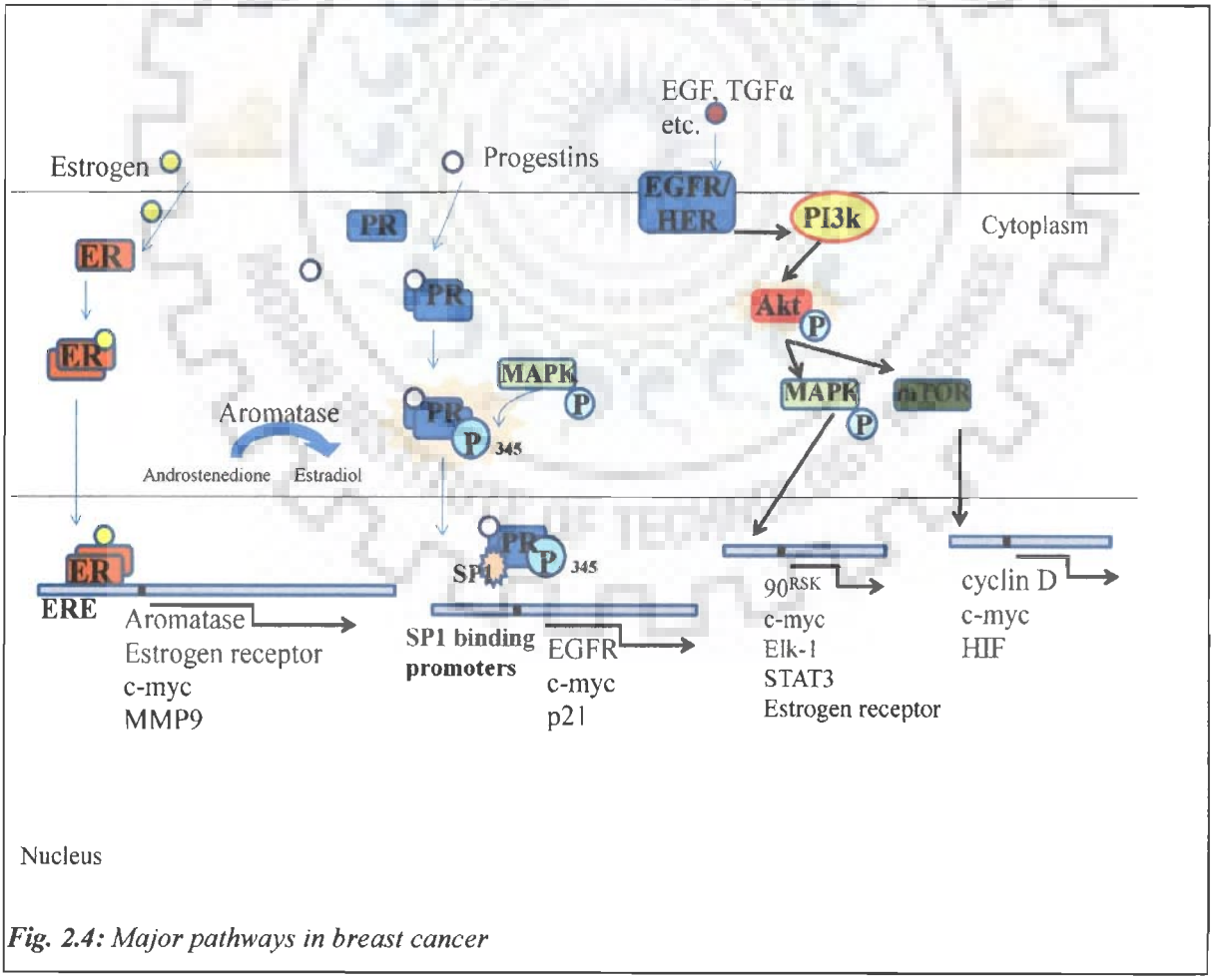


Fig. 2.4: Major pathways in breast cancer

In addition to its association with disease, over-expression of the HER-2/neu protein has been reported in 10 to 34% of breast cancers (Ross and Fletcher, 1998). There are at least 30 ligands with selectivity for one or more c-erbB receptors. EGF, TGFα, AR, HB-EGF, BTC, and EPR are ligands for EGFR and are involved in cell proliferation (Alroy and Yarden, 2000; Harari and Yarden, 2000; Yarden and Sliwkowski, 2001). It is therefore associated with oncogenicity and coupled to the ras-MAPK signaling pathway (Vazquez-Martin et al., 2007) (Fig. 2.4). Overexpression of c-erbB-2 is associated with up-regulated gelatinases (Yu et al., 1992). Hergulin a ligand of HER-2 and the TGF-β selectively increases expression of MMP-9 (Kwok et al., 2009; Xu et al., 1997), and also uPA and uPAR (Chambers et al., 2001).The latter was shown to be mediated via p38MAPK and MEK, and to enhance both cell motility and invasion. Increased MMP-2 expression and decreased TIMP-2 were found in human mammary epithelial cells (MCF10A) transfected with H-ras, c-erbB-2, or both (Giunciuglio et al. 1995).

2.9.4 Tumor suppressors BRCA1, BRCA2

The two most important breast cancer susceptibility genes, BRCA1 and BRCA2, were identified by linkage analysis and positional cloning in the 1990s (Miki et al., 1994; Wooster et al., 1995). Mutations in BRCA1 and BRCA2 are rare, but confer high risks of breast cancers. Well over 1000 different mutations have been identified in BRCA1 and BRCA2, and genetic testing for mutations in these genes in high-risk families is now well established (Walsh et al., 2006). Recent studies reveal that the BRCA proteins are required for maintenance of chromosomal stability in mammalian cells and function in the biological response to DNA damage (Venkitaraman, 2001). BRCA1 interacts of with the BACH1 and BARD1 proteins, and pleiotropic mutations in BRCA1 may be associated with defects in protein–protein interactions. In contrast, the role of BRCA2 in DNA repair may be more defined by its direct interaction with the RAD51 recombinase (Liu and West, 2002).

2.9.5 Other mutations

Studies reveal that either growth factor receptors like insulin like growth factor binding protein (IGFBP) bears polymorphism located in the promoter region of the gene which may increase serum IGF binding protein 3 (IGFBP3) levels (Deal et al., 2001) and increased signaling. Others like steroid metabolism enzymes like Catechol-O-methyltransferase (COMT) develops valine to methionine (Val158Met) polymorphism which decreases enzyme activity and increase in catechol estrogen metabolites thereby causing DNA damage (Lachman et al., 1996;

Singh et al., 2005b). Also the other wound response signature (380 genes) (Chang et al., 2005), hypoxia-associated transcriptional response (123 genes) (Chi et al., 2006) are linked to breast cancer progression as found in the microarray analysis of the transcriptome.

2.10 Types of cancer cell death

Cell death occurs either by apoptosis, autophagy or necrosis.

2.10.1 Apoptosis

Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The understanding of apoptosis has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy (Lowe and Lin, 2000). Apoptosis occurs through two main pathways. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily (Zapata et al., 2001). The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal (Hockenbery et al., 1990). Both these pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell. Apoptosis is marked by rounding-up of cells, retraction of pseudopodes, reduction of cellular and nuclear volume (pyknosis), nuclear fragmentation (karyorrhexis), plasma membrane blebbing and engulfment by resident phagocytes (in vivo) – according to the definition of Kerr et al. (1972). Although the features of apoptosis are not a synonym to programmed cell death but it is obviously a sequestration of events definitely leading to cell death. Biochemically apoptosis can be distinguished by the activation of proapoptotic family of proteins (Bax, Bak, Bid etc.), activation of the apoptotic enzyme cascade of caspases, mitochondrial membrane potential dissipation resulting in the mitochondrial membrane permeabilization, oligonucleosomal DNA fragmentation and phosphatidyl serine exposure (Galluzzi et al., 2007; Golstein and Kroemer, 2007; Tasdemir et al., 2008).

2.10.1.1 The Extrinsic Pathway

This pathway comprises several protein members including the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases

8 and 10, which ultimately activate the rest of the downstream caspases leading to apoptosis (Zapata et al., 2001) (Fig 2.5). Activation of the extrinsic pathway is initiated with the ligation of cell surface receptors called death receptors (DRs) to Fas ligand of an adjacent cell. Fas is a member of the tumor necrosis factor receptor superfamily and is also called Apo-1 or CD95. Other TNF receptors include TNF R1, DR3 (Apo 2), DR4 (tumor necrosis factor related apoptosis-inducing ligand receptor 1 [TRAIL R1]), DR5 (TRAIL R2), and DR6 (Zapata et al., 2001). Regulators of the pathway include transcription factors such as NFκB and activating protein 1 (AP1) that regulate the FasL gene, because it is a transcriptionally inactive gene (Wajant, 2002).

2.10.1.2 The Intrinsic Pathway

One of the most important regulators of this pathway is the Bcl-2 family of proteins. The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1 (Reed, 1994). Antiapoptotic Bcl-2 members act as inhibitors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members act as activators. These effects are more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone (Reed, 1997). Following a death signal, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis (Scorrano and Korsmeyer, 2003).

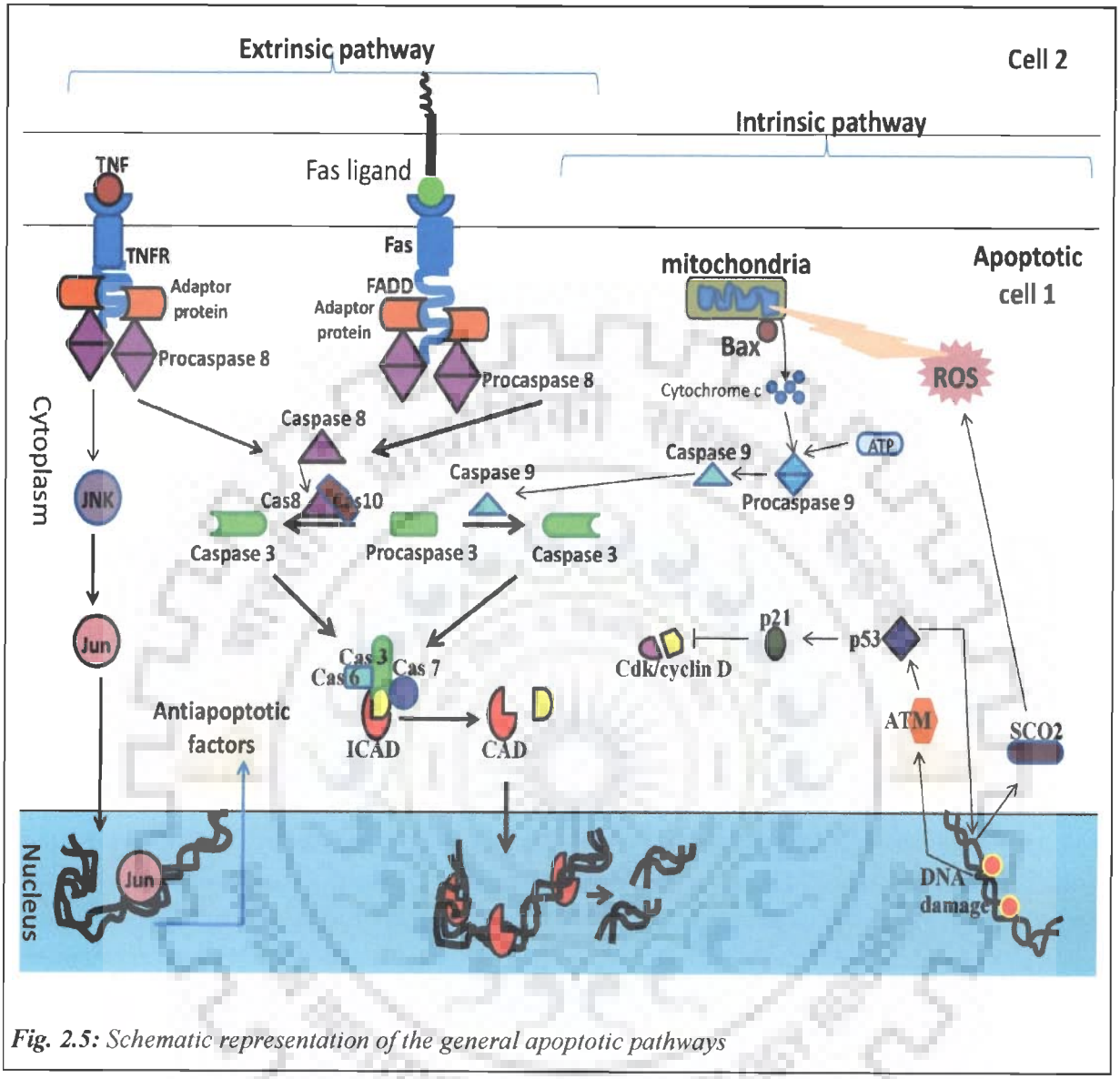


Fig. 2.5: Schematic representation of the general apoptotic pathways

All BH3-only molecules require multidomain BH3 proteins (Bax, Bak) to exert their intrinsic proapoptotic activity (Korsmeyer, 1995; Scorrano and Korsmeyer, 2003). In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome c and second mitochondria-derived activator of caspase (called direct IAP-binding protein with low pI). Cytochrome c, once released in the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis. Activated caspases lead to the cleavage of nuclear lamin and breakdown of the nucleus through caspase-3 (Reed, 1997) (Fig. 2.5).

2.10.1.3 The Final Pathway: Caspases

The final pathway that leads to execution of the death signal is the activation of a series of proteases termed caspases. Not all caspases are involved in apoptosis. The caspases that have been well described are caspases-3, -6, -7, -8, and-9 (Mancini et al., 1998; Thornberry and Lazebnik, 1998). The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease (ICAD), and the caspase-activated deoxyribonuclease (CAD) becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases-9 and -8 in the intrinsic and extrinsic pathways, respectively. The downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleaes (CIDE family), and finally, destruction of "housekeeping" cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing. (Mancini et al., 1998; Thornberry and Lazebnik, 1998).

The extrinsic and intrinsic apoptotic pathways are regulated by proteins such as p53, NFκB, the ubiquitin proteosome system, and the PI3K pathway (Fig. 2.6). The protein p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest, DNA repair, and apoptosis. This protein is mutated in a large number of cancers including breast. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood (Benchimol, 2001; Yu et al., 2003).

NFκB is a nuclear transcription factor that regulates expression of a large number of genes involved in the regulation of apoptosis, tumorigenesis, inflammation, and many autoimmune diseases (Maldonado et al., 1997). NFκB is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, radiation, pharmacologic agents, and stress. In its inactive form, NFκB is sequestered in the cytoplasm, bound inhibitor proteins of the IkB family. The various stimuli that activate NFκB cause phosphorylation of IkB, which is followed by its degradation. This results in exposure of the nuclear localization signals on NFκB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NFκB binds with the consensus sequence of various genes and thus activates their transcription (Maldonado et al., 1997). Under physiological conditions the activation of NFκB induces resistance to apoptotic stimuli through the activation of many complex proteins including TNF receptor-associated

factor, IAP, and X-linked IAP. However, in response to certain stimuli, NFκB activation may lead to induction of apoptosis (Kuhnel et al., 2000).This may be explained by the activation of some proapoptotic proteins such as interferon-regulated factor-1, c-myc, p53, and caspases such as caspase 1.

The ubiquitin/proteosome system is composed of a large proteinase complex that is responsible for the turnover of most intracellular proteins and consequently regulates cell growth and apoptosis (Myung et al., 2001). Protein degradation is a highly coordinated process that involves recognition of the protein by attaching it to multiple ubiquitin molecules and then its digestion by the 26S proteosome. Many cell cycle regulators and transcription factors such as p53, cyclins and cyclin-dependent kinase inhibitors, and NFkB are regulated by the ubiquitin/proteosome system (Adams et al., 2000; Myung et al., 2001). Many of the Bcl-2 family members are substrates of the ubiquitin/proteosome (Adams et al., 2000).

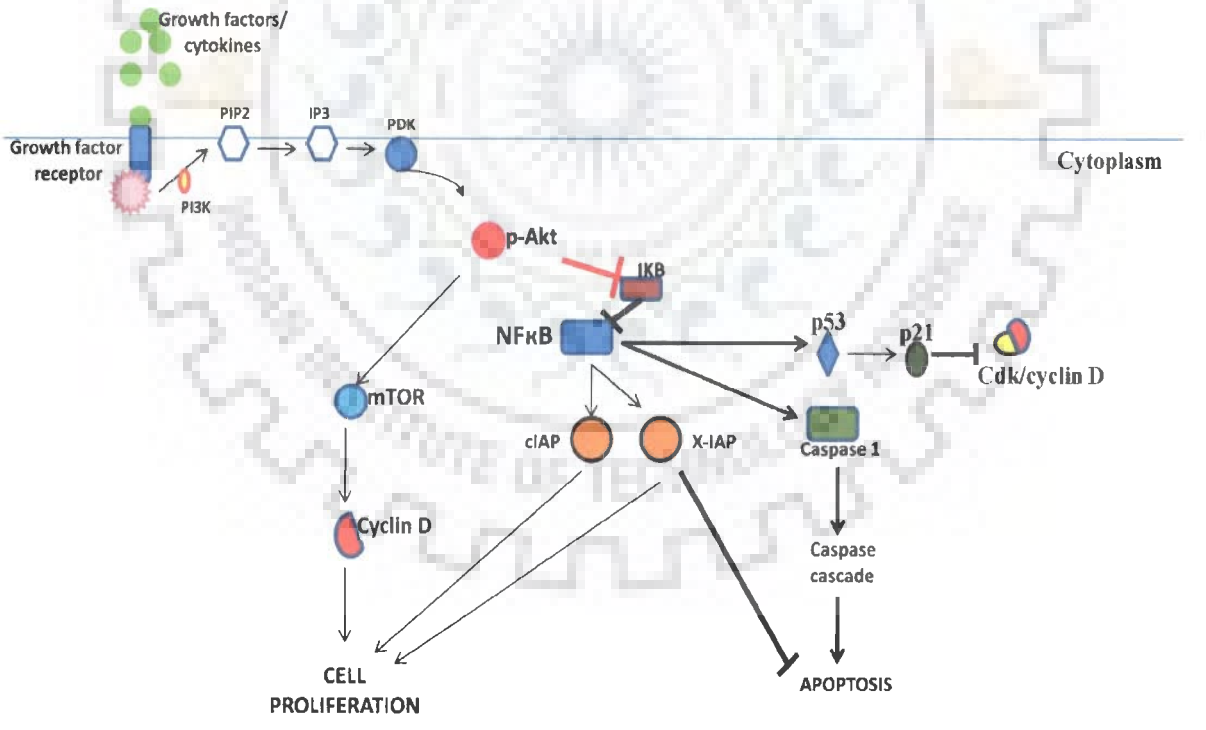


Fig. 2.6: Schematic diagram representing differential regulators of apoptosis

2.10.2 Autophagy

Autophagy is a massive death of individual cells (self-digestion process) due to the accumulation of double-membraned autophagic vacuoles, which finally fuse with the lysosomes

and little or no uptake by the phagocytic cells in vivo (Levine and Yuan, 2005). Autophagy is marked by the association Beclin 1 to the autophagic vesicles and the conversion of LC3-I to LC3-II. Autophagy is known as programmed cell death type II. The physiological function of autophagy is the control of cellular nutrient and organelle homeostasis and can be regulated by various extracellular and intracellular cues (Klionsky and Emr, 2000; Levine and Klionsky, 2004). Although not well-understood, current knowledge suggests that, the autophagy machinery includes several functional groups that regulate the life cycle of autophagosomes. They include Ulk1/2 protein kinase complex, VPS34-Beclin 1 lipid kinase complex, two ubiquitin-like protein conjugation systems and Atg protein retrieval and recycling system (Ohsumi, 2001; Xie and Klionsky, 2007). The VPS34-Beclin 1 kinase complex mediates localization of other autophagy proteins to the preautophagosomal structure and participates in the nucleation of autophagosome formation (Kihara et al., 2001; Suzuki et al., 2001) (Fig. 2.7). Many new components in this network are associated with vesicle trafficking, protein or lipid phosphorylation and protein ubiquitination. Autophagy is of two types: microautophagy and macroautophagy (Wang and Klionsky, 2003).

Microautophagy involves the direct engulfment of cytoplasm at the surface of the degradative organelle by protrusion, septation, and/or invagination of the limiting membrane. On the other hand, macroautophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, termed an autophagosome (Wang and Klionsky, 2003).

The mammalian homologue of yeast tor protein, mTor, can sense the environmental change due to starvation and cellular metabolic stress (Codogno and Meijer, 2004; Klionsky, 2005). The phosphorylation of a cascade of different autophagic proteins (Atg, yeast hololoque) by phosphatidylinositol (PtdIns) 3-kinase, PDK1, and Akt/PKB leads cytoplasm to vacuole targeting. This in turn results in membrane nucleation depending on the PtdIns 3-kinase complex I, and Atg9. These Atg proteins (including the Atg3) ubiqutinates and covalently attaches LC3 (light chain proteins of microtubulin structures) to phosphatidylethanolamine, thereby attracting cysteine protease that cleaves the C-terminus of Atg8/LC3 to expose a glycine residue (LC3-II form) for subsequent conjugation with Beclin 1, the class III phosphotidylinositol-3-kinase components of the vesicle-forming machinery to form the preautophagosomal structures. Lipid conjugation leads to the conversion of the soluble form of LC3 (named LC3-I) to the autophagic-vesicle-associated form (LC3-II). LC3-II is used as a marker of autophagy because of its lipidation (Maiuri et al., 2007). The Atg12–Atg5-Atg16 complex mediates the expansion

of the vesicle possibly by acting as a coat. LC3–phosphatidylethanolamine-Atg12–Atg5-Atg16 complex also acts in the elongation of the vesicle as a structural component. Atg9 cycles between the preautophagosomal structures and mitochondria, thereby supplying the lipid for the expanding membrane. Upon, or just prior to, vesicle completion, the coat proteins and the Atg proteins, except for the LC3– phosphatidylethanolamine that is oriented toward the lumen (and Atg19), are dissociated from the vesicle. Finally, the completed vesicle can fuse with the vacuole to form the autophagosome (Yorimitsu and Klionsky, 2005). Once formed, the autophagosome eats up the cell organelles and thus cause gradual cell destruction (Fig. 2.7). In cancerous cells Bcl-2 binds Beclin 1 and inhibits its availability and kinase activities for the formation of macroautophagosome (Chen et al., 2010). This in turn protects the cancer cells from the deleterious effects of cell death.

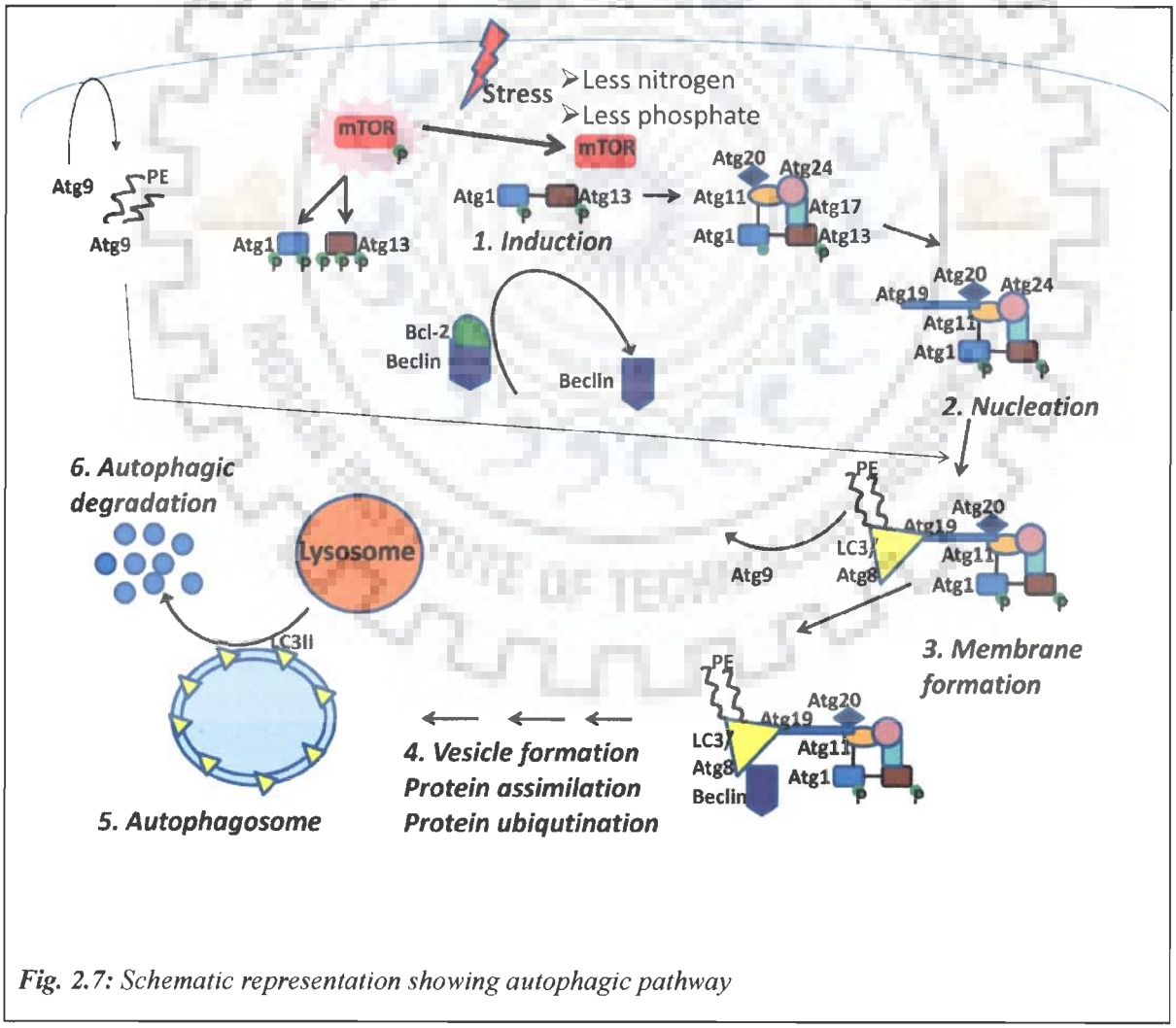


Fig. 2.7: Schematic representation showing autophagic pathway

Autophagy stimulators may be therapeutically useful to either promote autophagic cell death or to prevent the damaging effects of autophagy deficiency and mismanagement of metabolic stress leading to DNA damage and tumor progression. Reactive oxygen species have been known to oxidize the cellular sterols. The oxysterols thus produced when not expelled from the cell, stimulates autophagy (Medina et al., 2009; Payre et al., 2008). By limiting protein, organelle and ultimately DNA damage, autophagy stimulators may suppress tumor progression. In human breast, ovarian and prostate cancers, where allelic loss of Beclin 1 occurs with high frequency, correction of the autophagy deficiency with autophagy stimulators may delay tumor progression by reducing the rate at which tumor-promoting mutations accumulate (Mathew et al., 2007).

2.10.3 Necrosis

Necrosis, on the other hand causes cell death by cytoplasmic swelling (oncosis), rupture of plasma membrane and swelling of the cytoplasmic organelles. Necrosis is also characterized by the activation of calpains and cathepsins, RIP 1 protein phosphorylation and ubiquitination and drop of ATP levels. This form of cell death is only a result of an external stimuli causing cell death very fast (Nicotera and Melino, 2004). Necrotic cells do not fragment into discrete corpses as their apoptotic counterparts do. Moreover, their nuclei remain intact and can aggregate and accumulate in necrotic tissues.

2.11 Anticancer drugs targeting multiple pathways

Anticancer drugs may be divided into two broad subtypes: synthetic molecules and natural compounds, mainly phytochemicals (Fig. 2.8). Anticancer compounds from natural animal origin are very less except for melanin (Sharma et al., 2007; 2008), chitosan and its derivatives (Jiang et al., 2011). Hence natural anticancer molecules are considered as phytochemicals in the present thesis.

2.11.1 Common synthetic molecules

2.11.1.1 Platinum compounds

One of the great success stories in the field of cancer chemotherapy is the discovery of cisplatin (cis-diamminedichloroplatinum (II)) (Rosenberg et al., 1969), as a curative treatment for testicular tumors. Approved by the U.S. Food and Drug Administration in 1978, cisplatin is also administered for several other forms of cancer including ovarian, cervical, head and neck,

esophageal and non-small cell lung cancers (Patole et al., 2005; Pisani et al., 2011; Zhang et al., 2011). Inspite of these, the drug is only effective in testicular cancers to an extent of almost 90% cure rates (Allardyce et al., 2005). However, the treatment with cisplatin is limited to the toxic side effects including nephrotoxicity, emetogenesis, ototoxicity and nephrotoxicity (Zhang et al., 2011). Since the serendipitous discovery of its anti-neoplastic activity, many research groups have focused on revealing the molecular details of the mechanism of action of cisplatin and related compounds (Richard, 1992; Top et al., 2003). The next generation platinum drugs that came up are carboplatin,

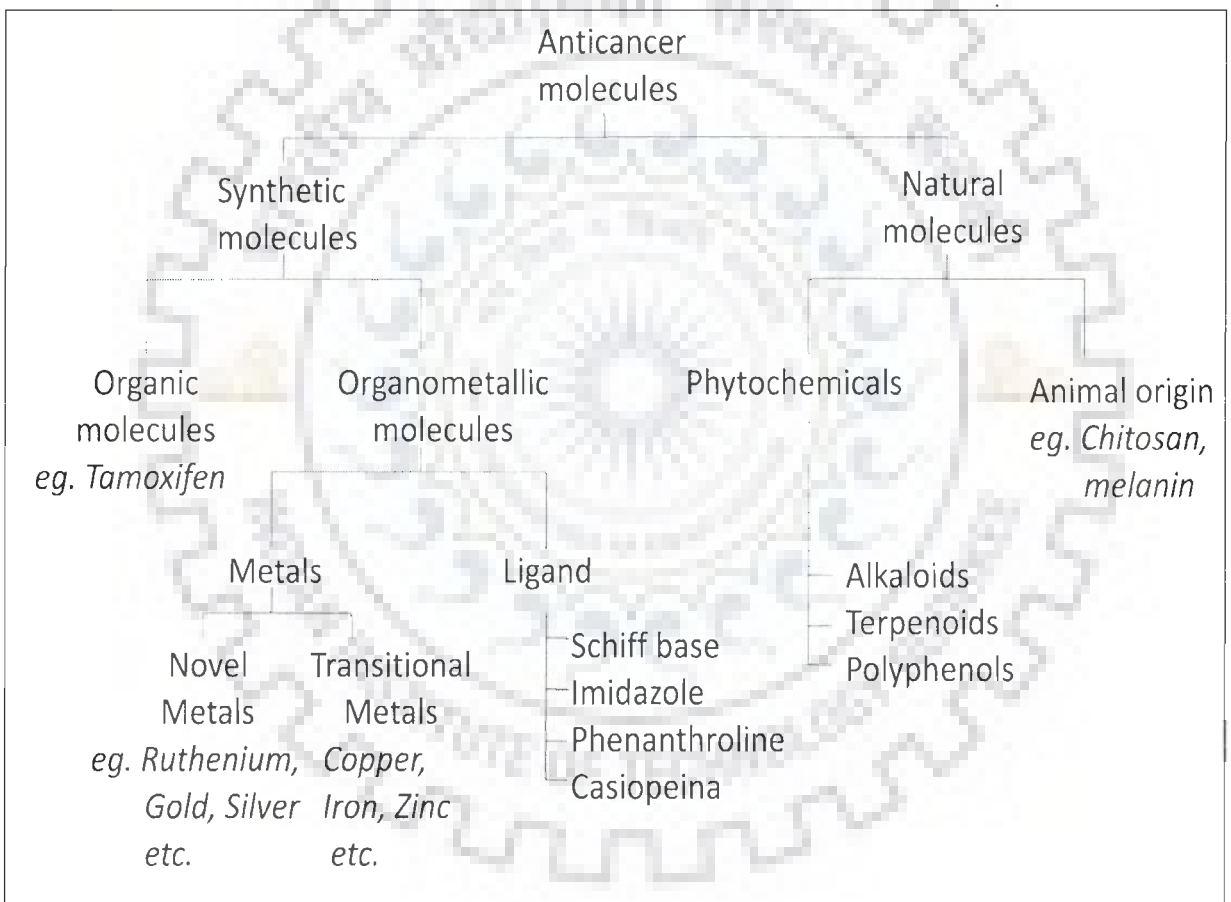


Fig. 2.8: Major classification of anticancer agents

oxaliplatin, satraplatin etc. (Nguyen et al., 2007; Viotte et al., 1995) and were used in clinical trials but still had the drawbacks of minor toxicity and major resistance. The main mode of the anti-neoplastic action of these drugs is by (i) massive cellular accumulation by both ionic diffusion and active transport by the copper transporters like CTR1; (ii) the univalent ions in these compounds gets easily displaced by the hydroxyl ions in the cellular ionic environment

and (iii) they form covalent bonds with the nucleic acids to form a variety of platinum-DNA adducts which finally cause severe irreversible DNA damage for replication and transcription leading to cytotoxicity (Huang, 2008; Lemmer et al., 1999). Therefore, new family of platinum compounds with structural modification, which is selective to only the cancer cell type, will be the next generation drugs.

2.11.1.2 Ruthenium compounds

Novel metals are another class of compounds that have been proved very effective in a number of multidrug resistant cancer cells. Ruthenium(III) complex salts of the general formula HL^+ trans-$[RuCl_4L_2]^-$ with nitrogen-containing heterocyclic ligands L, exhibit moderate cytotoxicity in vitro (Galeano et al., 1992), but surprisingly remarkable anti-neoplastic activity in vivo against chemically induced autochtonous colorectal tumors in rats (Berger et al. 1989; Seelig et al. 1992). The heterocyclic ruthenium(III) complexes indazolium trans-[tetrachlorobisindazole-ruthenate(III)] (KP1019) and sodium trans-[tetrachlorobisindazole-ruthenate(III)] (KP1339) induced apoptosis in colorectal cancer cell lines SW480 and HT29 cells predominantly by the intrinsic mitochondrial pathway as indicated by the loss of mitochondrial membrane potential. Correspondingly, the sensitivity of the cells paralleled expression of Bcl_2 while it was only slightly affected by mutations in Ki-ras (Kapitza et al., 2005) which is usually a common mutation in the colorectal cancers. Recently, ruthenium biochemists reported new ruthenium(II)-arene complex, which bearing a carborane unit, ruthenium and ferrocenyl functional groups at the nanoscale level and found to induce apoptosis in lung cancer cell line (HCC827) by stimulating proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP) in vitro and in vivo (Zhang et al., 2011). Attempts are being made for the targeted delivery of these novel synthetic compounds to the cancerous cells. Major advancement in the development of the ruthenium complexes as anticancer drugs has been made by Pisani et al. (2011). The discovery of dinuclear ruthenium(II) complexes ΔΔ/ΛΛ-[{Ru(phen)(2) }(2) {μ-bb(n) }](4+) (Rubb(n)), where phen is 1,10-phenanthroline, bb(n) is bis[4(4'-methyl-2,2'-bipyridyl)]-1,n-alkane (n=16) has been reported to be accumulated more than 16 times in leukemia cells as compared to the healthy B cells (Pisani et al., 2011).

2.11.1.3 Iron Compounds

Iron is known to be a biological metal. It acts as a co-factor in a large number of metalloproteins. The transition-metal π-complex, the central Fe^{2+} atom has gained importance

since way back when Ferrocene was first described in 1951 (Kealy and Pauson 1951). Ferrocene is a remarkably stable molecule that undergoes numerous reactions. Some ferrocene derivatives have been shown to exhibit anticancer activity against various cancer cells (Allardyce et al. 2005; Kopf-Maier and Erkenswick 1984; Viotte et al. 1995), and there are already some ferrocene-derived drug candidates, most prominent being ferrocifen, an activity-enhanced analog of the breast cancer drug tamoxifen (Nguyen et al. 2007; Top et al. 2003). In the same line of study, reports showed that unsaturated metal-thiosemicarbazonato iron centered compounds with planar structures demonstrated effective anti-proliferative effects against glioma cancer cell lines (Patole et al. 1995). Although iron based anti-proliferative compounds are still in use in the recent discoveries as a mediator of the drug-delivery system, reports from various studies show that iron overload is related to colon (Huang, 2003, Richard, 1992), breast (Dai et al., 2008; Huang, 2008; Jian et al., 2011), liver (Guo et al., 2006; Huang, 2003; Lemmer et al., 1999; Youn et al., 2009), lung (Elias et al., 1995; Rodriguez et al., 2000), kidney (Zainal et al., 1999) and skin cancers (Bhasin et al., 2002 a,b). These have led to the use of iron chelators as an anticancer agent (Richardson, 2002; Richardson et al., 2009; Rodriguez-Lucena et al., 2010).

2.11.1.4 Zinc Compounds

Zinc is also a biological metal and plays an essential role in cell membrane integrity and is a component of more than 300 different enzymes that function in many aspects of cellular metabolism, involving metabolism of proteins, lipids and carbohydrates (Parkin, 2004). The functions of zinc comprise the stabilization of conformation in transcription factors and modulation cellular signal transduction processes. Milacic et al. (2008) found that zinc (II) chloride inhibit the chymotrypsin-like activity of a purified 20S proteasome at an IC_{50} value of 13.8 μM. This led to the development of pyrrolidine dithiocarbamate (PyDT)-zinc(II) complex which could inhibit cellular proteasomal activity, suppress proliferation and induce apoptosis in various human breast and prostate cancer cell lines (Milacic et al., 2008). The Zn(II) complexes $[ZnCl_2(Fo4Npypipe)]$ and $[ZnCl_2(Ac4Npypipe)]$ (where 2-formyl pyridine N(4)-1-(2-pyridyl)-piperazinyl thiosemicarbazone is represented as Fo4Npypipe and 2-acetyl pyridine N(4)-1-(2-pyridyl)-piperazinyl thiosemicarbazone, represented as Ac4Npypipe) has been reported to be anti-tumorigenic against leukemia P388-bearing mice apart from the in vitro inhibitory effect on the cell proliferation of MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) (Kovala-Demertzi et al., 2008). The efficacy of these

compounds on the other hand is dependent on their structural similarities to the thiosemicarbazonato which are known as the most potent inhibitors of ribonucleotide reductase activity. Ribonucleotide reductase activity catalyzes the synthesis of deoxyribonucleotides from their ribonucleotide precursors and as such is responsible for maintaining a balanced supply of the deoxyribonucleotides required for DNA synthesis and repair. Strong positive correlation has been established between this activity and the rate of replication of cancer cells (Finch et al., 2000; Richardson, 2002). The mechanism of action of thiosemicarbazone complexes is due to their ability to inhibit the biosynthesis of DNA, possibly by (i) blocking the enzyme ribonucleotide diphosphate reductase, (ii) binding to the nitrogen bases of DNA, (iii) hindering or blocking base replication and (iv) creation of lesions in DNA strands by oxidative rupture (Kovala-Demertzi, 2008). Other zinc conjugated to 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine, 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine, or 2-(3-hydroxypropylamino)-6-benzylamino-9-isopropylpurine have been reported to inhibit the cyclin dependent kinase activity of the tumor cells (Trávníček et al., 2006). However, inspite of these activities, one of the major drawbacks of zinc in comparison to copper (which is another biological trace element) is that it lacks its oxidation state transition property which makes the copper compounds more effective as anticancer agents when compared to zinc.

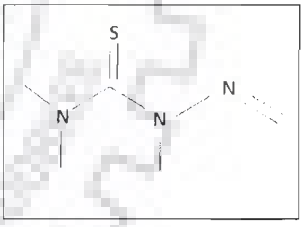
2.11.1.5 Copper Compounds

Various types of cancers arise with association with disturbed homeostasis of the cellular copper and zinc pool. The trace element copper is known as a micronutrient. The transitional metals have a large variety of coordination numbers and geometries, accessible redox states in physiological conditions and a wide range of thermodynamic and reactivity properties which can be successfully tuned by selection of suitable ligands. However, copper being a biological metal which has its own transporters is tightly regulated in the cellular enzymatic machinery. Copper has been shown to be capable of mediating the action of several plant-derived compounds through production of reactive oxygen species (ROS). The copper (I) and ROS mediated efficacy of plumbagin (*Plumbago zeylanica*) is especially effective for skin (Nazeem et al., 2009) and other cancers (Ahmad et al., 2008a; Padhye et al., 2010). This effect was further studied by Chen et al. (2009b). The reaction with [Cu(PLN)(2)].2H(2)O and with 2,2'-bipyridine (bipy) as a co-ligand, plumbagin reacts with Cu(II) to give [Cu(PLN)(bipy)(H(2)O)](2)(NO(3))(2).4H(2)O. These copper of plumbagin were reported to be highly potent in killing the cancer cells and inhibiting the topoisomerase I activity in DNA

replication (Chen et al., 2009b). Apart from the induction of an oxidative stress, copper coordinated compounds have recently emerged to inhibit the proteasome, a protein complex whose proteolytic activity is needed by several cellular processes. On the contrary, since copper is indispensable for the formation of new blood vessels i.e. angiogenesis, a different antitumor approach based on the administration of copper sequestering agents has been attempted and its effectiveness is currently under evaluation by clinical trials. The proven essentiality of copper for angiogenesis, together with the marked sensitivity shown by several cancer cell lines to the copper toxicity, open a new perspective in the anticancer strategy: exploiting the tumor need of copper to accumulate toxic amount of the metal inside its cells. Considering the fact that copper being an endogenous metal and the copper complexes can mimic various cellular processes as well, it is worth to investigate some novel copper complexes to check for the biological characteristics.

Apart from the choice of transition metals, the efficacy of a ligand is very important to stabilize the functional state of the metal. The followings are some potent ligands which are used in the designing of recent anticancer synthetic compounds.

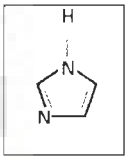
2.11.1.5.1 Schiff base ligand: Thiosemicarbazone complexes



Schiff base (or azomethine), named by the chemist Hugo Schiff, is a functional group that contains a double bond between carbon and nitrogen. Schiff base ligands are able to coordinate metals through imine nitrogen and stabilize many different metals in various states of oxidation (Cozzi, 2004). Schiff bases and their metal complexes have intensive antibacterial and antitumor properties (Adsule et al., 2006; Cerchiaro et al., 2005; Zhong et al., 2006). Thiosemicarbazones exhibit various biological activities and have therefore attracted considerable pharmaceutical interest (Hu et al., 2006b). They have been evaluated over the last 50 years as antiviral, antibacterial and anticancer therapeutics. Thiosemicarbazones belong to a large group of thiourea derivatives, whose biological activities are a function of parent aldehyde or ketone moiety (Du et al., 2002; Lovejoy and Richardson, 2002). Conjugated N–N–S tridentate ligand system of thiosemicarbazide (NH_2–CS–NH–NH_2) seems essential for anticancer activity. The structural alterations that hinder a thiosemicarbazone's ability to function as a chelating agent tend to destroy or reduce its medicinal activity (Liberta and West, 1992). Furthermore, the most active thiosemicarbazones are those which possess the *trans* isomer (i.e., hydrogen bonding involving O...HN). Several mechanisms of antitumor action of

thiosemicarbazones were proposed. For example, they could stabilize cleavable complexes formed by topoisomerase II (topoII) and DNA leading to apoptosis. The stabilizing effect is mainly due to the alkylation of thiol residues on the topo II–DNA complex (Chen et al., 2004). Besides, thiosemicarbazones could inhibit ribonucleotide reductase activity. The ribonucleotide reductase catalyzes the synthesis of deoxyribonucleotides required for DNA synthesis. Since deoxyribonucleotides are present in extremely low levels in mammalian cells, it is a crucial and rate-controlling step in the pathway leading to the biosynthesis of DNA. Although the transitional metal conjugated thiosemicarbazone complexes are reported for their anti-tumor properties (Belicchi-Ferrari et al., 2010; Ferraz et al., 2011; Prathima et al., 2010; Rosu et al., 2010; Yang et al., 2007), studies by Li et al. (2010) report that 3-carbaldehyde chromone thiosemicarbazone Cu(II) complex possesses better antioxidant activity than the ligand, Zn(II) and Ni(II) complexes.

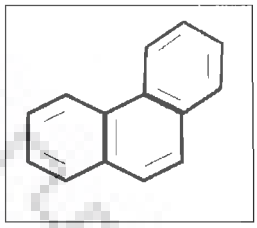
2.11.1.5.2 Imidazole



Imidazoles are common scaffolds in highly significant biomolecules, including biotin, the essential amino acid histidine, histamine, the pilocarpine alkaloids (Grimmett, 1996), and other alkaloids, which have been shown to exhibit interesting biological activities such as antimicrobial, anticryptococcal, inhibition of nitric oxide synthase, and cytotoxic activities (Luca, 2006). Imidazole derivatives have also been found to possess many pharmacological properties and are widely implicated in biochemical processes. Members of this class of diazoles are known to possess NO synthase inhibition (Sennequier et al., 1999), antibiotic (Nanivadekar et al., 1982) and antiulcerative activities (Brimblecombe et al., 1975) and include compounds, which are inhibitors of 5-lipoxygenase (Mano et al., 2003; 2004) and substances with CB_1 receptor (Dyck et al., 2004; Hunkeler et al., 1981), VEGF receptor I and II (Kiselyov et al., 2006), and neuropeptide Y antagonistic activities (Blum et al., 2004). In addition, these heterocycles include several inhibitors of p38 MAP kinases (Bolós, 2005; Diller et al., 2005; Jackson and Bullington, 2002; Wilson et al., 1997), a subgroup of mitogen-activated protein kinases, JNK3 (Takle et al., 2006), B-Raf kinase (Kalmes et al., 1999), transforming growth factor β1 (TGF-β1) type 1 activin receptor-like kinase (Byfield et al., 2004; Singh et al., 2005a) and acyl-CoA:cholesterol *O*-acyl transferase (ACAT) (Bani et al., 1995). Some derivatives such as cimetidine, etomidate and ketoconazole are already in use in drug therapy (Delgado and Remers, 1998; Coura and Castro, 2002). Synthesis of amino substituted imidazoles (Kostakis et al., 2008) and platinum imidazoles (Ravera et al., 2011) are

respectively effective in breast and colon cancer cells. In addition, the results also showed that the copper imidazole has a very low IC_{50} value in the range of 8-15 μM (Collins et al., 2000; Sandbhor et al., 2004) towards melanoma. This data confirmed that at this concentration the copper imidazole complex is more effective in 50% cell death of the melanoma cells.

2.11.1.5.3 Phenanthroline



Sigman and co-workers (1979) discovered the first efficient artificial nuclease that acts via an essential, non-covalently bound intermediate, and they showed that Cu(1,10-phenanthroline)2 was the precursor (Sigman et al., 1979; 1993; 1996). The serendipitous finding, while investigating the zinc requirement of a DNA polymerase the copper-phenanthroline (phen), led the discovery of these artificial nucleases. In presence of a copper ion, phen, and a reducing agent such as a thiol the compounds show enhanced nuclease activity. The cleavage mechanism involves the reversible binding of the $[Cu(phen)_2]^+$ complex within the minor groove of DNA to form an essential non-covalent intermediate finally to cleave the nucleic acid (Johnson and Nazhat, 1987). The efficiency of this system to cleave DNA has led to the synthesis of systems like photo-dynamic therapy with these phenanthroline drugs to achieve the target of specific drug delivery only at the site of cancer at least in solid tumors (Patra et al., 2007). The $[Cu(phen)_2]^+$ complexes has a very low inhibitory concentrations in the range of nanomolar and to a maximum of 10 μM. It has been found that $[Cu(phen)_2(mal)] \cdot 2H_2O$ among $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ ($malH_2$ = malonic acid) is most active against kidney and liver carcinoma cell lines (Thati et al., 2009) to an extent of even 18 times more potent than cisplatin. The inhibitory concentration of copper(II) complex, [Cu(4-Mecdoa)(phen)$_2$] (phen = phenanthroline, 4-Mecdoa = 4-methylcoumarin-6,7-dioxactetate) is between 1.3-11 μM when tested in different types of cell lines (Thati et al., 2007). Although phenanthroline is an excellent artificial nuclease to cause cancer cell DNA damage but due to the lack of cell specificity its cytotoxic effects cannot be levied from the normal cells. The copper-valine-phenanthroline has recently been reported to cleave DNA very efficiently (Dong et al., 2011). It is hypothesized that the presence of such amino acids may induce some sort of specificity to cancer cells. These copper phenanthroline are still under investigation.

2.11.1.5.4 Casiopeína

In the light of the successful application of metal coordinated complexes in medicine, particularly as anticancer drugs and in the search of more effective, less toxic and wider spectrum chemotherapeutic agents, a group of copper-coordinated complexes, (casiopeina), have been developed (Mendoza-Diaz et al., 1991; Ruiz-Azuara, 1996; 1997). The ligand frame of these compounds is worth understanding to synthesize future anticancer molecules. Some members of this series of compounds have shown a 50% growth inhibition in transformed human epithelial cervix (HeLa) and CaLo colon cancer cells, respectively as well as L1210 murine leukemia cancer cells with doses 10–100 times lower doses than cisplatin (Gracia-Mora et al., 2001). Also apoptosis induction in L1210 murine leukemia and CH1 human ovarian carcinoma in cisplatin-resistant and sensitive cells with a member of Casiopeinas' family, casiopeina II (Cu(4,7-dimethyl-1,10-phenanthroline)(glycine)NO_3) (CII), was reported (Ruiz et al., 2000). Casiopeina III-ia [(4,4′-dimethyl-2,2′-bipiridine)(acetylacetonate) Copper (II) nitrate] when tested on colon carcinoma (Carvallo-Chaigneau et al., 2008), medulloblastoma (Mejia and Ruiz-Azuara, 2008) cells was found to cause apoptosis by increasing Bax levels and suggesting a caspase-dependent mechanism of death, as verified by nucleosomal fragmentation of DNA (Carvallo-Chaigneau et al., 2008). The casiopeinas, were found to bind to DNA and to degrade DNA and RNA in the presence of reducing agents (e.g. ascorbic acid). Casiopeinas show a high affinity for binding to DNA which remains inseparable with even harsh in vitro wash conditions. The reaction requires oxygen, probably involved in the generation of .OH (hydroxyl) radicals, which would be responsible for the strand breakage. The reaction was diminished by catalase, and was completely abolished by copper chelators (e.g. trientine, EDTA); however, superoxide dismutase (SOD) had no significant effect on casiopeina-mediated DNA degradation. Casiopeina IIgly (casIIgly) in the presence of ascorbate was capable of degrading RNA, plasmid and genomic DNA, and chromatin and intranuclear genetic material (Kachadourian et al., 2010; Rivero-Müller et al., 2007). Metal toxicity can be associated to oxidative tissue damage, due to the catalytic role of metals in the oxidative deteriorating effect to biological macromolecules (Okada, 1996). Also the presence of lipid peroxidation, DNA damage, depletion of sulphydryl homeostasis and altered calcium levels are reported to be produced as a consequence from metal reactivity and reactive oxygen species (ROS) generation (Sarkar et al., 1995). Reports on the toxicity of copper indicate that the acute response to unbound copper present in blood is the manifestation of hemolytic anemia (Barceloux, 1999; Linder and Hazegh-Azam, 1996), which is mainly caused by the oxidative stress on erythrocytes (E) by copper (Rolfe and Twedt, 1995). In

addition and as a consequence of hemolytic damage, slight morphological changes in the spleen can be manifested from the effects of drugs or chemicals on the hematopoietic and lymphoid system, also the measurement of splenic weight serves as guidance for treatment-related response to xenobiotics (Rosse, 1987).

In this thesis the activities of a series of copper compounds have been studied. Chapter 4 initially screens a potent copper complex with respect to the cytotoxicty results against breast cancer cell lines and subsequently the next chapter (Chapter 5) clearly investigates the mode of anticancer action of the copper complex in vitro and in vivo against breast tumor.

2.11.2 Phytochemicals

Although new and improved discoveries to the synthetic anticancer compounds are a topic of research, the search of phytochemicals with anticancer properties is in very high demand. The reason mainly remains that these phytochemicals (also designated as neutraceuticals) are a part of the regular dietary regime and human nutritional status can effectively ward off complex diseases like cancer (as reviewed by Sarkar et al., 2010). On the basis of this idea, and numerous epidemiological findings, attention has centered on dietary phytochemicals as an effective intervention in cancer development. However, the failure of large-scale clinical trials in 1990s, including the Alpha-Tocopherol, Beta-Carotene trial (Heinonen and Albanes, 1994) and the Carotene and Retinol Efficacy Trial (Omenn et al., 1996), has raised doubts whether diet-based cancer prevention can be successful. But as these trials were not designed on mechanism-based preclinical studies, identifying cancer preventive dietary agents that have specific molecular or cellular targets is thought to be an essential way forwards. The next generation drugs were developed from the lead molecules from a number of plant extracts. Phytochemicals like camptothecin, etoposide and topotecan are potent topoisomerase inhibitors, and vinorelbine, paclitaxel, docetaxel, vinblastine and vincristine are known to act against tubulin thus inhibiting the replication or cell division of cancer cells are under clinical trials (Canel et al., 2000; Heijden et al., 2004; Leveque and Jehl, 1996; Lorence and Nessler, 2004; Montecucco and Biamonti, 2007; Sirikantaramas et al., 2007; Vaishampayan et al., 1999; Zhou et al., 2009). The overall strategy for discovering molecular targets of phytochemicals can involve several approaches depending on (i) whether the starting point is a known oncoprotein or (ii) a phytochemical with proven anticancer activity. In brief, the first approach beginning with target protein selection is based on the data ideally obtained from RNA interference (RNAi)

screen (Boutros and Ahringer, 2008; Iorns et al., 2007; Moffat and Sabatini, 2006), as well as in vitro, ex vivo and in vivo models. Potential phytochemical candidates can then be selected by in silico virtual screening, based on natural chemical libraries. The X-ray crystallographic structures of a protein of interest either alone or bound to an inhibitor or ligand are crucial in the process of identifying protein–small molecule interactions and designing more specific inhibitors. The in silico drug screening gives rise to millions of structural alterations towards a single target. Although this approach is very specific but a biologically acceptable molecule is a matter of trial (Fig. 2.9). The second approach is a compound-based method that begins with the selection of putative cancer preventive phytochemical candidates based on previous research studies. From the selected compounds, the most effective candidates are taken forwards as lead compounds using screening methods such as cell transformation, cell proliferation and kinase assay or reporter gene assays (Fig. 2.9). In this approach the identification and functional stability maintenance and characterization of the lead molecule is at first crucial and the next milestone is identification of its exact cellular target to understand its cellular mechanism of action. Finally the exact mode of molecule to protein interaction is proved by the interaction of the lead compounds with candidate target proteins by 'reverse docking' on known protein crystal structures or proteomic tools (Cheng et al., 2010; Rix and Superti-Furga, 2009).

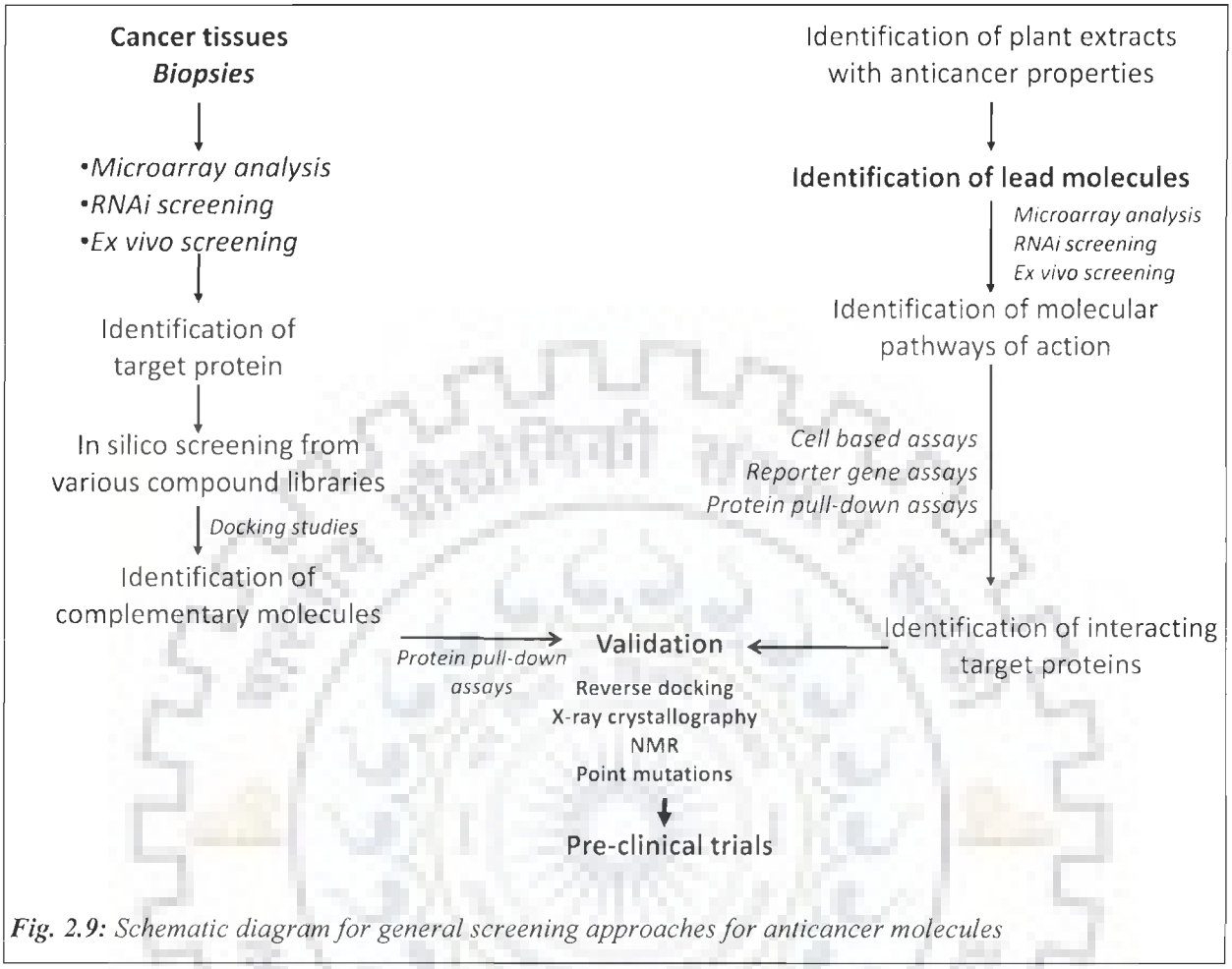


Fig. 2.9: Schematic diagram for general screening approaches for anticancer molecules

2.11.3 Protein targets of phytochemicals

Cancerous cells have active cell proliferation machinery and they require active source of metabolism. The first factor to cause such effect is the growth stimuli. Three main pathways are broadly responsible in cancer characteristics and needs to be targeted.

2.11.3.1 The Growth Factor mediated pathway: MAPK pathway

The mitogen activated protein kinase (MAPK) belongs to a large family of serine-threonine kinases (Dong et al., 2002). The mitogen or the growth factors that lead to the activation of these kinases are mainly the cytokines present in the tumor microenvironment. There are three major subfamilies of MAPK: the extracellular-signal-regulated kinases (Ras/Raf1/MEK/ERK); the c-Jun N-terminal or stress-activated protein kinases (JNK or SAPK); and MAPK14 (p38α). Each of these proteins ERK, JNK and MAPK14 are activated by MEK and they in turn associate with their respective cellular regulatory proteins (transcription factors) and thus express the cell proliferation proteins (Fig. 2.10). Several phytochemicals, including

quercetin (Lee et al., 2008a), myricetin (Lee et al., 2007) and equol (Kang et al., 2007) could dock with the allosteric pocket adjacent to its ATP-binding site (Ohren et al., 2004) in MEK. In fact, quercetin showed a much stronger inhibitory effect against MEK1 kinase activity than the well-known MEK1 inhibitor, PD098059 (Lee et al., 2008a). Experimentally, an analogue of resveratrol (RSvL2) was shown to strongly bind MEK1, whereas resveratrol bound only very weakly (Lee et al., 2008a). When PD098059 binds MEK1, it is

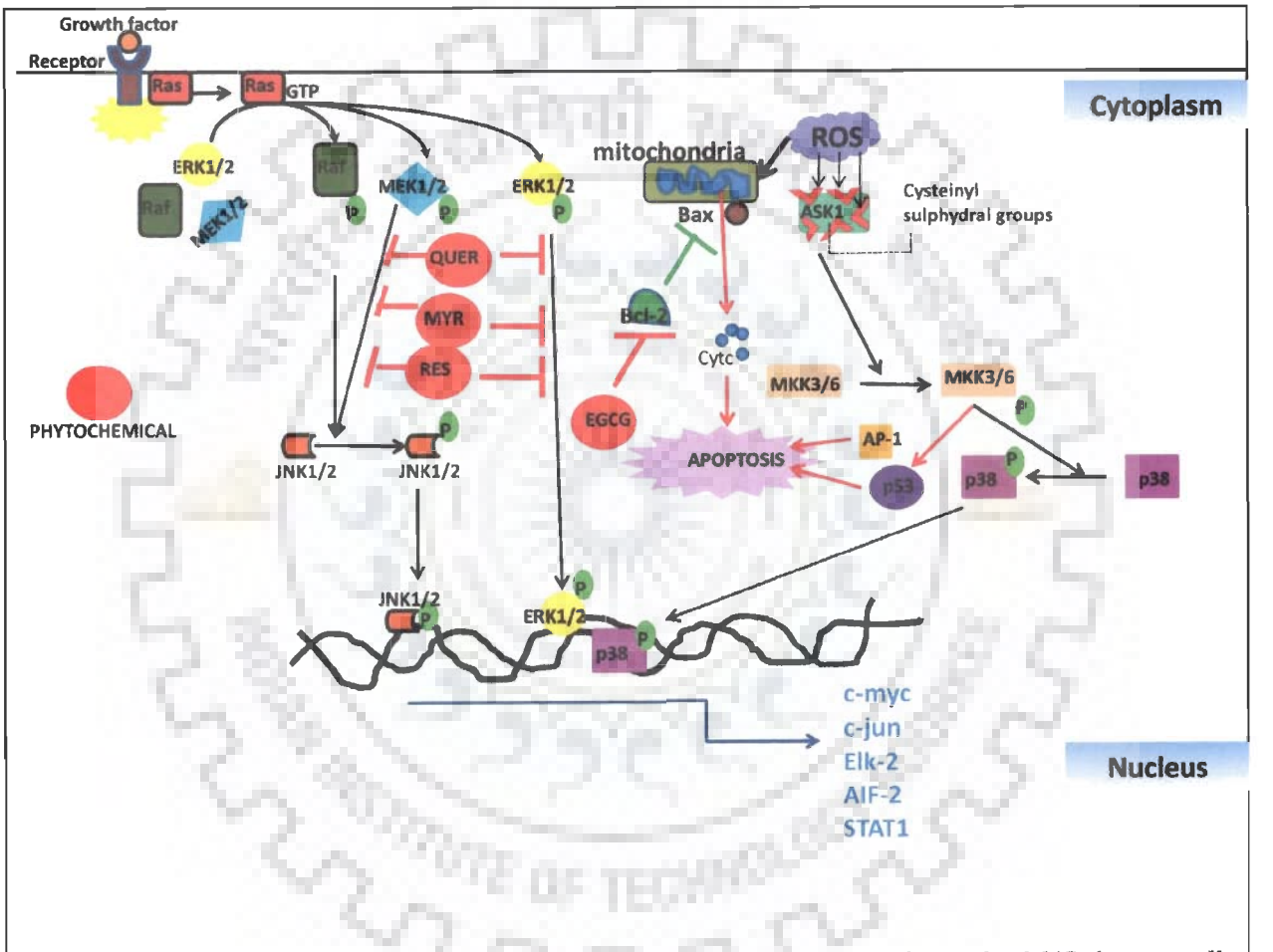


Fig. 2.10: *Schematic diagram showing multiple targets of phytochemicals in the MAP kinase cell proliferation pathway*

oriented in the allosteric site so that it forms a hydrogen bond with the backbone amide of Ser212 with its carbonyl oxygen, an electrostatic interaction with the backbone of Val127 and edge-to-face aromatic stacking with Phe209 (Ohren et al., 2004). The mechanism of allosteric inhibition of MEK is attributed to the inhibitor being able to stabilize the inactive conformation of the activation loop and deform the catalytic site (Fischmann et al., 2009). The flavones, quercetin and myricetin, which contain numerous polar hydroxyl groups, were not expected to

preferentially bind in the extremely hydrophobic pocket in which other MEK inhibitors bind. These compounds were found to bind in the opening of the allosteric site and interact with the metal ion. In fact phenolic phytochemicals like caffeic acid strongly suppressed mitogen-activated MEK1 and PKB activities and bound directly to either MEK1 or PKB in an ATP-noncompetitive manner and inhibit ERKs phosphorylation, AP-1, and NF-κB transactivation and subsequently inhibited TPA-, EGF- and H-Ras-induced neoplastic transformation of JB6 P+ cells (mouse epidermal cell lines) (Kang et al., 2011). Using a combination of nuclear magnetic resonance, binding assays, fluorescence polarization assay, and computational docking studies, Leone and his colleagues found that EGCG and black tea theaflavins are potent inhibitors (Ki in the nanomolar range) of the anti-apoptotic factors, Bcl-XL and Bcl-2, suggesting a direct binding of EGCG to the BH3 pocket of these proteins, at concentrations very close to physiological plasma levels, supporting a realistic use of EGCG as anticancer and pro-apoptotic drug in humans (Leone et al., 2003). Not only the MAPK, flavonones like 8-prenylnaringenin decrease the serum insulin like growth factor in animals under physiological conditions (Bottner et al., 2008). These results support the idea that a subtle difference in phytochemical structure, such as the addition of hydroxyl groups, will affect the binding affinity of a compound with a target protein.

2.11.3.2 The Energy Activation pathway: Akt pathway

Akt signaling promotes cell survival, proliferation and invasion. Hence blocking this pathway could impede the proliferation of tumor cells. Akt (protein kinase B) has been shown to play major role in the therapeutic resistance of tumor cells, because it acts against apoptotic mechanisms to promote cell survival (Fig 2.2). For example, ovarian cancer cell lines with either constitutive Akt1 activity or Akt2 gene amplification were highly resistant to paclitaxel, in contrast to cells with low Akt levels (Page et al., 2000). In cells, Akt is widely involved in glucose metabolism to supply nutrients. Tumor specific changes in metabolism were discovered by Warburg in the 1920s. These metabolic changes consist of increased glucose consumption, decreased oxidative phosphorylation and lactate production — characteristics of aerobic glycolysis in cancer cells (Engelman and Cantley, 2010). Akt and mTor are the key players in reprogramming metabolic pathways in cancer cells. Akt mobilizes glucose transporters to the cell surface to enhance glucose uptake and activates hexokinase 2 to phosphorylate and trap intracellular glucose. Through these effects, Akt is able to enhance glycolytic flux without affecting mitochondrial oxidative phosphorylation, thereby presumably contributing to the

Warburg effect (Elstrom et al., 2004) (Fig. 2). mTOR, a substrate of Akt, broadly mediates cell growth and proliferation by regulating ribosomal biogenesis and protein translation (Ruggero and Sonenberg, 2005) and can regulate response to nutrients by restricting cell cycle progression in the presence of suboptimal growth conditions (Plas and Thompson, 2005). FOXO, another very prominent substrate of Akt, is also a transcription factor for the glucose metabolic enzymes. The myc oncogene, which is widely activated in human cancers, has also been implicated in the direct activation of aerobic glycolysis. The myc transcription factor activates virtually all glycolytic enzyme genes and directly binds numerous glycolytic genes, including those encoding HK2, enolase, and LDHA (Kim and Dang, 2005). Aerobic glycolysis may provide cancer cells growth advantage in the tumor microenvironment (Gatenby and Gillies, 2004). Phytochemicals are organic small molecules which can interact with a number of intracellular proteins. Based on X-ray crystallography, quercetin and myricetin (Walker et al., 2000) have been shown to directly bind and suppress PI3K activity, but so have other protein targets, as discussed above.

2.11.3.3 The cell cycle regulators

The connection between the cell cycle and cancer is obvious: cell cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. Regulating cancer cell proliferation is crucial for chemoprevention. At least two types of cell cycle control mechanisms are recognized: first, a cascade of protein phosphorylations that relay a cell from one stage to the next and second, a set of checkpoints that monitor completion of critical events and delay progression to the next stage if necessary (Fig. 2.11). The first type of control involves a highly regulated kinase family (Morgan, 1995). Kinase activation generally requires association with a second subunit that is transiently expressed at the appropriate period of the cell cycle; the periodic ''cyclin'' subunit associates with its partner ''cyclin-dependent kinase'' (CDK) to create an active complex with unique substrate specificity. CDKs bind with cyclins to form CDK–cyclin complexes (Fisher, 1997). Regulatory phosphorylation and dephosphorylation fine-tune the activity of CDK–cyclin complexes, ensuring well-delineated transitions between cell cycle stages. A second type of cell cycle regulation is checkpoint control. It is not an essential part of the cycle progression machinery. Cell cycle checkpoints sense flaws in critical events such as DNA replication and chromosome segregation (Elledge, 1996). When checkpoints are activated, for example by under replicated or damaged DNA, signals are relayed to the cell cycle-progression machinery. These signals cause a delay in cycle progression, until

the possibility of mutation has been averted. Because checkpoint function is not required in every cell cycle, the extent of checkpoint function is not as obvious as that of components integral to the process, such as CDKs. Some CDKs like CDK5 inhibits MAPK and is responsible for apoptosis (Sharma et al., 2007). Many CDK inhibitors (CDKIs), such as the p21 and p27 proteins, attenuate formation of these complexes and block cell cycle progression (Sherr and Roberts, 1999). These proteins are activated in cancer cells by both the above signaling pathways MAPK and AKT and other the oncoproteins. Several phytochemicals can also function as CDKIs. For example, 3′,4′,7- trihydroxyisoflavone, which is a metabolite of the soybean isoflavone daidzein, is a direct inhibitor of CDK2 and CDK4 (Lee et al., 2010a).

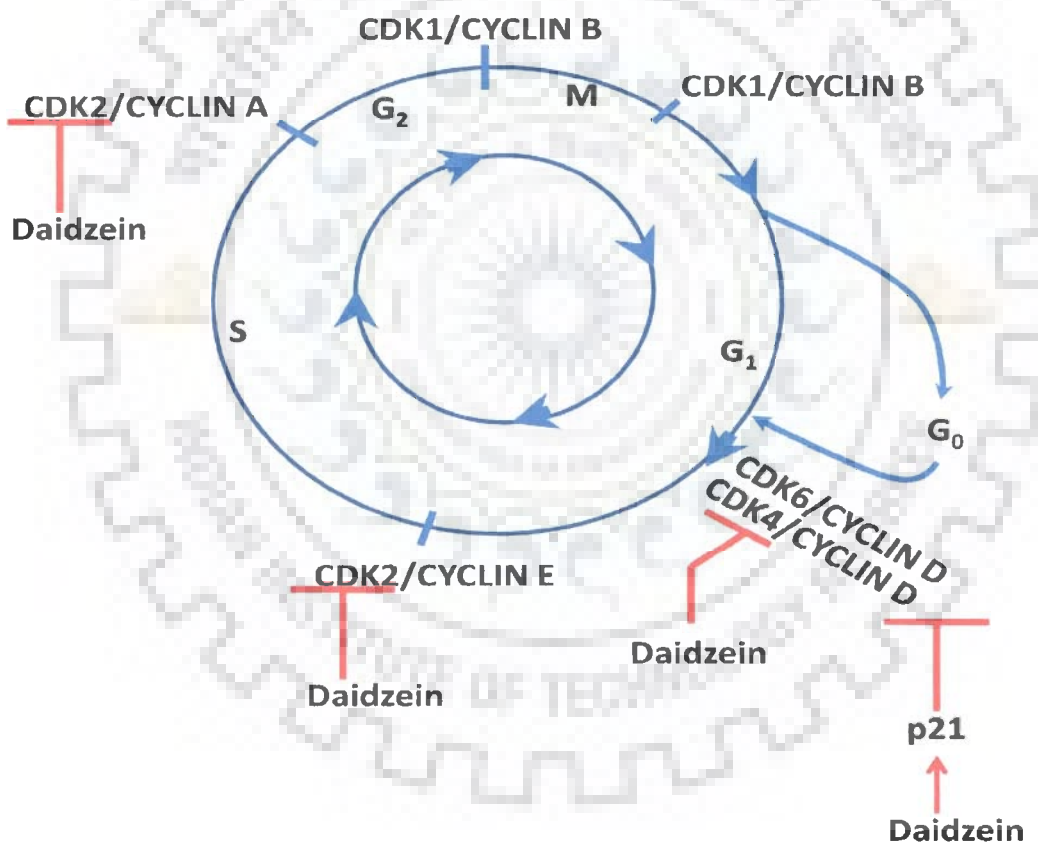


Fig. 2.11: Schematic representations showing that phytochemicals act as inhibitors of cell cycle regulators

In the tumor milieu the associated cancer cells have all the three pathways are activated. There are a lot of mutations in the genes which leads to either gain of function of the cell proliferation factors and/or there is a loss of function of the tumor suppressor proteins. Thus the concept of "oncogene addiction," a phenomenon in which mutant genes not only provide

transformed cells with a survival advantage over their normal counterparts, but are also to avoid cell death (Weinstein and Joe, 2006). The existence of such redundant survival pathways could also explain why inhibitors of a single dysregulated pathway may be insufficient to induce cell death in tumor cells. In support of this notion, recent studies demonstrated that in cells of glioblastoma, inhibition of multiple, rather than a single, receptor tyrosine kinase(s) was necessary to induce cell death (Stommel et al., 2007). The phytochemicals have some specificity towards a particular target but they have, in general, lesser affinity towards a single protein. These molecules on the other hand activate a large number of different proteins which may play active role in different pathways. Given below are some examples of the targets of few selected phytochemicals.

2.11.4 Some known phytochemicals and their targets

2.11.4.1 Alkaloids

These are complex molecules which have potent anticancer properties. In fact a number of alkaloids like vinblastine, topotecan, paclitaxel, docetaxel etc. are already in clinical trials against lymphoma, sarcoma, breast, non-small cell lung carcinoma etc. They mostly have their affinity to inhibit the cytoskeletal structure tubulin (Heijden et al., 2004; Leveque and Jehl, 1996; Vaishampayan et al., 1999; Zhou et al., 2009). These are essential for the synthesis of spindle network during mitosis. But these molecules have very high cytotoxicity but less specificity for cancer cells and hence they cause some systemic toxicity like hair loss, anemia, neutropenia, leucopenia and other such effects of chemotherapy. Not only this docetaxel is effluxed from the cancer cells by ATP-binding cassette transporters (ABCs) and also it is metabolized intracellularly by glutathione-S-transferase P1 which results in its resistance in breast cancer cells (Gilbert et al., 1993; Huang et al., 2003; Silvestrini et al., 1997; Su et al., 2003).

2.11.4.2 Terpenoids

Terpenoids, also referred to as terpenes or isoprenoids, are the largest group of natural compounds found in plants and are synthesized from two five-carbon building blocks. Based on the number of building blocks, terpenoids are classified as monoterpenes, sequiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, and polyterpenes (Rabi and Bishayee, 2009) with almost 40,000 different terpenoids have been isolated from plants, animals and microbial

species (Rohdich et al., 2005; Withers ST and Keasling, 2007). Among the terpenoids, triterpenoids have recently emerged as a unique group of phytochemicals with multifunctional anticancer activities as demonstrated by promising results in preclinical studies. The emergence of resistance to anticancer drugs, in particular multidrug resistance (MDR), resulted in many of the available anticancer drugs ineffective (Borowski et al., 2005). MDR is a complex multifactorial phenomenon that can result from a number of biochemical mechanisms, including a decreased drug uptake or an increased drug efflux; the perturbed expression of target enzymes or altered target enzymes; the altered metabolism of drugs; the increased repair of drug-induced DNA damage; or a failure to undergo apoptosis (Szakács et al., 2006; Teodori et al., 2006). The most important and widely studied members of ABC transporters are MDR1/P-glycoprotein (MDR1/P-gp, ABCB1), multidrug resistance associated protein (MRP1/ABCC1) and breast cancer resistance protein (BCRP, ABCG2), (Lage 2003; Teodori et al., 2006; Szakács et al., 2006). Several macrocyclic diterpenes from *Euphorbia* species, which were shown to be very strong modulators of P-glycoprotein in resistant cancer cells (Duarte et al., 2006; 2007; 2008; Ferreira et al., 2005). Lathyrane diterpenes, latilagascenes and jolkinol B have been recently found to be very active against mouse lymphoma cell lines (Duarte et al., 2006). In the model of combination chemotherapy, the interaction between the doxorubicin and latilagascene was studied in vitro, on human MDR1 gene transfected mouse lymphoma cells, which showed that the type of interaction was synergistic. Recently preliminary cytotoxicity studies were carried out with clerodane diterpenoid isolated from *Polyalthia cerasoides* on intestinal cell line CaCO-2 which revealed that the cancerous cells underwent apoptosis even at nanomolar drug concentrations of these phytochemicals (Ravikumar et al., 2010). Triterpenoids are biosynthesized in plants by the cyclization of squalene a precursor of all steroids (Phillips et al., 2006). Two triterpenoids, enoxolone, and celastrol, have been recently reported to have Shp2 PTP (protein tyrosin phosphatase) inhibitor activity (Scot et al., 2011). New studies reveal that steroids and triterpenoids with negatively charged phosphate, carboxylate, or sulfonate groups serve as novel pharmacophores of selective PTP inhibitors. Cucurbitacin I have been found to inhibit the proliferation of MDA-MB-468 human breast cancer cells that express constitutively activated STAT3 (Blaskovich et al., 2003). Cucurbitacin I also reduced the levels of phosphotyrosine of constitutively activated STAT3 and induced apoptosis. These triterpenoids class is also reported to inhibit COX-2, interlukin-1β- and tumor necrosis factor-α (TNF-α)-induced IκB phosphorylation and reduce the expression of matrix metalloproteinase-9 (MMP-9)

and urinary plasminogen activator (uPA) leading to inhibition of migration of W256 cells (Idris et al., 2009). COX (Ding et al., 2003) and TNF-α (Pandey et al., 2010) are linked to cholesterol biosynthetic pathways and thereby linked to cancer cell proliferation. On the other hand synthetic triterpenoid, 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole diminished the invasive growth of 4T1 breast cancer cells with a parallel accumulation of cells in G_2-M phase, inactivation of STAT3, Src and Akt as well as inhibition of c-Myc (Place et al., 2003). Thus MAPK, Akt and cell cycle regulatory pathways of a cancer cell are under the control of these molecules.

2.11.4.3 Polyphenols

Polyphenols are a group of chemical substances found in plants that are characterized by the presence of more than one phenol unit or building block per molecule. Polyphenols can be divided into several classes according to the number of phenol rings that they contain and the structural elements that bind these rings to one another (Stevenson and Hurst, 2007). The main groups of polyphenols are: flavonoids, stilbenes, phenolic acids, phenolic alcohols and/or lignans and chalcones (D'Archivio et al., 2007) (Fig. 2.12).

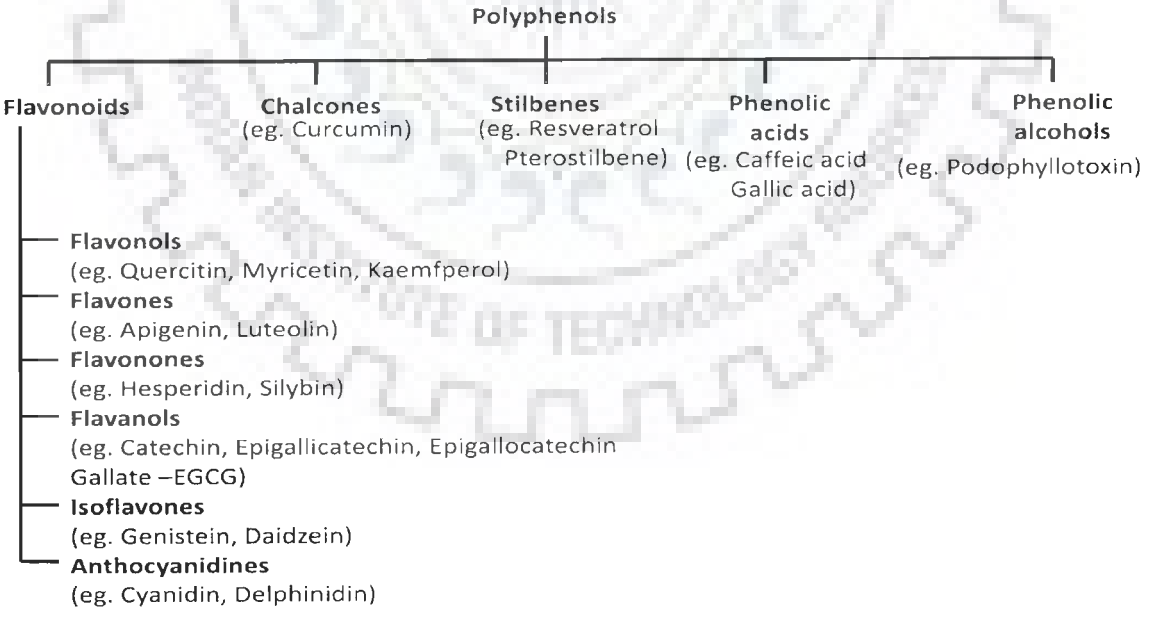


Fig. 2.12: *Diagramatic representation of classification of polyphenols*

Polyphenols are an important part of the human diet, and are found in berries, grapes/wine, tea, chocolate/cocoa, coffee, soybeans, and other fruits and vegetables (Manach et

al., 2004). Indeed, fruits and vegetables are excellent sources of polyphenols. Diets rich in polyphenols are epidemiologically associated with a lower risk of developing cancer (Cao et al., 1998; Garcia-Closas et al., 1999; Knekt et al., 1997; 2002; Levi, 1999;). Among these, the largest and best studied polyphenols are the flavonoids, which comprise several thousands of compounds, including flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavones (Lotito and Frei, 2006).

At the molecular level, polyphenols affect three major signaling pathways that are related to cancer: (i) the mitogen-activated protein kinase (MAPK), (ii) the phosphatidylinositol-3-kinase (PI3-K), and (iii) NF-κB pathways. Among various flavonoids, 5-deoxykaempferol (5-DK), kaempferol, quercetin, myricetin, fisetin and quercetagetin are known to target some key regulator-switches of the above mentioned pathways in different models. 5-DK suppressed the UVB-induced expression of COX-2 and VEGF in mouse skin epidermal JB6 P+cells (Lee et al., 2010b) thereby inhibiting inflammatory responses (and/or induction of cytokines at the tumor millieu) and angiogenesis. Kaempferol, which is found in tea, propolis and grapefruit, has an antioxidant and anti-inflammatory effects. Cell-based experiments and computer modeling showed that kaempferol inhibited RSK2-mediated (ribosomal S6 kinase, a direct substrate which is located between the ERK and its own target transcription factors) cancer cell proliferation, and that the flavonol is able to bind with RSK2 directly (Cho et al., 2009). The direct binding of kaempferol with Src leads to MAPK pathway blockage. In addition, it is elucidated that PI3-K is a novel target molecule for the inhibitory effects of kaempferol on neoplastic cell transformation of mouse epidermal JB6 P+ cells (Lee et al., 2010b). Quercetin is one of the strongest anti-carcinogenic agents (Waterhouse, 2002).

Kinase assay demonstrated that quercetin inhibits MEK1, PI3-K (Hwang et al., 2009) and Raf-1 kinase activities (Lee et al., 2008a). These results were confirmed with docking data showing that quercetin can bind with both MEK1 and Raf-1. These studies clearly show that quercetin is able to inhibit both the MAPK and PI3-K pathways involved in cancer development. Another flavonol, myricetin, is easily docked to the ATP-binding site of regulating protein Fyn. As a result of the binding, myricetin attenuated UVB-induced COX-2 expression in mouse skin (Jung et al., 2008). Luteolin (a major component of broccoli) suppresses COX-2 expression and AP-1 and NF-κB activity in JB6 P+ cells (Byun et al., 2010).

The isoflavones are another group of flavonoids. Isoflavones in soybean and their metabolites, such as genistein, 7,3′,4′-trihydroxyisoflavone (7,3′,4′-THIF) and equol, reversed

DNA hypermethylation and retinoic acid receptor β (RARβ), p16INK4a (cyclin dependent kinase inhibitor) and MGMT (O6-methylguanine DNA methyltransferase) gene reactivation in human esophageal cancer KYSE 510 and KYSE 150 cells, and prostate cancer LNCaP and PC3 cells (Fang et al., 2005). These methyltransferases are required after every cell division for the gene silencing. But in cancer cells activation of these enzymes mostly leads to the silencing of the tumor suppressors (Robertson, 2001). It was also proved that genistein directly repressed DNA methyltransferase activity. In addition to regulating the expression of cell cycle-regulatory proteins, 7,3′,4′-THIF also bound strongly to CDK4 and CDK2 and inhibited their kinase activities. Genistein is also known to inhibit the enzymes for steroid biosynthesis although the molecule itself is mild estrogenic (Barnes, 2010). These isoflavones can modulate the steroid receptor functions in vivo rat models but their mode of action is different from the natural steroidal moieties. They in general do not share their effects through the hypothalamo-pituitary unit (Rimoldi et al., 2007).

Cyanidin and delphinidin, which are both classed as anthocyanidin, inhibit carcinogenesis by targeting the MAPK pathway. Catechin EGCG is a small-molecule inhibitor of insulin-like growth factor-I receptor (IGF-IR) activity (Li et al., 2007). (−)-Epigallocatechin gallate (EGCG) directly interacted with GRP78 at the ATP-binding site of protein in an ATP-competitive manner, resulting in the inhibition of ATPase activity (Ermakova et al., 2006).

Curcumin, a major curcumanoid in turmeric, is a potent inhibitor of NF-κB and Akt mediated pathways (Balasubramanyam et al., 2004; Liao et al., 2011). It shows direct binding and inhibition of p300/CBP pathways (Balasubramanyam et al., 2004). It is also found to inhibit NF-κB by inhibiting the proteasome function. Thus curcumin is a natural ubiquitin proteasome inhibitor (Dikshit et al., 2006a,b).

Stilbenes on the other hand is present in less quantities in various fruits and vegetables but they posses high potential of anticancer activity. These molecules have been shown to inhibit almost all the major pathways linked to cancer apart from its effect on the hormone dependent cancers. Resveratrol shows direct binding to COX-2 and Ref-1 proteins (Yang et al., 2005; Zykova et al., 2008) and is found to inhibit cyclin D1and E, MAPK, Akt, JNK, COX-2, VEGF. Interestingly resveratrol is found to inhibit androgen receptor (AR) (Benitez et al., 2007; Mitchell et al., 1999; Wang et al., 2010; Yuan et al., 2004). The variety of different types of targets of this molecule made it attractive for the scientists to understand more on the mechanism of action and the treatment in different types of cancers. Resveratrol is found to

inhibit cyclin D, CDK, tumor growth factors (TGF), ERK, cyclooxygenase, lipooxygenase, β-catenin and upregulates caspases, p53, p21 and other cell apoptotic factors (Aggarwal et al.; 2004). It also inhibits the tubulin polymerization pathway and suppresses angiogenesis, invasion and metastasis and has immune-modulatory effects (Aggarwal et al.; 2004). Resveratrol is found to antagonize the estrogen receptor at higher concentrations and act as an agonist at lower concentrations (Bowers et al., 2000). Resveratrol is also found to be protective against the estrogen metabolism induced DNA adduct formation (Zahid et al., 2008). Almost all the possible derivatives of resveratrol are being synthesized in different laboratories and also the natural analogues of this molecule are widely studied. Other natural analogues like pterostilbene, pinostilbene, pinosylvin, and several synthetic like 2,3′,4,4′,5′-Pentamethoxy-*trans*-stilbene, 3,3′,4,5,5′-pentahydroxy-*trans*-stilbene are also being studied presently (Lee et al., 2008b).

Cancer cells are known to accumulate a number of genetic mutations. So it can be speculated that if a compound targets different pathways simultaneously, the molecule will be effective in controlling the disease. Polyphenols are effective for overcoming this difficulty because of its two distinct features, low toxicity and multi-signaling inhibitory effect. Apart from this, bioavailability and metabolism of polyphenol needs to be considered. Ellagitannins, for example, are conjugated in the liver to be transformed into urolithins which can inhibit prostate cancer cell growth (Heber, 2008).

This broad review inspires the study of the efficacy and complete signal transduction of these selected wonder-molecules. In the Chapter 4 of the present thesis polyphenol fraction from some known medicinal plants are screened for anticancer properties against breast cancer cells.

Among the polyphenol molecules the stilbenes have been reported not only to activate the growth arrest pathways in cancer cells but are special to inhibit several hormone regulated pathways. These molecules can therefore be used to target hormone related cancers.

2.12 Polyphenol: Stilbene

Stilbene is a di-aryl ethene which is a hydrocarbon consisting of a trans ethene double bond substituted with a phenyl group on both carbon atoms of the double bond. Resveratrol is a prominent and extremely well studied member of phytochemical. Other than resveratrol not many of the members have been studied so far in the biological system to reveal the molecular pathways as anticancer agents. As described in the previous section all possible derivatives of resveratrol moiety is under investigations for their potency as cancer preventive molecules in different cell lines.

The trans-3,5,4'-trihydroxystilbene, otherwise known as resveratrol, is generally a byproduct of the anthocyanin biosynthetic pathway in the plants (Chong et al., 2009). The starting molecule being phenylalanine, is converted to 4-coumaroyl-CoA by various enzymatic pathways. This molecule is then converted to resveratrol by resveratrol synthase (Melchior and Kindl, 1991; Schoppner and Kindl, 1984). The stilbenes are further modified in plants. Although known for its medicinal properties in 'darakchasava', a well known Indian herbal preparation in Ayurveda (Paul et al., 1999), stilbenes remained in obscurity for few decades (Takaoka, 1999). It is present in grapes, peanuts and mulberries and in plants affected by fungus *Botrytis cinerea* (Langcake and McCarthy, 1979; Langcake and Pryce, 1976). Extensive data in different human cell cultures indicate that resveratrol can modulate multiple pathways involved in cell growth, apoptosis and inflammation (Athar et al., 2007). The anti-carcinogenic effects of resveratrol appear to be closely associated with its antioxidant activity and have been shown to inhibit cyclooxygenase, hydroperoxidase, protein kinase C, Bcl-2 phosphorulation, Akt, NF-kB, matrix metalloproteinases and cell cycle regulators (Seve et al., 2005). It is much well absorbed following oral administration to humans (Soleas et al., 2001). However, resveratrol is metabolized into sulphated and glucoronidated forms within 15 min of entering the blood stream (Saiko et al., 2008). All previous studies showed that resveratrol can thus target a number of different diseases with diverse cellular targets but its shortcomings need to be further replaced by other stilbenes of this type with better efficacy.

2.12.1 Pterostilbene

Pterostilbene is trans-3,5-dimethoxy-4'-hydroxystilbene which is a natural methoxylated analogue of resveratrol. It is present in the heartwood of *Pterocarpus marsupium* (Maurya et al., 1984), *Pterocarpus santalinus* (Sehadri, 1972), leaves of *Vitis vinifera* (Langcake et al., 1979), fungal infected grape berries of varieties Chardonnay and Gamay (Adrian et al., 2000) and in healthy and immature berries of varieties Pinot Noir and Gamay (Pezet et al., 1988). Initially pterostilbene was established as an antioxidant molecule along with resveratrol. Moreover, pterostilbene was reported to be more effective in scavenging peroxyl-radical scavenging than the artificial free radical scavenger, Trolox (Rimando et al., 2002). In the same study it was established that pterostilbene inhibits cyclooxygenase enzyme in murine macrophages and colon cancer cell lines. Cyclooxygenase enzyme catalyzes the rate-limiting step in prostaglandin synthesis, converting arachidonic acid into prostaglandin H2, which is the further metabolized to prostaglandin E_2 (PGE_2) PGF_2, PGD_2 and other eicosanoids thereby producing inflammation.

The production of these inflammatory molecules are however linked to the signal transduction of Ras/MEK/ERK, epidermal growth factor receptor (EGFR), PI3K/GSK, STAT and is also linked to the local release of interleukin 6 (IL-6) in the tumor tissue which aids in enhanced growth of the tumor (Ding et al., 2003). In this context the report that at a very low concentration of ~5μM, this stilbene inhibits the carcinogen induced preneoplastic lesions in mouse mammary gland organ culture (Rimando et al., 2002) confirms the promising potency of

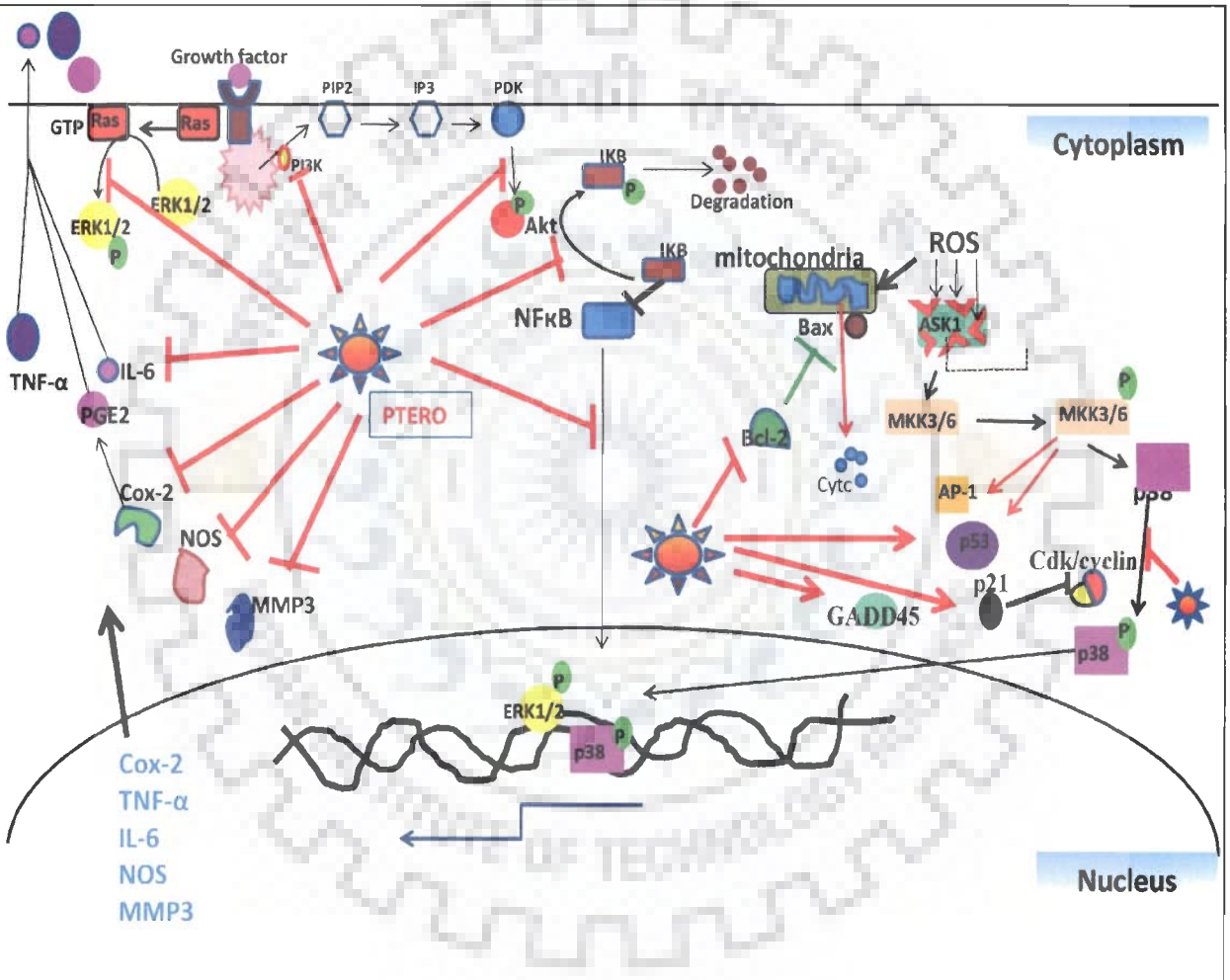


Fig. 2.13: *Schematic representation of the recent targets of pterostilbene in colon and gastric carcinoma and inflammatory macrophage models.*

this molecule as an anticancer agent which needs to be further investigated. Pterostilbene has also been reported to inhibit the lipopolysaccharide induced activation of PI3K/Akt and NFκB, thus found to bear anti-inflammatory effect on murine macrophages (Pan et al., 2008). The generalized targets of pterostilbene in gastric, colon and inflammatory macrophages are represented as Fig. 2.13. In the murine macrophages cyclooxygenase-2 and inducible nitric

oxide synthase gene is inhibited both at mRNA and protein level by the molecule. The anti-inflammatory activity of pterostilbene was also evidenced in the decreased release of prostaglandin E2 (PGE2) in the media by colon cancer cells HT-29 and low release of TNFα by canine chondrocytes after the treatment with the drug (Remsberg et al., 2008). Prostaglandin E2, an eicosanoid, was recently shown to induce growth factor receptor phosphorylation and mitogenic signaling in colon cancer cells (Pai et al., 2002). Similar to the inflammatory models, the PI3K/Akt pathway is also upregulated in cancer cells for its growth and active metabolism. The carcinogen (azoxymethane) induced colonic aberrant cryptic foci is a preliminary marker of colon carcinogenesis. Pterostilbene is found to inhibit the azoxymethane inducible nitric oxide synthase in these colon cancer cells (Suh et al., 2007). Further, the phytochemical was found to be apoptotic in other gastric cancers, B16 melanoma and also in multidrug resistant BCR-ABL-1 expressing leukemia cells (Ferrer et al., 2005; Pan et al., 2007; Tolomeo et al., 2005). Due to the fact that pterostilbene have shown longer half-life in vivo and the highly potent inhibition of the B16M-F10 melanoma cells in the liver (a common site for metastasis development) Ferrer et al., (2005) claimed that this stilbene has potency to be developed as anticancer drug.

Chapter 4 of the present thesis shows that the methanolic extract of *Pterocarpus marsupium* is most potent among other phytochemical extracts against the breast cancer cell lines. Pterostilbene was isolated and purified and then the mechanism of anticancer properties of this molecule was investigated further in Chapter 5 and the following chapters. The present thesis shows that pterostilbene activates the reactive oxygen species (ROS) in breast cancer cells.

2.13 ROS manipulation in cancer cell therapy

To elucidate the mechanism of phytochemicals, a basic understanding is needed of what stimulates cancer cell proliferation. Cancer cells, particularly those that are highly invasive or metastatic, may require a certain level of oxidative stress to maintain a balance between undergoing either proliferation or apoptosis. They constitutively generate large but tolerable amounts of H_2O_2 that apparently functions as signaling molecules in the mitogen-activated protein kinase pathway to constantly activate redox-sensitive transcription factors and responsive genes that are involved in the survival of cancer cells as well as their proliferation. Therefore, there can be two possible contradictory mechanisms to manipulate these cells. Either of them may be adopted to kill the cancer cells. (i) If the excess H_2O_2 can be scavenged by

phenolic phytochemicals having antioxidant activity, the oxidative stress-responsive genes can be suppressed and consequently cancer cell proliferation inhibited; and (ii) if phenolic phytochemicals can induce the formation of H_2O_2 to achieve an intolerable level of high oxidative stress in cancer cells so that the stress genes are activated. This second mechanism is interesting and is presently being studied by the cell biologists. In this case there is less scope for the development of drug resistance. When the critical threshold for cancer cells to cope with the induced oxidative stress has been reached, key cellular components such as DNA are damaged irreparably. In conjunction, genes involved in initiating cell cycle arrest and/or apoptosis are activated. There are several studies which reported diverse biological activities of the stilbene scaffolds. These activities are attributed to the fact that they are mainly polyhydroxylated molecules which bear the capacity to form hydrogen-bonds and to generate stable resonance structures in presence of oxidative electrophilic molecules in the biological system (Stivala *et* al., 2001). The generation of phenoxyl radical of polyphenols by peroxidase-H_2O_2 system which oxidizes both cellular glutathione and NADH, accompanied by oxygen uptake to form ROS has been demonstrated by Galati et al., (1999; 2002). A review by Filomeni et al., (2005) has shown that MAPK mediated phosphorylation pathways are exhaustively needed for apoptosis in case of oxidative stress. The oxidative stress depletes the intracellular glutathione and converts it to oxidized form. The phenoxyl radicals can generate the free radical ions in the intracellular environment (Loo, 2003). If the polyphenol-backbone mediates the over-production of these hydroxyl ions, the cancer cells having low anti-oxidant enzymes and depleted glutathione (Meves et al., 2001) cannot tolerate the oxidant stress (Loo, 2003). The ROS molecules thus generated oxidize the cysteinyl sulphydryl groups on the protein tyrosin kinase which in turn activates the epidermal growth factor receptor mediated MAPK and finally activator protein (AP)-1 and p53 are activated to induce apoptosis (Loo, 2003). Intolerable amounts of ROS cause oxidative damage to DNA in conjunction with activation of the MAPK cascade and turn on the growth arrest and DNA damage inducible genes (such as GADD45 and GADD153), as well as genes that are involved in initiating cell cycle arrest (such as waf-1), and apoptosis (such as Bax) (Loo, 2003). This phenomenon of ROS mediated cancer cell apoptosis is a recent topic of investigation with the natural polyphenols as they may act differentially on the normal as well as the cancer cells. Theaflavin monogallates inhibited the growth of Caco-2 colon cancer cells but not their normal counterparts (Lu et al., 2000). It is shown in the studies of Khan and Wilson that ROS depencent c-jun NH2-terminal kinase/c-jun signaling cascade mediates cell death

induced by diallyl disulfide in neuroblastoma cells and not specifically the normal nerve cells probably due to their normal anti-oxidant enzyme systems (Khan and Wilson, 1995).

2.14 Summary of the literature review

In brief, previous literature depicts that cancer cells have growth factor (mostly regulated by cytokines present in the tumor microenvironment or in some cases growth hormones) mediated cell proliferation, stress tolerance (hypoxic conditions) and active metabolic machinery. They co-operate among the surrounding non-cancerous stromal tissues. Targeting cancer cells is a bit tricky as these altered cells have developed more than one survival pathway over a long time which has escaped the normal control of apoptotic machinery of the cellular physiology. The final goal to attack these cells is to activate the apoptotic and/or autophagic machinery in these cells due to the systemic infusion of some drugs. When targeting a marker protein with an inhibitor has led to some form of success but on the other hand has developed stimulation of the specific drug resistance due to the activation of remaining alternative growth pathways which were otherwise present per se. Microarray analysis have also revealed that one signature pathway like the stress-resistance hypoxia, wound healing pathways etc. has more than hundreds of genes activated. But the trick lies in the phosphorylation-dephosphorylation and the energy metabolic pool of a cancer cell which distinguishes it from the normal cells. Again the phosphate ion transfer during phosphorylation-dephosphorylation reactions is highly co-ordinated with respect to the oxidation state of a cell. Among the endocrine related cancers, breast cancer is a hormone dependent/independent form of cancer among middle aged women in general. The disease is highly metastatic. Anti-estrogens like tamoxifen have led to the development of drug resistance. Other known chemotherapeutic drugs have other side effects. Hence in-depth research is emphasized for the discovery of new molecules and understanding of their molecular mode of action targeting breast cancer. Among the synthetic and natural compounds, the former in general acts as nucleases or protease inhibitors. But a lot of them have caused systemic toxicity due to the fact that they could not be metabolized and excreted properly, in spite of their efficacy at in vitro cell culture systems. Hence more emphasis is given to the groups which are more biological. The second category of natural anticancer agents is mainly phytochemicals. The advantage of these molecules over the previously designed artificial synthetic compounds is that they are easily taken up by the cells and they have diverse cellular targets. Their targets may be different cellular proteins from distinctly separate pathways. Moreover, they are in general non-toxic to the normal body cells although they bear cytostatic

effects on the cancer cells. Hence the challenge lies in unveiling the molecular pathways of a synthetic and a natural compound bearing potency for breast cancer growth arrest.

2.15 The problem addressed in present thesis

The present thesis deals basically with two approaches. First, is to identify of some potent anticancer molecules either from synthetic or natural sources. Not only this, it is also important to check whether the synthetic molecule causes any form of systemic toxicity. Second is to understand the mode of action of the most potent compounds to inhibit breast cancers. Towards the end, pterostilbene, a natural phytochemical, was selected for their anticancer properties among the other plant extracts tested. Finally the successive chapters reported the mode of their action. Pterostilbene, although established as an anti-hyperglycemic and anti-inflammatory agent, but to understand and link the different targets of this stilbene specifically in breast cancer is a new finding that is dealt with in the present thesis. It was also quite interesting to find that not only this stilbene has different modes of action, but it differentially activates some pathways in normal mammary cells and mammary tumor cells.

CHAPTER 3

MATERIALS & METHODS

3 Materials & Methods

3.1 Introduction

This chapter deals with the overall experimental details in order to prove a molecule to be an anticancer agent. It also deals with the various techniques that are employed to unveil the underlying molecular pathways of action of the compound.

3.2 Reagents

The different cell lines used in this study were obtained from National Center for Cell Science, Pune, India. All the cell culture reagents were purchased from GIBCO (Invitrogen, USA) unless otherwise mentioned. The antibiotics (penicillin and streptomycin), actinomycin D, cycloheximide, dimethyl sulphoxide (DMSO), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], 5-florouracil, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 5',5'- dithiobis-2-nitrobenzoic acid, horseradish peroxidase (HRP) chromogen – diamino benzidine (DAB) and all analytical grade chemicals were obtained from HiMedia (Mumbai, India). Tamoxifen was obtained from Calbiochem (Darmstadt, Germany). Injectable cisplatin was obtained from Ranbaxy (Mumbai, India). Phytochemical standards: resveratrol and pterostilbene, N-Methyl-N-Nitroso Urea (MNU), glutathione reductase, BCA total protein quantification kit and sterol standards: lathosterol, cholesterol, desmosterol and 7-dehydrocholesterol were obtained from Sigma (St. Louis, MO, USA). RT-PCR quantification kit, p53 ready-to-use primers, the alkaline phosphatase (ALP) chromogen - BCIP-NBT and calf-thymus DNA were obtained from Genei (Bangalore, India), primary antibody for Beclin 1 was kindly gifted by Dr. Marja Jäättelä (Professor, Apoptosis Department, Institute of Cancer Biology, Denmark). Primary antibodies for Bax, Bcl-2, cytochrome c, caspase 3,caspase 9, p38, p-Akt, Akt, PR, SRC1, GRIP-1 TIMP-3 and β-actin and the caspase 3 assay kit were kindly gifted by Dr. Arun Bandyopadhyay (Senior Scientist, Indian Institute of Chemical Biology, Kolkata, India). Primary antibody for LC3B was obtained from Abcam (Cambridge, UK). Nanofect transfection reagent was purchased from QIAGEN (Qiagen,Valencia, CA). Estrogen, progesterone, RU 486 and pCMV-beta-gal constructs were kindly provided by Dr. Ilpo Huhtaniemi, Professor, Imperial College, London, UK. The pEGFP-PR and pCMX-LXRβ constructs were kindly donated by Dr. Gordon Hager (Professor, National Cancer Institute, Rockville, USA) and Dr. Steven A. Kliewer (Professor, Southwestern Medical center, USA),

respectively. The pGL3-PRE-luc and pSG5-PR plasmids were a kind gift from Dr. Jan J. Brosens (Professor, Imperial College of London, London, UK).

3.3 In vitro DNA binding assay

The procedure checks whether binding affinity of a compound to DNA is better than that of ethidium bromide (EB). For this, varying concentrations of the complexes (0, 10, 20, 30, 40 and 50 μM) were added to 50 μM calf thymus-DNA solution in phosphate buffer (pH 7.2) which was previously saturated with 50 μM of EB. Excitation wavelength of the sample was set at 480 nm and the emission spectra were scanned from 700 to 490 nm (Ghosh et al. 2008). Fluorescence quenching of EB were measured for the entire emission spectra with increasing concentrations of synthetic compounds. Stern-Volmer quenching constants were calculated using the following equation:

$I_0/I = 1 + K_{SV}r$, where I_0/I is the fluorescence intensities in absence and presence of test compound respectively, K_{SV} is a linear Stern-Volmer constant and r is the ratio of the total concentration of the complex to that of the DNA.

3.4 *In vitro* experimental models

3.4.1 Cell lines and cell culture

Breast cancer cells MCF-7, T47D and MDA-MB-231, prostate cancer cells PC3, Cervix cancer cells SiHa and a normal human embryonic kidney cell line HEK293 were used in this study.

MCF-7 cells (from passage number 14-20) were grown in DMEM with 10% fetal bovine serum (heat inactivated) and 1% antibiotic (100 U/ml of penicillin and 100 μg/ml streptomycin) mix at 37 °C in humidified atmosphere in a CO_2 incubator. The medium of the cells was changed every day to avoid nutrient stress. Drugs were added after washing the cells twice with PBS followed by addition of complete medium.

T47D cells (from passage number 65-70) were grown in high glucose RPMI with 10% fetal bovine serum, 2 mM L-glutamine, 0.2 U/ml of insulin (porcin) (Sigma, St. Louis, MO, USA) and 1% antibiotic (100 U/ml of penicillin and 100 μg/ml streptomycin). The culture flasks were maintained in humidified atmosphere in a CO_2 incubator at 37 °C. The spent medium was replaced by fresh medium on every third day.

MDA-MB-231 (from passage number 75-78) was grown in Leibovitz L-15 supplemented with 10% fetal bovine serum and 1% antibiotic (100 U/ml of penicillin and 100 µg/ml streptomycin) mix in a hypoxic environment at 37 °C in humidified atmosphere in a CO_2 incubator. The medium was changed by fresh media every day.

The prostate cancer cells PC3 (from passage numbers 30-32) were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (heat inactivated) and 1% antibiotic (100 U/ml of penicillin and 100 µg/ml streptomycin) mix at 37 °C in humidified atmosphere in a CO_2 incubator. The culture medium was changed every alternate day with fresh DMEM-F12 (complete).

The cervix cancer cell lines, SiHa, were grown in low glucose RPMI supplemented with 10% fetal bovine serum and 1% antibiotic (100 U/ml of penicillin and 100 µg/ml streptomycin). The cells used in this study were from passage numbers 67-70. The cell cultures were maintained in humidified atmosphere in a CO_2 incubator at 37 °C and the culture medium was changed with fresh medium every alternate day.

The immortalized human embryonic kidney cell line, HEK-293 (from passage number 60-63) were maintained in DMEM medium supplemented with 10% fetal bovine serum (heat inactivated) and 1% antibiotic (100 U/ml of penicillin and 100 µg/ml streptomycin) mix at 37 °C in humidified atmosphere in a CO_2 incubator.

In this thesis when the interactions of test compounds with progesterone receptor function was assayed *in vitro*, the cells were washed twice with PBS followed by the addition of steroid free complete media (with charcoal stripped serum), 24 h prior to the addition of drugs.

3.5 *In vivo* experimental models

Female laboratory rats: Phylum: Chordata

Subphylum: Vertebrata

Class: Mammalia

Order: Rodentia

Family: Muridae

Genus: *Rattus*

Species: *R. norvegicus*

Subspecies: *R. norvegicus albinus*

were purchased from National Institute of Pharmaceutical Education and Research (NIPER), Chandigarh and All India Institute of Medical Sciences (AIIMS), New Delhi, India. The animals were housed in a well-ventilated animal house with 12 h light : 12 h dark with proper food and water *ad libitum* and were acclimatized for about 10 days prior to initiation of any experiments. The experiments were carried out with the approval as well as guidelines of Institutional Animal Ethical Committee (Regn no. 563/02/a/CPCSEA). After the experiments, the rats were sacrificed by cervical dislocation under ether anesthesia and their blood was obtained by cardiac puncture for further biochemical analyses.

3.5.1 Development of rat breast tumor model with MNU

Breast tumors were developed in female rats, *Rattus norvegicus*, with the approval of Institutional Animal Ethical Committee and according to the protocol of Thomson and Adlakha (1991). Healthy animals (about 40 days old) were purchased from the similar source as mentioned above and maintained in the Institutional Animal House as mentioned above. Initially, a group of animals (n = 40) received a single i.v. injection in the tail vein with N-Methyl-N-Nitroso Urea (MNU) at the dose of 50 mg/kg body weight while other group (n = 10) was injected with normal saline and both the groups were monitored for further 8-12 months. During this time the animals were fed with a balanced animal diet available commercially (Ashirwad Animal Feed Industries, Punjab, India) and had free access to normal drinking water. The rats were weighed weekly since injection of MNU and their mammary glands were palpated twice a week after the start of 5^{th} month. Once the tumor volume reached about 12 mm^3, the rats were finally divided into the following experimental groups. The animals of MNU treated group (as discussed earlier) that did not develop any tumor were not included in this study.

Group I : Healthy female, non-tumor model (n=10)

Group II : Breast tumor model (n=11)

Group III : Drug treated breast tumor model (n=11)

Group IV : Cisplatin treated breast tumor model (n=11)

The tumor volumes of tumor bearing rats (drug treated/non-treated) were measured with slide calipers twice a week in accordance to the formula $0.44 \times A \times B^2$ where A is the base diameter on elongated side of the tumor and B is its perpendicular value (Davoodpour et al., 2007). The specific growth rate (SGR) and the tumor doubling time (DT) were thereafter calculated as:

SGR = $\ln(V_2/ V_1) / (t_2 - t_1)$ and

DT = $(t_2 - t_1)$ ln2 / ln (V_2/ V_1), where t_2 and t_1 represent final and initial time for the measurement of tumor and V_2 and V_1 were the final and initial tumor volumes, respectively (Mehrara et al., 2007).

Approximately after 24 h of final treatment, the rats were sacrificed as described earlier. The blood was collected by cardiac puncture for determination of the hematological parameters and the tumor was excised out for further analysis. Their body weight and the weight of the major organs namely, liver, kidney and spleen were recorded separately.

3.5.2 Determination of lethal dose

Before treatment with novel drugs (like synthetic copper complexes), the determination of its dose at which there was no physiological side effects were required. In order to determine this, the dose which caused the death of 50% of the animals in a group (designated as LD_{50}) was calculated. All doses below this concentration were taken as sub-acute doses for testing various properties of a compound. Thus for determining LD_{50}, healthy female rats (60 days) were divided into the following groups (Table 3.1). The synthetic test molecules were injected intraperitoneally to the rats at a range of concentrations varying from 0.5-50 mg/kg body weight. Each group consisted of 10 animals (n=10) and they were dosed twice per week for 21 days. The number of deaths/group was counted and the equivalent dose at which 50% of the animals died (LD_{50}) was determined.

Table 3.1: Grouping of animals for determination of LD_{50}

Animal groups (n=10)	**Test compounds (*i.p.*) (mg/kg body weight)**
I	0.5
II	1
III	10
IV	20
V	30
VI	40
VII	50

3.5.3 Development of rat uterine C3 model for in vivo screening of anti-progestagen

Female albino rats (60-day-old) were ovariectomised following the guidelines of Institutional Animal Ethics Committee (Reg^{n}. No. 563/02/a/CPCSEA, Govt. of India). After 10 days rats were randomized and placed in groups of six (n=6). The animals were then treated once daily for 2 days:

Group I : Sham control
Group II : Vehicle control (DMSO)
Group III : Progesterone (0.3 mg/kg bw) gavaged
Group IV : Pterostilbene (10 mg/kg bw) s.c. injection in the nape of the neck (0.02 ml)
Group V : Pterostilbene (20 mg/kg bw) – do –
Group VI : Pterostilbene (30 mg/kg bw) – do –
Group VII : RU 486 (3 mg/kg bw) – do –
Group VIII : Estadiol (0.3 mg/kg bw) gavaged

On the second day of treatment, all animals were also treated with estradiol (0.3 mg/kg bw) orally by gavages (following Lundeen et al., 2001). After 24 h the animals were euthanized by 30 mg/ kg body weight of ketamine. The uteri were then removed, stripped of remaining fat and mesentery, weighed, and snap-frozen in liquid nitrogen. Total RNA was isolated from the uteri using the established protocol (Chomczynski and Sacchi, 1987) and the expression of uterine C3 mRNA transcript was checked.

3.6 Cytotoxicity assay

A sub-confluent layer of cells were trypsinized and collected in the respective cell culture media. Cells were counted and seeded at a density of 5×10^3 cells/ml. A volume of 200 µl of the cell suspension (in complete medium), was added to the wells of 96-well plates (Griener, Germany). Serial dilutions of drugs to be tested were dissolved in ethanol (or DMSO) and were added to the monolayer. The final ethanol concentration for all dilutions was 0.1% which was used as vehicle control. The effects of compounds on different cancer cell lines were tested along a range of concentrations under similar conditions. Cultures were assayed after 24 h by the addition of 50 µl of 5 mg/ml MTT and incubating for 4 h at 37 °C as described previously (Mosmann, 1983). The MTT-containing medium was aspirated and 200 µl of DMSO and 25 µl of Sorensen glycine buffer (0.1 M glycine and 0.1 M NaCl, pH 10.5) were added to lyse the cells and solubilize the water insoluble formazan. Absorbance of the lysates was determined on a

Fluostar optima (BMG Labtech, Germany) microplate reader at 570 nm. The percentage inhibition was calculated as:

[(Mean OD of vehicle treated cells – mean OD of treated cells) × 100]÷Mean OD of vehicle treated cells.

The IC_{50} values were calculated using graph pad prism, version 5.02 software (GraphPad Software Inc., CA, USA).

3.7 Acridine orange/ ethidium bromide staining

In order to check the plasma-membrane permeability, nuclear morphology and the chromatin condensation, the cells were stained with Acridine orange (AO) / Ethidium bromide (EB) dye mixture (100 µg/ml AO and 100 µg/ml EB) prepared in PBS according to the protocol described earlier (Takahashi et al., 2004). This protocol gives a visual distinction of the nucleus of healthy cells as well as cells undergoing death due to apoptosis or necrosis. 0.5×10^6 cells were seeded for the assay in a 6-well plate. Test compounds were added to the cell monolayers when they had reached a confluency of 60%. After 24 h of treatment, the cells were properly washed with PBS. Then 500 µl AO/EB dye mixture was added to the plate and incubated for 2 min. The cells were again washed with PBS and immediately observed under fluorescent microscope (Zeiss, Axiovert 25, Germany).

3.8 DNA fragmentation assay

The DNA cleavage pattern due to the cytotoxicity of the compound was analyzed by agarose gel electrophoresis according to the protocol described earlier (Alcazar et al., 1997). 3×10^6 cells were plated in 9 cm plate. When sub-confluent, the cells exposed to respective drugs for 24 h. Thereafter cells were washed with PBS, scraped, and the pellet was lysed with 400 µl of hypotonic buffer solution (10 mM Tris pH 7.5, 1 mM EDTA, 0.2% triton X-100) for 15 min at room temperature followed by 15 min centrifugation at 16,000 ×g at 4 °C. In order to isolate the whole-cell genomic DNA, 350 µl of the supernatant was again lysed in 106 µl of the second lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 40 mM EDTA, 1% SDS, 0.2 mg/ml Proteinase K (at final concentration) for 4 h at 37 °C. The DNA was extracted with phenol/chloroform/isoamyl alcohol (25:25:1) and the pellet obtained was washed with ethanol and resuspended for RNase digestion in 15 µl of 10 mM Tris, 1 mM EDTA, pH 8.5, and 50 µg/ml RNase for 1 h at 37 °C. The DNA was analyzed by electrophoresis at 50 V/cm in a 2% agarose gel.

3.9 Single cell gel electrophoresis (Comet assay)

Apart from the previous DNA fragmentation assay, cyto-toxicity leading to DNA damage was further validated by single cell gel electrophoresis. The protocol was followed in accordance with the method described earlier (Tice et al., 2000), with minor modifications. 3×10^6 cells were plated in 9 cm plate, when sub-confluent, treatment was given with respective drug concentrations for 24 h, thereafter cells were washed with PBS and harvested. The cells were counted and a cell suspension was prepared at a density of 6×10^6 cells/ml. Glass slides coated with a thin layer of 1% normal melting agarose were prepared previously. Equal amount of cell suspension and 0.65% low melting agarose were mixed at 37 °C in PBS and quickly layered on the prepared slide. A topmost layer of 1% normal melting agarose was finally applied to the slide. The prepared slide was then stored at 4 °C for 10 min before incubating it in lysis buffer consisting of 2.5 M NaCl, 100 mM EDTA pH 10, 10 mM Tris-base, 1% Sodium lauryl sarcosinate and 1% Triton X-100 at 4 °C for 2 h. Electrophoresis was carried out for 5 min at 25 V in 300 mM NaOH, 1 mM EDTA. The slides were then neutralized with Tris at pH 7.5. The nuclei were stained with EB and the visualized under fluorescent microscope. Comet tail length was calculated as distance between the end of nuclei heads and end of each tail. Tail moments were defined (according to the equation of Bentle et al., 2006) as the product of the %DNA in each tail, and the distance between the mean of the head and tail distributions as:

%DNA(tail) = TA × TAI × 100 / [(TA × TAI) + (HA × HAI)] where, TA= tail area, TAI= tail area intensity, HA= head area and HAI= head area intensity.

3.10 Clonogenic assay

The protocol measured the colony formation capacity of the cancer cells after being exposed to drugs. This assay was a measure of the mitotic potential of the remaining cancer cells which tolerate the anticancer drug exposure and remain to be alive. Cells were initially seeded on 6 well plates at a low density of 0.5×10^4 cells/well in complete medium. When the cells reached a confluency of ~ 55%, they were washed in complete medium and treated with respective concentrations of drugs. The cells were then removed after different time points of drug exposure using trypsin-EDTA (Invitrogen, Carlsbad, CA, USA), counted and re-seeded in 9 cm plates in triplicates for further growth in drug free environment. Following 7-10 days of incubation at 37 °C in a humidified atmosphere in a CO_2 (5%) incubator, colonies were fixed and stained in 2% crystal violet in absolute ethanol and finally counted. Clones of at least 50 cells were counted as one colony. The plating efficiency was calculated for each well by

dividing the number of colonies by the original number of cells plated. The surviving fraction was normalized to control cell plating efficiency by dividing the plating efficiency of treated cells by that of control cells (Raffoul et al., 2006).

3.11 Soft Agar Assays

This protocol additionally estimated the metastatic capacity of the cancer cells after exposure to an anticancer drug. The soft agar assay was carried out as described earlier (Roh et al., 2008). About 10^5 cells/ml were obtained by trypsinization of a sub-confluent monolayer of cells. These cells were mixed with 0.3% soft agar and plated on top of 0.6% bottom agar previously seeded on each of a 6 cm plate. The medium was changed regularly after every 24 h. On the fifth day after seeding, the test chemicals were added to the medium and the cells were incubated for 72 h. The cells were additionally incubated at 37 °C for 10 days in drug-free complete media to allow three dimensional colony formations. The cells were then stained with trypan blue (0.25% in PBS) to check their viability. Images were captured with a Zeiss Axiovert40 inverted wide field microscope. Triplicate plating was carried out for each sample. The migration potential of the cells was calculated as :

[No. of Surface colonies x average size of a colony x Thickness of the agar layer] / Initial no. of cells seeded

3.12 Caspase assay

The caspase activity was measured using caspase 3 substrates kindly donated by Dr. Arun Bandyopadhyay, Indian Institute of Chemical Biology, Kolkata, India. Cell lysate was prepared using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA) for 10 min at 0 °C followed by centrifugation at 10,000 × g for 10 min at 4 °C. The total protein in the lysate was measured with BCA kit (Sigma-Aldrich) according to manufacturer's protocol. A total of 20 µl of lysate containing 100 µg of total protein was added to the assay buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol) containing 200 µM caspase substrates to make a total of 100 µl solution and incubated for 2 h at 37 °C. The absorbance was then monitored at 405 nm to determine the caspase activity.

3.13 Scanning electron microscopy

About 5 × 10^6 cells were plated in each of 9 cm tissue culture plates. When the cells reached sub-confluency, the test compounds were added. After 24 h, the cells were washed with

PBS and then fixed with sodium cacodylate buffer (100 mM sodium cacodylate, pH 7.2, 2.5% glutaraldehyde, 1.6% paraformaldehyde) (Arvidson et al., 2003). After removal of the fixative, the cells were again washed with PBS, scraped and pelleted at 900 ×g. The cell pellet was then post-fixed for 1 h in 1% osmium tetraoxide, 0.8% potassium ferricyanide in 100 mM of sodium cacodylate, pH 7.2. They were next thoroughly washed with water and dehydrated in increasing gradients of alcohol. Traces of alcohol were then removed and the cells were coated with gold film using a sputter coater (S150, Edwards high vacuum Co., Massachusetts, USA). Finally the surface morphology of the cells was examined by a Leo435VF (UK) scanning electron microscope.

3.14 Transmission electron microscopy

An equal number of 5×10^6 cells were plated in each of 9 cm tissue culture plates. When the plate reached ~ 55% confluency, the test compounds were added. The cells were fixed after various time intervals. Fixation was carried out with 2% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) for 1 h and then washed with Sorensen phosphate buffer (0.1 M) for 12 h. The standard 0.1 M Sorensen phosphate buffer was prepared by mixing 40.5 ml of $Na_2HPO_4.2H_2O$ (0.2 M) and 9.5 ml of $NaH_2PO_4.2H_2O$ (0.2 M) and finally it was diluted to 100 ml. The cells were then post-fixed with 1% OsO_4 in buffer containing 0.05 M Sorensen phosphate buffer and 0.25 M glucose for 1 h followed by washing them twice with distilled water and pre-stained with an aqueous solution of 2% uranyl acetate for 2 h (according to the standardized protocol followed by Sophisticated Analytical Instrument Facility for electron microscopy, All India Institute of Medical Sciences, New Delhi, India). The cells were then examined by a Morgagni 268D (Fei Company, The Netherlands) transmission electron microscope. The images were visualized by SiViewer Olympus Soft Imaging Solutions.

3.15 Cell lysate preparation for antioxidant enzyme assays

An equal number of 5×10^6 cells were plated in each of 9 cm tissue culture plates. The cells were exposed to test compounds for 24 h, washed with PBS and harvested as single cell suspension. In case of *in vivo* animals, 100 mg of rat breast tissue was taken to prepare the cell lysate. Cell lysates were prepared in ice cold PBS (pH 7.4) containing 2 mM PMSF with sonication (3 bursts of 20 sec each). Total protein was quantified with BCA protein quantification kit. Equal amounts of protein samples were taken for each enzyme assays.

3.16 Antioxidant enzyme assays

Catalase, glutathione peroxidase and glutathione reductase assays were carried out to determine the intracellular antioxidant enzyme status. Total glutathione was assayed as a measure of intracellular antioxidant molecular content. Catalase assay was performed according to the method described earlier (Aebi, 1984). An amount of 0.3 mg total protein/ml from the cell lysate (as described in the previous section) were added to the assay buffer containing 20 mM H_2O_2, 10 mM K-phosphate buffer at pH 7.0 and then incubated for 10 min at room temperature. The absorbance of H_2O_2 was measured in a spectrophotometer (Cary 100 Bio, Varian, USA) at 240 nM.

Glutathione peroxidase was estimated according to the earlier described method by Paglia and Valentina (1967). The assay buffer consisting of 50 mM K-phosphate buffer (at pH 7.0), 4 mM NaN_3, 5 mM GSH, 0.35 mM of NADPH, 1U glutathione reductase and 100 µl of H_2O_2 was added to total protein of 300 µg/ml in the lysate. The absorbance of NADP produced was estimated at 340 nM after 5 min of incubation.

Glutathione reductase assay was performed according to Bergmeyer and Garvehn, (1978). For this, 0.01 ml of 10 mM EDTA, 0.06 ml of 10 mM NADPH, 2 µl β-mercaptoethanol, 0.1 ml of 250 mM GSSG (glutathione oxidized) were added to 0.778 ml of potassium-phosphate buffer solution at pH 7.4. To this 0.05 ml of the total lysate consisting of 0.5 mg total protein/ml were added followed by thorough mixing. After incubation for 5 min the absorbance of NADPH was measured at 340 nm.

Total glutathione was determined according to the protocol described earlier by Pae et al. (2003). About 5×10^6 cells were seeded in a 9 cm plate and incubated with the test compounds for 24 h. After incubation, the cells were washed and scrapped with ice cold PBS and resuspended in 300 µl of buffer A containing 125 mM potassium dihydrogen phosphate, 6.3 mM EDTA, pH 7.5 and was subjected to rapid freeze-thaw processing followed by sonication for 1 min. The protein from the supernatant obtained thereafter was precipitated with 5% trichloro acetic acid for 15 min. After centrifugation at 14,000 ×g for 15 min, the protein free lysate was then analysed for the total glutathione content. The reduction of glutathione by glutathione reductase was carried out in a 1 ml cuvette containing NADPH (0.525 mM), 5',5'- dithiobis-2-nitrobenzoic acid (1.5 mM) and supernatant in the ratio of 2:2:1 and 1 U/ml of glutathione reductase. The absorbance of the reaction mixture was carried out in a spectrophotometer for 3

min for every 20 s interval and the glutathione content was calculated from the rate of change of absorbance.

All the enzyme activities were finally expressed as nM/mg total protein/min.

3.17 Alkaline phosphatase assay

The alkaline phosphatase assay was performed in order to understand the anti-progestagenic effect of a compound in PR positive T47D cells. The protocol was earlier described by Mukherjee (Mukherjee, 2003) and was further followed with slight modifications. 40 µg of total protein form cell lysate was added to pNPP-glycine buffer, prepared freshly (1:1) containing 0.1M glycine (pH 9.8), 0.5 mM $MgCl_2$ and 10 mM of pNPP (p-Nitrophenol phosphate). After incubation for 30 min the reaction was stopped by adding 0.05 N of NaOH. The absorbance was measured at 405nm.

3.18 Measurement of intracellular Rreactive Oxygen Species (ROS) levels

The generation of reactive oxygen species was detected using nitroblue tetrazolium (NBT) reduction assay as previously described (Ishii et al., 2008) with slight modifications. 100 µl of cell suspension containing 10^6 cells from various treatments were mixed with 100 µl of NBT solution (1.4 mM NBT, 1 mM EDTA, 1 mM phenylthiourea) and incubated at 37 °C for 4 h. The samples were centrifuged 1000 ×g for 10 min and then washed with 500 µl of 70% methanol and NBT reduction was measured by dissolving the pellet in extraction solution containing 1 ml of 2 M KOH and 1.5 ml of dimethyl sulphoxide followed by vigorous mixing. The samples were centrifuged at 3500 ×g for 20 min and the O.D was measured at 630 nm. In order to check the exact reactive oxygen species generated as a result of apoptosis, specific free radical scavengers NaN_3 (singlet oxygen scavenger), KI (hydroxyl ion scavenger) and catalase (hydrogen peroxide scavengers) were added to inhibit their further production (Azmi et al., 2005) in the cell culture medium along with the treatments with the test compounds. 24 h after the combined incubation of the compounds and the ROS scavengers, the intracellular ROS was measured similarly as mentioned above.

3.19 Oil Red O Staining Procedure

The production of intracellular neutral lipid was stained and quantified by oil red o (ORO) procedure. Approximately 10^5 cells were grown on glass coverslips in a 6-well plate. The cells were treated with test compounds for various time durations (24 to 72 h), followed by

fixing them with 3.7% paraformaldehyde for 1 h at room temperature. They were then washed two times with PBS. Thereafter the cells were stained by ORO dissolved in 60% isopropanol for 30 min. After washing the excess stain, the images were captured with a Zeiss Axiovert40 inverted wide field microscope. The lipids were quantified by dissolving the ORO stain in 100% isopropanol and the absorbance was measured at 510 nm according to published procedure (Vosper et al., 2001).

3.20 Triglyceride measurement

For the estimation of cellular triglycerides, 0.5×10^6 cells were grown in 6 cm plates and treated with drugs for 24, 48 and 72 h. After the drug exposure the cells were washed with PBS and cell lysate was prepared according to the protocol mentioned in section 3.15. The triglyceride level was estimated in the cell lysates using a commercially available kit (Erba, Germany) according to the manufacturer's instruction.

3.21 Extraction of lipids

For extraction of intracellular sterols, 5×10^6 cells were seeded in 9 cm plates. Following incubation with the various test compounds for 72 h cellular lipid contents were extracted according to the method described earlier (Bligh and Dyer, 1959) with slight modifications. After harvesting, the cells were washed three times in PBS and then sonicated 3 times for 15 s each (cycle 40, output 80%) and finally the cell lysate was cleared by centrifugation at 4000 ×g for 5 min. The lysate was diluted with 1 ml of deionised water and the lipids were extracted with 4 ml of n-hexane for 5min. Saponification was achieved by adding 0.5 ml of potassium hydroxide in ethanol (0.16 M) followed by heating at 55°C for 15 min. The samples were again centrifuged at 4000 ×g for 2 min, after which the organic phase was transferred to a glass tube and dried using gentle nitrogen stream at room temperature.

3.22 Thin Layer Chromatography

Thin layer chromatography was carried out with two different samples using different solvent phases.

3.22.1 TLC of phytochemical

In order to check the purity of pterostilbene isolated from the extracts of *Pterocarpus marsupium*, 10 µl of the partially purified fraction was spotted on 20 × 20 TLC silica gel 60 CF254 plates (Merck, Darmstadt, Germany). Authentic standards of trans-pterostilbene 2

(Sigma, St. Louis, USA) were used to monitor the movement of the pterostilbene spot. Linear ascending development of the samples were carried out using a mobile phase of EtOAc:AcOH:H2O (17:1:2) as described by Nepote et al. (2004). After a complete run the samples were incubated in an iodine chamber and visualized.

3.22.2 TLC of intracellular sterol

For detecting the intracellular sterol content, silver nitrate staining of the TLC plate was carried out with silica gel 60 from Merck (Darmstadt, Germany) according to the protocol described by Kedjouar et al. (2004). At the beginning, the plates were pulverized with a solution of 0.1% (w/v) silver nitrate in acetonitrile in the absence of light, and then wrapped in aluminum foil before drying in an oven under reduced pressure at 110 °C over 1 h. Intracellular sterols that were extracted, analyzed by silver nitrate thin layer chromatography with methanol/acetone (57:2, v/v) as the mobile phase. Samples were then detected by spraying with 50% sulfuric acid in methanol (v/v) and by heating the chromatogram on a hot plate. The standards used for calibration were cholesterol, lathosterol, 7-dehydrocholesterol and desmosterol at 1 mg/ml in ethanol. Retention factors (RF) were determined for each spot on the TLC as the ratio between the distance of migration of the eluate from the deposit and the distance of the solvent from the deposit.

3.23 Immunoflourescence

For immunoflourescence, 5×10^4 cells were plated onto 6-well plates. After drug treatment, the cells were washed twice with ice-cold PBS and fixed with methanol and acetone solution (1:1) for 15 s. The fixed monolayers were then washed with distilled water and blocked with 5% BSA in PBS solution. After blocking, the cells were incubated with the primary antibody of cytochrome c, TIMP-3 and PR (1:100 in 5% BSA in PBS) at 37°C for 1 h and washed three times with 1% BSA in PBS. Then the monolayers were incubated with fluorescein-conjugated secondary antibodies (1:200) at 37 °C for 1 h. Cells were then visualized and imaged under 40 x magnification (objective) by Zeiss Axiovert40 microscope.

3.24 Immunobloting

For immunoblotting, 5×10^4 cells were plated onto 6-well plates and exposed to test compounds for various time intervals. Cell lysates were prepared following cell harvesting in lysis buffer (20 mM Tris pH 7.2, 5 mM EGTA, 5 mM EDTA, 0.4 % (w/v) SDS and 1x protease inhibitor cocktail). The protein was quantified with BCA protein estimation kit according to the

manufacturer's protocol. About 40 µg of total protein samples were then analyzed by 12% polyacrylamide gel (Laemmli, 1970). This was followed by immunoblot analysis of the protein samples according the method described earlier (Park et al., 1999). The analyzed proteins were transferred to PVDF membrane and the blots were then blocked with TBST (20 mM Tris-Cl, pH 7.5, 150 mM sodium chloride, 0.05% Tween-20) containing 5% skimmed milk powder. They were then washed with TBST and incubated overnight at 4 °C with TBST buffer containing appropriate amounts of primary antibodies in a respective dilution as given in table 3.2. The blots were then washed and incubated with anti-rabbit or anti-goat secondary antibody (1:200) conjugated with either ALP (Alkaline phosphatase) or horseradish peroxidase, depending on the secondary antibody used. Color development was performed in dark with the alkaline phosphatase chromogen, BCIP-NBT substrate (Bangalore Genei, India) or 0.5% of DAB (chromogen for horseradish peroxidase). The images of the developed blots were captured with epi-illumination of the gel documentation system (Bio Rad, USA). Each of the blots were normalized with β-actin as internal control using ImageJ 1.43 software (NIH, USA).

Table 3.2: List of antibodies used for immunoblot analysis

Primary antibody	Dilutions	Secondary antibody	Dilutions	Chromogen
Caspase 3	1: 400	Anti-mouse	1:200	DAB
Caspase 9	1:200	Anti-rabbit	1:200	DAB
p38	1:400	Anti-rabbit	1:200	DAB
p-Akt,	1:400	Anti-rabbit	1:200	DAB
Akt	1:400	Anti-rabbit	1:200	DAB
Bax	1:400	Anti-rabbit	1:200	DAB
Bcl-2	1:400	Anti-rabbit	1:200	DAB
Beclin 1	1:1000	Anti-rabbit	1:200	BCIP/NBT
LC3	1:1000	Anti-rabbit	1:200	BCIP/NBT
PR	1:1000	Anti-rabbit	1:200	BCIP/NBT
SRC1	1:1000	Anti-rabbit	1:200	BCIP/NBT
GRIP-1	1:1000	Anti-rabbit	1:200	BCIP/NBT
β-actin	1:400	Anti-rabbit-HRP	1:200	DAB
		Anti-rabbit-ALP	1:200	BCIP/NBT

3.25 Dot-blot analysis

For dot-blot analysis, the nuclear and the cytoplasmic fractions were isolated separately according to the method of Caron-Leslie (Caron-Leslie et al., 1991). About 10^7 cells from each of experimental sets were subjected to hypotonic shock using ice-cold buffer (1.5 mM $MgCl_2$, 0.25% (v/v) Triton X-100, 1 mM PMSF, 2 mM EDTA, 0.5mg/ml leupeptin). The nuclei were then harvested by centrifugation (100 ×g), and the resulting supernatant was collected as cytoplasmic extracts. The nuclei pellet was washed twice with the above buffer, and nuclear protein was extracted with 0.3 M NaCl, 10 mM Tris-Cl, pH 7.4, 1 mM PMSF, 2 mM EDTA, and 0.5 mg/ml leupeptin at 4 °C for 1 h on a moving platform. The chromatin material was pelleted by centrifugation at 10,000 ×g for 45 min at 4 °C. The supernatant (nuclear extract) was collected and stored at -80 °C. The total protein was quantified from cytoplasmic and nuclear fractions of each of the treatments with BCA protein estimation kit according to the manufacturer's protocol (Sigma, St. Louis, USA). About 20 µg of total protein was transferred to the PVDF membrane as dots. Ponceau S staining was performed for total protein and visualized by a digital camera. The membrane was blocked overnight at 4°C in TBST (20 mM Tris–HCl pH 8.0, 137 mM NaCl and 0.1% Tween 20) and 5% dry milk. Following washing with TBST, the membrane was incubated with primary antibody for PR (1:2000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 1 h. The dot-blots were then washed and incubated with anti-rabbit secondary antibody (1:200) conjugated with ALP (Bangalore Genei, India). Color development was performed in dark with BCIP-NBT substrate (Bangalore Genei, India). The images of the developed blots were captured with epi-illumination of the gel documentation system (Bio Rad, USA). The blots were analysed by ImageJ 1.43 software (NIH, USA).

3.26 Reverse transcriptase PCR

Total RNA was extracted from the treated cells according to the method described earlier (Chomczynski and Sacchi, 1987). Initially, 10^4 cells were seeded in a 6-well plate. After the respective drug treatments the cells were washed twice with PBS and harvested. In case of *in vivo* model, 100 mg of the rat breast tissue were taken and powdered in liquid nitrogen. The cells therefore obtained from *in vitro* or *in vivo* were lysed with 0.5 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate and 0.1 M β-mercaptoethanol). 50 µl of 2 M sodium acetate (pH 4.0), 250 µl of phenol and 250 µl of choloform mix (chloroform:isoamyl alcohol, 49:1) were sequentially added to the lysate

followed by brief vortexing. The separation of the aqueous and orgainc phases was achieved by a centrifugation at 14,000 ×g for 30 min at 4°C. The upper aqueous phase which contained total RNA from the cell lysate was collected and the RNA was precipitated with isopropanol at -20°C overnight. Finally, the RNA pellet was washed twice with 75% ethanol and dissolved in DEPC (diethylpyrocarbonate) treated water. In order to finally obtain the DNA-free RNA , the samples were treated with DNase (5 U/20 µg total RNA) for 30 min followed by phenol-chloroform extraction. This was done in order to remove all traces of genomic DNA contamination in the total RNA isolated. 2 µl of the total RNA isolated was dissolved in nuclease free water and its O.D (optical density) was determined at 260 nm against nuclease free water taken as blank. The protein contamination in the sample is also measured at an absorbance of 280 nm. The RNA samples which had a ratio of OD_{260} : OD_{280} >1.8 was further analyzed by RT-PCR. Equal amount of the individual treatments were reverse transcribed with the RT-PCR kit (Bangalore Genei, Bangalore, India) according to the manufacturer's instruction. Similar patterns of treatment followed by RNA isolation and RT-PCR was carried out three times to determine the inter-assay variations. PCR was performed in thermocycler (PTC-200, MJ Research, USA) by initial denaturing at 94 °C for 2 min, denaturing 30 s, annealing at various temperatures (depending on primer pairs used) for 30 s and extension at 72 °C for 45 s followed by number of cycles for amplification. A final extension at 72 °C for 2 min was programmed to complete the product extensions. The PCR amplification products were stored at - 20 °C until further use. They were then separated in 1% agarose gel and visualized in a gel documentation system (Bio Rad, USA). The intensity of the bands on gels was converted into digital image with a gel analyzer. Primers were designed with the help of Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998, Primer3) and standardized in the laboratory. Cyclophilin, GAPDH and/or β-actin PCR products were used as internal standards. Primer sequence, product size, annealing temperature, number of cycles used and gene bank accession number of all primers are presented in table 3.3.

Table 3.3: List of primers used for the semi-quantitative RT-PCR in the present study

Primers	Sense	Antisense	Annealing Temp. (°C)	Length Base pairs	NCBI Acc. No	Cycles
ABCA1 (Human)	TTAAACGCCCTCAC CAAAGAC	AAAAGCCGCCATA CCTAAACT	47.8	394	NM_0 05502. 2	30
ABCG1 (Human)	ACTGCAGCATCGTG TACTGGA	CGTCTCGTCGATG TCACAGTG	47.8	275	NM_2 07627. 1	30
Akt (Human/Rat)	GTAGAGGTTGCCCA CACGCTTAC	GTAGTCGTTGTCC TCCAGCACCT	63	410	NM_0 01014 431.1	30
AMACR (Human/Rat)	ATCTTTGACGGGAC AGATGC	CTTTTGGCAGAAG GAACAGC	55	169	NM_0 12816	30
β-actin (Human)	TCACCCACACTGTG CCCCATCTACGA	CAGCGGAACCGCT CATTGCCAATGG	59	300	NM_0 01101. 2	30
Bax (Human)	TGCAGAGGATGATT GCTGAC	GAGGACTCCAGCC ACAAAGA	60	317	NM_1 38761. 2	30
Bax (Rat)	TGCAGAGGATGATT GCTGAC	GAGGACTCCAGCC ACAAAGA	60	330	NM_0 07527. 3	30
Bcl-2 (Human)	GTAAACTGGGGTC GCATTGT	TGCTGCATTGTTC CCATAGA	58	168	NM_0 01191	30
Bcl-2 (Rat)	CGACTTTGCAGAGA TGTCCA	ATGCCGGTTCAGG TACTCAG	60	222	NM_0 16993	30
C3 (Rat)	CCACGTCAGGGTCC CAGCTACTAG	ACGAAAGCTGTCC CGTCCACG	57	805	NM_0 16994. 2	30

Caspase3 (Human/rat)	TTTTTCAGAGGGGA TCGTTG	ACAAAGCGACTGG ATGAACC	49	237	NM_0 32991	30
C/EBPα (Human)	TGGACAAGAACAG CAACGAG	CTGGCCCCCTCAT CTTAGAC	47.8	446	NM_0 04364. 3	30
CHOP (Human)	TGCCTTTCTCCTTC GGGACACTGT	CGGTCAATCAGAG CTCGGCGA	59	498	NM_0 04083. 4	30
Cyclophilin (Human)	CGCCTCTCCGAACG CAACATGAA	TCCACCTCGATCT TGCCGCAG	57.5	617	NM_0 00942. 4	30
DHCR-7 (Human)	CAGCGCCAGAGAC TGCAAA	TGAAGAACAGCTT GAAGTCAAACC	50.8	136	NM_0 01360. 2	30
GAPDH (Rat)	AGACAGCCGCATCT TCTTGT	CTTGCCGTGGGTA GAGTCAT	58	207	NM01 7008	30
KLK4 (Human)	AACAGACCCTTGCT CGCTAA	CCAGAGTTAACTG GCCTGGA	48	447	NM_0 04917. 3	30
LXRβ (Human)	CGCCCCTTCTTCTT CACCCACTGTAA	GCCTGCACGGTGG AAGTCGT	55.8	967	NM_0 07121. 4	30
MMP9 (Human)	TTGACAGCGACAA GAAGTGG	CCCTCAGTGAAGC GGTACAT	52	148	NM_0 04994 .2	30
MMP9 (Rat)	TTCGACGCTGACAA GAAGTG	AGGGGAGTCCTCG TGGTAGT	52	136	NM_0 31055	30
Nkx3.1 (Rat)	GGCACTTGGGGTCT TATCTG	GGGGCTGACAGGT ACTTCTG	58	166	NM_0 06167	30
ORP1L	TGATTGCCTTAATC TCTTCACC	ACTCAGGGACCTT TCGGACTC	47.8	223	NM_0 80597.	30

(Human)					2	
PR (Human)	AACACGTCAGTGG GCAGATG	GCAGCAATAACTT CAGACATC	45.2	418	NM_0 00926. 4	30
TNFα (Rat)	CTCTTCAAGGGACA AGGCTG	TCGCTTCACAGAG CAATGAC	57	290	NM_0 12675. 2	30
Wnt (Rat)	TGT ATC AGG ACC ACA TGC AG	GCT CGG CTC ATG GCA TTT AC	52	392	NM_0 22631	30

3.27 Transfection

The day before transfection, 8×10^5 cells were seeded in 6 cm cell culture plates in complete medium. The cells were incubated at 37 °C in a 5% CO_2 incubator until they grew to 50-80% confluency on the day of transfections. Then, 2.5 µg of DNA was dissolved in TE buffer (pH 7.0) (minimum DNA concentration being 0.1 µg/ml) and diluted with cell growth medium without serum and antibiotics to a total of 150 µl. In case of co-transfections with two plasmids, the receptor containing plasmid and the reporter plasmid DNA were taken in a ratio of 1:4 concentrations, respectively, keeping the total DNA constant (2.5 µg). 15 µl of polyfect and/ nanofect transfection reagent (Qiagen, CA, USA) were added to the DNA solution and mixed well by slow pipetting, according to manufacturer's instructions. The DNA-dendrimer complex formation is achieved in 10 min at room temperature. During the incubation of the complex, the cell medium was aspirated from the culture plates and the cells were washed twice with PBS. To the plate, 4 ml of complete medium was added. 1 ml of complete media was added to the DNA-dendrimer complex and very gently mixed. The whole transfection complex was layered on the cell monolayer dropwise and the plate was gently swirled to ensure uniform distribution of the complexes. The cells were incubated for 24 h at at 37 °C in a 5% CO_2 incubator before treating them with test compounds. In this study pCMX-LXRβ, pEGFP-PRA, pEGFP-PRB, pGL3-PRE-luc and pSG5-PR were transfected to cells under different experimental requirements. Each experiment was performed in triplicate and varied by less than 10%. All the transfection experiments were normalized with β-galactosidase expression vectors (pCMV-beta-gal). The transfection efficiency was checked by normalizing the values with respect to β-galactosidase activity.

3.28 Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay (ChIP) was carried out in order to check the interaction of PR (progesterone receptor) to its response element in presence of test compound. The assay was carried out according to the protocol described earlier by Weinmann et al. (2001) with minor modifications. T47D cells were seeded in 9 cm plates and grown till 80% confluency. Thereafter the complete medium was replaced with charcoal stripped serum containing medium for 24 h prior to the addition of compounds. The cells were incubated with pterostilbene (30 μM), with or without the presence of progesterone (10 nM), RU486 (10 nM) with or without progesterone (10 nM) or left only with vehicle treatments for 24 h. After treatment the cells were washed twice with phosphate buffered saline (PBS) and then cross-linked with 1% formaldehyde in PBS. Next, the cells were again washed twice with PBS at 4°C, collected and resuspended in 500 μL of lysis buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH6.5) followed by 500 μL of lysis buffer II (200 mM NaCl, 1mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5) and the pellet was collected by centrifugation at 5000 ×g for 2 min at 4°C. The nuclear pellet thus obtained was resuspended in 200 μL of lysis buffer III (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) in presence of protease inhibitor cocktail and left on ice for 10 min. Then, the suspension was sonicated three times for 10 seconds at 40% maximal power (Fisher Sonic Dismembrator, Pittsburgh, PA), followed by centrifugation at 4°C for 10 minutes at 14,000 ×g. Supernatant was removed and diluted in 600 μl of immunoprecipitation buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0). The chromatin was immunoprecipitated with PR-antibody (Santa Cruz Biotechnology) at a final concentration of 2 μg/ml at 4°C overnight. Protein A agarose (45 μl of 50% slurry in 10 mM Tris-HCl pH-8.0, 1 mM EDTA) was pre-cleared with 2 μl of sheared salmon sperm DNA (10 mg/ml) and 1 μL BSA (100 mg/ml) for 1 h at 4 °C. 45 μl of precleared protein A agarose beads were added to the immunoprecipitated complex for DNA adhesion and further incubated for 4 h at 4 °C. The agarose beads were collected by centrifugation at 5000 ×g for 2 min and were sequentially washed for 5 minutes with the following buffers: wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, and 150 mM NaCl), wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, and 500 mM NaCl), and wash C (0.25 M LiCl, 1% Nonidet P40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris-Cl, pH 8.1). The precipitates were then washed twice with TE buffer. The immune complexes were eluted with the buffer containing 1% SDS and 0.1 M $NaHCO_3$. The elutes were

reverse cross-linked by heating at 65°C for 6 h and the DNA was obtained by phenol/chloroform extractions followed by a suspension in TE buffer. 1 µl of each sample were used for PCR with c-myc promoter sequences containing progesterone response element: upstream, 5'-AACAAGGAGGTGGCTGGAAACTTG-3' and 5'CCGCGTTCAGGTTTGCGAAAGTAA-3' primer pair (nucleotides -1895 to -2078), yielding an amplified product with a size of 184 base pairs. The PCR amplification conditions were as follows: initial denaturation at 94 °C (3 min); 33 cycles of incubations at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and a final one-step elongation at 72 °C for 2 min. As a negative control, PCR amplification was also carried out under the same conditions with chromatin DNA prior to immunoprecipitation with PR antibody. The amplification products were analyzed in 2% agarose gel and visualized by ethidium bromide staining.

3.29 Histopathology

The tissues from different organs were collected from all groups of animals and fixed in 10% neutral buffered formalin solution. The fixed tissues were dehydrated in series of ethanol gradations from 30 to 100%, then cleared in xylene for 1 h with two changes and finally embedded in wax at 60 °C (two changes). Paraffin blocks of tissues were sectioned at 5 µm thickness and fixed on glass slides. The sections were de-paraffinated in xylene followed by hydrations in gradations of alcohol (100-70% ethanol). The slides were kept of 2 min in each of alcohol gradation. Thereafter, the sections were stained with Harry's haematoxylin (30 s), washed with tap water (1 min). These sections were again dehydrated in ethanol (70-90%) 2 min each and counter stained with eosin (2 min), followed by dehydration again in 90% and absolute ethanol for 2 min each. The sections were finally passed through xylene (1 min) and mounted with DPX (according to the methods described earlier by Mukherjee, 2003).

In case of tumor tissues, the mitotic and apoptotic indices were calculated by counting the total number of mitotic cells and the cells undergoing apoptosis respectively in at least 10 microscopic fields per tumor tissue section and analyzed as follows:

Mitotic index = [(Total number of mitotic cells) × 100] ÷ Total number of cells in the microscopic field,

Apoptotic index = [(Total number of apoptotic cells) × 100] ÷ Total number of cells in the microscopic field.

3.30 Primary breast culture

The mammary gland culture was carried out according to the procedure of Bhat et al. (2001) with minor modifications. The rats were killed by cervical dislocation, and the thoracic pair of mammary glands and/tumors were dissected out and incubated for 2 days in RPMI high glucose media at 37 °C in 5% CO_2 environment. The medium was supplemented with 350 µg/ml glutamine, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), and growth-promoting hormones (5 µg of insulin, 1 ng of E_2, and 1 ng of progesterone per ml of medium; Sigma Chemical Co.). The pterostilbene (30 µM) was added on the second day and incubated for an additional 72 h before analyzing the plates.

3.31 Statistical analysis

For all the quantitative estimations the statistical analysis were carried out with Origin 6.1 software (Origin Lab Corporation, USA). The data was expressed as mean ± S.E.M. (standard error of mean). The values in the result were initially calculated as an average of duplicates and then the experiments were repeated thrice and the mean ± S.E.M. were calculated. For statistical analyses of data, ANOVA (one-way analysis of variance), followed by multiple two-tail comparison *t*-test were used. The maximum level of significance as $P < 0.05$ was considered but lesser levels of significance were always mentioned wherever applicable.

CHAPTER 4

SCREENING OF ANTI-CANCER COMPOUNDS

4 Screening of anti-cancer compounds

4.1 Introduction

Anticancer compounds are screened by their potency to induce cytotoxicity on cancer cells *in vitro*. The definition of cytotoxicity varies, depending on the nature of the study and whether the cells are killed or simply have their metabolism altered. To study the effect of anticancer agents, the molecules are expected to induce cell killing by means of apoptosis distinguishing it from necrosis, which generally is caused by some stress. In cancer cells the events of apoptosis are triggered only when the cell loses its mitotic potential along with jeopardized metabolism (Freshney, 2005). Hence, mere cell viability measurement cannot exactly prove a drug to be non-tumorigenic. Mosmann, (1983) adapted a direct estimation of a population of cells that are viable and metabolically active after being exposed to the drug concentrations. MTT is a yellow water soluble tetrazolium dye that is reduced by mitochondrial succinate dehydrogenase of the live and metabolically active cells to form a purple coloured insoluble formazan. Estimation of the formazan thus produced is direct measure of the cytotoxicity of the highly dividing cancer cells following the respective drug exposure. In order to screen new compounds *in vitro*, various cancer cell lines of breast, prostate and cervix were used in microtitration assay. Similar cytotoxicity tests are usually performed in order to screen novel compounds with anticancer activities. Further, the anticancer potential of a compound is validated by the analysis of the nuclear morphology by acridine orange/ ethidium bromide (AO/EB) staining (Karande et al. 2006). Fluorescent microscopic images reveal characteristic chromatin condensation for the apoptotic cells, which are otherwise absent in the healthy cells (Renvoize et al., 1998).

In the present thesis the anticancer compounds were divided into two major categories: (i) the synthetic planar copper complexes and (ii) the natural plant extracts.

The systemic metal toxicity is a serious hurdle in the discovery of novel synthetic anticancer compounds. However, a biological transitional metal co-ordinated with a suitable ligand to stabilize its functional properties can be hypothesized to cause less systemic toxicity. Copper is a biologically relevant metal as it is associated with various biomolecules related to essential physiological activities (Blain et al., 1998; Gennis et al., 1995; Petrovic et al., 1996; Petris et al., 2000). Anticancer compounds with copper as a metal center is therefore hypothesized to be less toxic and more potent. In the present chapter, the efficacy of a family of Schiff base copper complexes $[Cu(Pyimpy)X_2]$ or [Cu(Pyimpy)Y], where Pyimpy is a tridentate

ligand containing two pyridine and one imine nitrogen donor, and X and Y are monodentate and bidentate ligands respectively were tested.

In the second part of the present chapter, a group of phytochemicals were tested for similar cytotoxic properties against cancer cells. The plants were selected based on the existing literature (Table 4.1).

Table 4.1: List of some medicinal plants screened.

Plants	Major constituents	Biological targets	References
Swertia chirayita	Amarogentin (glycoside)	Effective against mouse skin cancers	Saha et al., 2006
Saussurea costus	Costunolide (Sesquiterpenes)	Effective against HL-60 human leukemia cells, inflammation and stomach ailments	Lee et al., 2001; Panday et al., 2007
Picrorrhiza kurroa	Apocynin (4-acetovanillone)	Acts against breast cancer cells	Klees et al., 2006
Rauwolfia serpentine	Serpentine, β-carboline (Alkaloid)	Effective against prostate cancer, induces topoisomerase II mediated DNA cleavage	Bemis et al., 2006; Dassonneville et al., 1999
Pterocarpus marsupium	Pterostilbene (Stilbene)	Effective against leukemia and colon cancer cells	Suh et al., 2007; Tolomeo et al., 2005
Podophyllum hexandrum	Podophyllotoxin (Lignin)	Protective against radiation toxicity in mice	Gupta et al., 2007; Arora et al., 2005
Aconitum heterophyllum	Lycaconitne (Alkaloid)	Effective against multidrug resistant human fibrocarcinoma, neuroblastoma and possesses antibacterial activity	Ahmad et al., 2008b; Kim et al., 1998
Nardostachys grandiflora	Lignans	Protective against doxorubicin induced cardiac damage, acetylcholinesterase inhibitory activity, antibacterial and antifungal activity	Kumar et al., 2006; Subashini et al., 2006; Vinutha et al., 2007

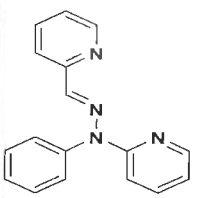
Indian regular food spices contain a number of phytochemicals which have been well established to induce growth arrest in various cancer cells in culture. Garlic, turmeric, capasain, fenugreek etc are some of the most prominent in this aspect (Arunkumar et al., 2006; Choi et al., 2010; Gunadharini et al., 2006). Phytochemicals in regular dietary regime thereby designated as neutraceuticals can effectively ward off complex diseases like cancer (as reviewed by Sarkar et al., 2010). Many of them regulate the cancer development by Akt/FOXO3a/GSK-3 beta pathways in different cell lines. These pathways are active in hormone dependent prostate

cancers and isoflavones, a group of polyphenol generally inhibit prostate cancers by inhibiting several members of these pathways (Li et al., 2008). Various polyphenolic compounds were found to show multiple targets of action as described in chapter 2. Based on the available literature, the present study was aimed to screen some of the natural phytochemicals for their anticancer properties. In order to isolate the polyphenolic fractions, methanolic extracts were prepared from *Swertia chirayita, Saussurea costus, Picrorrhiza kurroa, Rauwolfia serpentina, Pterocarpus marsupium, Podophyllum hexandrum, Aconitum heterophyllum, Nardostachys grandiflora*, from aerial parts (trunk/stem) and tested *in vitro* by microtitration assays using multiple cancer cell lines.

4.2 Compounds

4.2.1 Synthetic compounds

The complexes used in this study were mononuclear copper complexes having a Schiff base ligand containing two pyridine and one imine donors. The tridentate ligand (named as pyimpy) along with other monodentate ligands bind to copper and produce a new family of copper complexes. The compounds were designed, synthesized and spectroscopically and structurally characterized by Dr. Pramod Kumar and Dr. Kaushik Ghosh, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee India as collaboration (Kumar, 2011). All the mononuclear copper-pyimpy complexes (CuP) were named numerically as CuP1 to CuP10 on the basis of the variation of monodentate ligands viz. Cl^-, OH_2, N_3^-, SCN^-, CN^-, OAc^-, OBz^-, NO_2^- etc. In addition to this, a bis-complex of tridentate ligand of pyimpy i.e., $[Cu(Pyimpy)_2](ClO_4)_2]$, named as CuP11 was also tested (Fig. 4.1).



Pyimpy

CuP1	$[Cu(Pyimpy)(Cl)_2]$	CuP7	$[Cu(Pyimpy)(OAc)](ClO_4)$
CuP2	$[Cu(Pyimpy)(N_3)](ClO_4)$	CuP8	$[Cu(Pyimpy)(OBz)](ClO_4)$
CuP3	$[Cu(Pyimpy)(SCN)](ClO_4)$	CuP9	$[Cu(Pyimpy)(CN)](ClO_4)$
CuP4	$[Cu(Pyimpy)(2, 2' bipy)](ClO_4)_2$	CuP10	$[Cu(Pyimpy)(Im)](ClO_4)_2$
CuP5	$[Cu(Pyimpy)(Cl)(ClO_4)]$	CuP11	$[Cu(Pyimpy)_2](ClO_4)_2$
CuP6	$[Cu(Pyimpy)(H_2O)(ClO_4)](ClO_4)$		

2, 2' bipy = 2, 2' bipyridine OAc = acetate ion OBz = benzoate ion Im = imidazole

Fig. 4.1*: General structures of the family of copper complexes.*

4.2.2 Selected plants

All plant materials were obtained locally and identified as per the literature of Ayurveda and by local in-charge of herbal garden and also confirmed by Dr. H. S. Dhaliwal, Professor of Plant Biotechnology, Indian Institute of Technology Roorkee.

Fig. 4.2: *The plants selected for screening of anticancer properties. (A: Swertia chirayita; B: Saussurea costus; C: Rauwolfia serpentina; D: Picrorrhiza kurroa; E: Podophyllum hexandrum; F: Aconitum heterophyllum; G: Nardostachys grandiflora; H: Pterocarpus marsupium)*

The herbs were obtained from herb nursery of Indian Institute of Technology Roorkee, and heartwood of *Pterocarpus* was purchased from a local vender of Roorkee, India. Before extraction, the stem of the plants were washed with water, dried, crushed and extracted with 3 volumes of methanol for 6 h. The supernatant was pooled for the respective plants and the filtrate was extracted similarly thrice. The methanolic extract was dried under vacuum evaporator and the residue was dissolved in ethanol and labeled as a main stock of 10g/ml for the respective plants.

4.3 Brief experimental protocols

4.3.1 Standard anticancer compounds used

Cisplatin, 5-floro uracil (5-FU), actidione and resveratrol were used as positive control for this assay. In order to maintain uniformity in the positive control between both synthetic and natural chemical screenings; cisplatin is used as a reference anticancer drug for all other assays.

4.3.2 Cell lines

In order to test the cytotoxicity of synthetic compounds / extracts, a series of 3 cancerous cell lines from different organs were used. MCF-7 (breast carcinoma cells), PC3 (prostate cancer cell line), and SiHa (cervical cancer cell line) were used along with a normal cell line, HEK293 (human embroyonic kidney cell line) were used.

4.3.3 Cytotoxicity screening assays

Cytotoxicity is determined by the MTT assay as described in chapter 3 (3.6).

4.3.4 Acridine orange/Ethidium bromide screening assays

The MCF-7 cells were incubated with few selected test compounds which showed good toxicity against the cancerous cells but were non-toxic to the normal cells in the MTT assay. These were then analyzed for chromatin condensation by AO/EB staining and observed under fluorescent microscope. Detailed protocol of the assay is mentioned previously in chapter 3 (3.7).

4.3.5 In vitro *DNA binding assay*

Compounds which showed chromatin condensation were further validated for the *in vitro* binding to calf-thymus DNA in order to understand if the prior DNA damage causes chromatin condensation or the opposite. Detailed protocol of the assay is given in chapter 3 (section 3.3).

4.4 Results

4.4.1 Cytotoxicity assay

The drugs were added to the cell monolayer in microtitre plates in serial-dilutions ranging from 0.05-100 μM for each of CuP1-CuP11 (detailed procedure is given in Chapter 3). The drugs were dissolved in ethanol and added to the monolayer of cells in triplicates. The final ethanol concentration used for all dilutions (0.1%) was used as vehicle control. Cultures were assayed after 24 h and the comparative IC_{50} values of the series of copper complex compounds as tested in MCF-7, PC3, SiHa and HEK 293 cells are shown in table 4.2.

Table 4.2: The level of cytotoxicity *in vitro* by various Schiff-base copper complexes in cancerous and normal cell lines.

Compounds	IC_{50} (μM)			
	MCF-7	**PC3**	**SiHa**	**HEK293**
CuP1	4.29 ± 0.42	6.34 ± 0.58	4.89 ± 0.48	39.12 ± 1.28
CuP2	16.54 ± 1.04	11.10 ± 0.50	28.19 ± 1.27	28.19 ± 2.09
CuP3	7.58 ± 0.61	13.68 ± 2.59	11.91 ± 0.67	37.19 ± 2.19
CuP4	10.19 ± 0.34	8.64 ± 0.48	12.40 ± 1.02	56.23 ± 3.29
CuP5	7.54 ± 0.65	7.98 ± 0.70	4.59 ± 0.45	15.19 ± 2.39
CuP6	14.75 ± 1.81	7.99 ± 0.88	12.34 ± 1.32	47.19 ± 1.29
CuP7	7.55 ± 0.18	5.17 ± 1.05	10. 68 ± 0.66	37.15 ± 3.21
CuP8	14.93 ± 1.38	11.41 ± 0.49	3.46 ± 0.98	50.18 ± 2.48
CuP9	9.04 ± 0.36	5.36 ± 0.50	7.21 ± 0.50	17.24 ± 1.89
CuP10	48.85 ± 1.27	10.47 ± 0.57	72.18 ± 5.35	89.10 ± 3.18
CuP11	11.03 ± 0.58	10.69 ± 0.35	12.14 ± 1.20	67.21 ± 2.37
$CuCl_2$	>100	>100	>100	>100
Pyimpy (ligand)	89.57±0.27	96.66 ± 2.02	>100	>100
Cisplatin	64.13±5.98	80.00 ± 5.19	34.10 ± 2.35	79.29 ± 1.39

Many compounds of the series like CuP1, CuP3, CuP5 and CuP7 showed comparatively lower IC_{50} values as compared to other complexes of the series in all the three cell cancerous lines. The parent compound $CuCl_2$ or the ligand Pyimpy did not show any remarkable cytotoxicity in any of the cell lines tested. On the other hand, these compounds did not show any toxicity below 10 μM concentrations on normal cell lines.

The methanolic extracts of the stem of the above mentioned plants were filtered with 0.2 micron filters before testing them in cell cultures. Each of the plant extracts were added to the cell monolayer in microtitre plates in serial-dilutions ranging from 0.05-500 μg/ml. The final concentration of ethanol used for all dilutions (0.1%) was used as vehicle control. Cultures were assayed after 24 h as described previously and are shown in table 4.3.

Table 4.3: The level of cytotoxicity *in vitro* by various phytochemical extracts in cancerous and normal cell lines.

Test Materials	IC_{50} (µg/ml)			
	MCF-7	**PC3**	**SiHa**	**HEK-293**
Swertia chirayita	74.29 ± 2.44	86.34 ± 5.8	95.32 ± 2.3	123.86 ± 2.98
Saussurea costus	86.54 ± 2.04	91.10 ± 3.51	100.08 ± 2.15	207.67 ± 6.88
Rauwolfia serpentine	50.19 ± 1.43	67.64 ± 4.8	49.60 ± 4.91	68.56 ±7.87
Picrorrhiza kurroa	67.58 ± 1.21	73.68 ± 2.59	74.51 ± 1.37	107.47 ± 4.98
Podophyllum hexandrum	57.54 ± 4.65	77.98 ± 2.70	70.16 ± 5.42	53.67 ± 3.98
Aconitum heterophyllum	61.75 ± 1.81	57.99 ± 3.54	78.28 ± 3.29	85.87 ± 1.60
Nardostachys grandiflora	68.55 ± 3.87	87.17 ± 3.59	81.86 ± 1.66	90.98 ± 2.22
Pterocarpus marsupium	54.93 ± 1.48	61.41 ± 1.94	68.26 ± 3.68	118.27 ± 4.5
Resveratrol	98.3 ± 1.89	85.23± 2.15	>100	176.67 ± 6.37
5-FU	69.04 ± 0.36	65.36 ± 0.50	56.40 ± 0.51	96.57 ± 2.75
Actidione	18.85 ± 1.27	15.47 ± 1.57	27.3 ± 0.64	87.95 ± 4.87
Cisplatin	71.13±3.98	78.00 ± 5.19	35.30 ± 3.55	164.48 ± 11.96
Vehicle	-	-	-	

Cytotoxicity data showed that the extracts of *Rauwolfia serpentina, Podophyllum hexandrum, Aconitum heterophyllum and Pterocarpus marsupium* had comparatively lower IC_{50} values as compared to other phytochemicals in the series against all the three cancerous cell lines as tested here. The extract from *Podophyllum*, *Rauwolfia*, *Aconitum* and *Nardostachys* were found cytotoxic to the normal cell (HEK293) at a concentration of < 100 µg/ml whereas others had no effects on the normal cells at this concentration.

Since the main aim of the present thesis is to characterize anticancer molecules towards breast cancer cell lines, some of the potent CuP complexes and the extracts were further analyzed by the visual changes of the nucleus and the cell membrane by AO/EB staining.

4.4.2 Acridine orange staining

MCF-7 cells were treated for 24 h with the concentrations equivalent to the IC_{50} values for compounds CuP1, CuP3, CuP5 and CuP7 and the methanolic extracts of *Rauwolfia*

serpentina, Podophyllum hexandrum, Aconitum heterophyllum and Pterocarpus marsupium. Thereafter the cells were stained with acridine orange: ethidium bromide (1:1) as described in Chapter 3. There was a clear induction of apoptosis in all the treatment sets with the formation of chromatin fragmentation and cell membrane blebbing (Fig. 4.3). Live cells under the stress of early and late apoptosis could be easily distinguished by the clear chromatin condensation and the percentage uptake of AO: EB. Early apoptotic cells have bright green nucleus with condensed or fragmented chromatin (Fig. 4.3, marked as '1') while the late apoptotic cells display condensed and fragmented orange or yellowish orange chromatin (marked as '2'). EB, on the other hand, was only taken up by cells when cytoplasmic membrane integrity is lost as in late apoptosis or in necrosis, staining the nucleus red (Figure 4.3, marked as '3'). These results were analyzed in accordance with the earlier report by Renvoize et al. (1998). The AO/EB staining showed that in CuP1 treated cells there was clear chromatin condensation and nuclear blebbing. While the other compounds also caused active apoptosis but the effective concentration of CuP1 (4 μM) at which it showed the effect was better than the other compounds.

Among the methanolic extracts of the plants, extract from *Rauwolfia* sp. showed that it contains potent cytotoxic agents which killed the cells most prominently by necrosis. In acridine orange staining there was more number of dead cells (mainly by necrosis) as marked by the red coloured staining of ethidium bromide (Fig. 4.3). Extracts of *Podophyllum* caused more of chromatin condensation intracellularly even after 24 h of exposure but no marked cell death as shown in figure 4.3. While *Aconitum* extract showed rounding off of the cells and accumulation of membrane blebbings, *Pterocarpus* extract showed early and late apoptosis, membrane blebbings and also apoptotic death (Fig. 4.3). Therefore it could be concluded that only *Pterocarpus* extract could induce characteristic morphologies of apoptosis in breast cancer cells among all other extracts. This result was comparable to that of cisplatin which also showed all the apoptotic morphologies. The control MCF-7 cells on the other hand showed green colored nucleus but with no chromatin condensation.

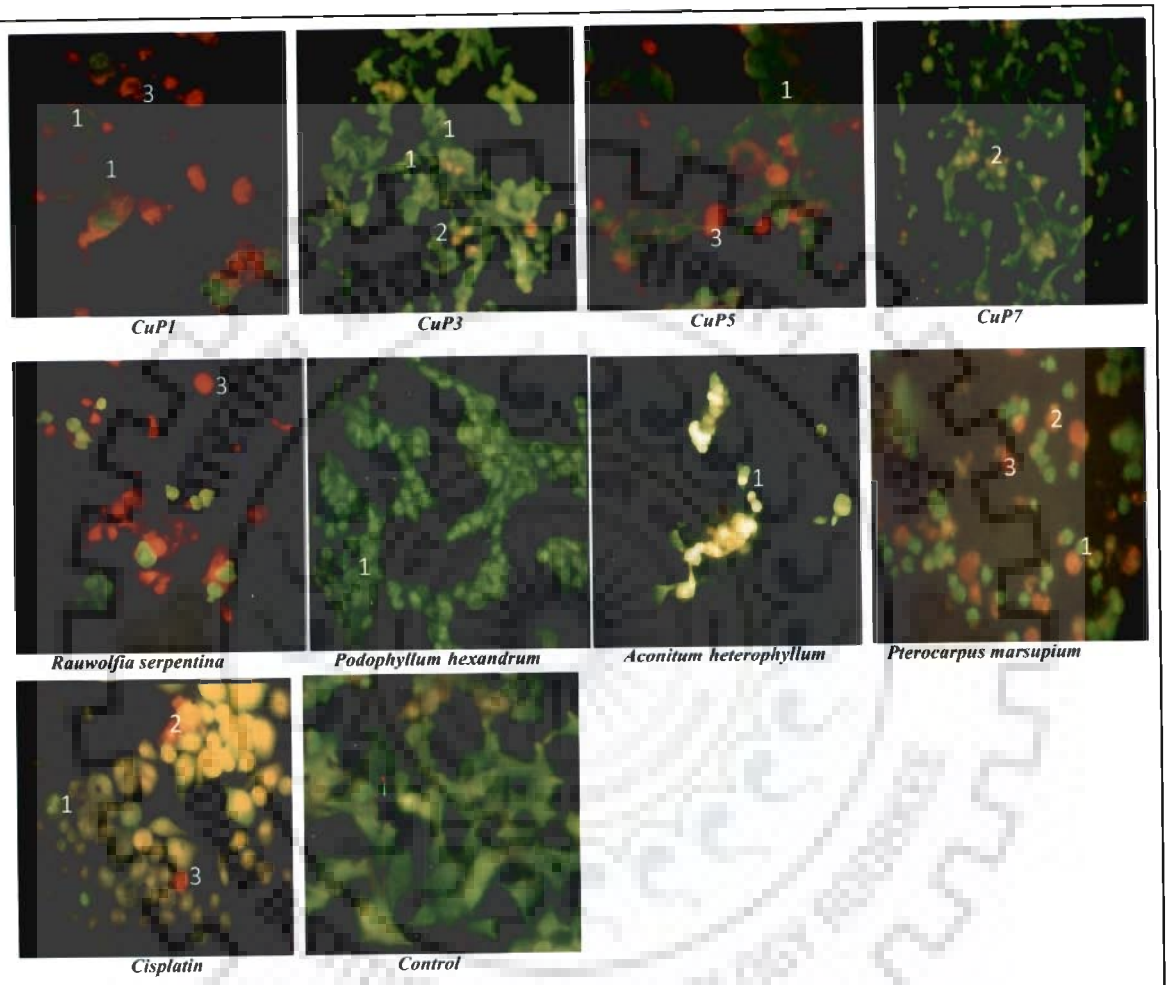


Fig. 4.3: *Induction of apoptosis and chromatin condensation after incubation of MCF-7 cells with some potent copper based synthetic compounds and some methanolic extracts. Nuclear staining was visualized under fluorescent microscope at 10X objective. The numerical: 1, 2 and 3 represent early apoptotic, late apoptotic and necrotic cell nucleus, respectively. The experiment was performed in triplicates and a representative experiment is presented.*

4.4.3 CuP1 induces DNA fragmentation in vitro

To understand the mechanism of development of characteristic apoptotic nucleus by both CuP1 and *Pterocarpus* extract, it was obvious to check whether these compounds had any effect

on the DNA binding in vitro. As a general trend of these metal co-ordinated complexes, they show replacement of the ethidium bromide (EB) in the solution thus quenching its fluorescence (Ghosh et al., 2008). As seen in Figure 4.4, the fluorescence of EB and calf thymus DNA complex was quenched with increasing concentrations of CuP1 from 0-50 μM. The quenching of EB fluorescence could be attributed to the displacement of DNA bound EB to its free state. No such quenching patterns were observed in the absence of DNA or EB even in the presence of CuP1. Further, neither only ligand (pyimpy) nor the copper salt ($CuCl_2$) showed any quenching effects. The Stern-Volmer quenching constant K_{SV} obtained from the slope of the regression curve in the linear range (Figure 4.4 inset) was found to be 0.28.

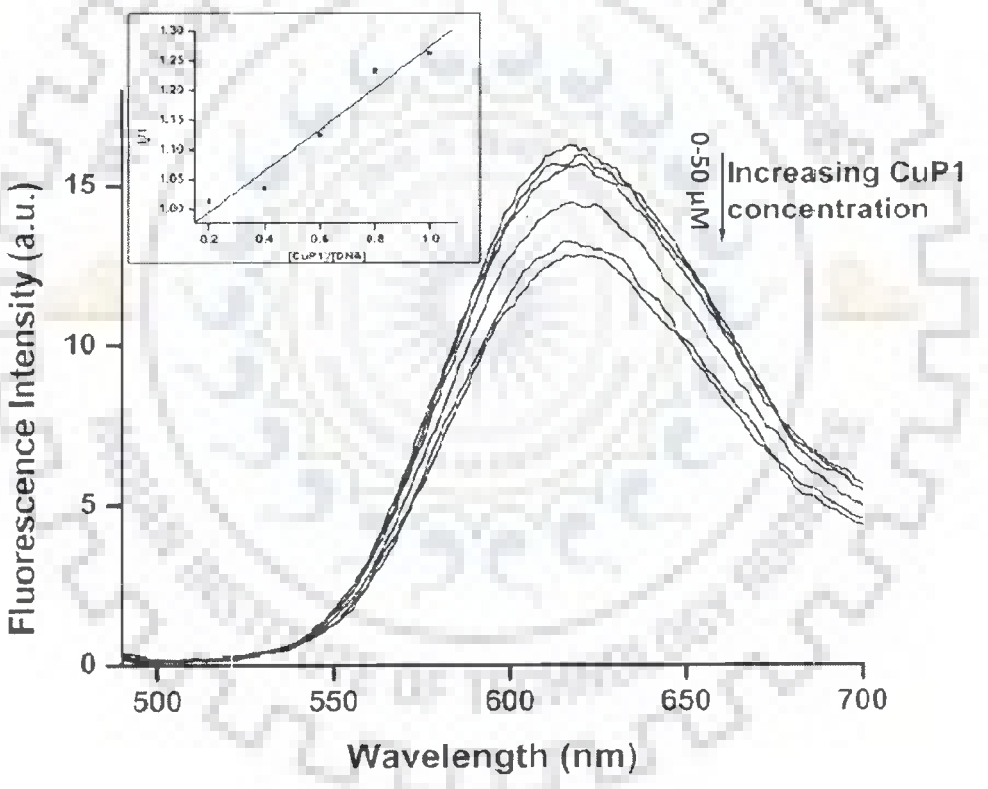


Fig. 4.4: *Quenching of ethidium bromide-calf thymus DNA complex fluorescence by increasing concentrations of CuP1. Inset shows Stern-Volmer plot with I_0/I (the fluorescence intensities in absence and presence of CuP1 respectively) vs [CuP1]/[DNA].*

None of the phytochemical extracts showed any such DNA binding activity.

4.5 Discussion

The present study was aimed to screen synthetic and phytochemicals for their potency as future anticancer agents. Transitional metal chemistry and phytochemicals are evolving for their capacity to target biological molecules. In a recent study by Ullah et al. (2011), it is reported that the isoflavone genistein, isolated from soy, bear apoptotic potential against breast cancer cell lines mainly due to their mobilization of endogenous copper. Therefore, there exists considerable interest for the discovery of new metal based anticancer drugs.

Here the anticancer activities of a series of copper complexes containing Schiff base ligands were checked. These series of compounds showed varying degrees of anti-proliferative effects on MCF-7, PC3 and SiHa cells as shown by different IC_{50} values which were consistent with those of similar other copper complexes already studied for their anticancer activities (Adsule et al., 2006). From a series of eleven different copper-complex compounds, one of the complexes, CuP1, showed prominent anticancer activities, as evident by its lower IC_{50} values (as compared to other members of the family). The potency of CuP1 was almost 12-folds higher in breast cancer cell line as compared to the well known drug cisplatin. Copper conjugated compounds (copper-moxifloxiacin) are found to be active against both hormone dependent and independent breast cancer cell lines (Patitungkho et al., 2011). It is known that cisplatin acts at the N7 site of guanine base in the DNA where it forms DNA-adducts and activates hMSH2 which is a member of the mismatch repair complex (Donahue et al., 1990; Fink et al., 1998; Chaney and Vaisman, 1999). Thus the irreparable damage to DNA causes the cells to accumulate at the G2 phase (Demarcq et al., 1994) following which p53 molecules initiate apoptosis in a caspase dependent manner (Bullock and Ferst, 2001; Morgan and Kastan, 1997). Although further studies are required to define the exact mode of action of this newly synthesized copper molecule, the present data confirms that CuP1 interacts with DNA in vitro. The chromatin condensation caused by CuP1 was almost comparable to that of the positive control drug i.e., cisplatin, when tested in MCF-7 cells at their respective IC_{50} equivalent values. But no cytotoxic effect was observed in the parent compound (neither the ligand nor the metal salt) at equimolar concentration. Moreover, their IC_{50} values are higher than the acceptable range of 20 μM for synthetic compounds. CuP1 was found to be soluble in physiological saline at a concentration of 5 mg/ml. This property of CuP1 might make the molecule biologically more acceptable. Although the toxicity caused by the molecule is studied in the subsequent

chapter, the data from the present study revealed that CuP1 had some prominent anticancer properties and therefore, needs further investigation.

On the other hand when the methanolic extracts of the different plants were compared it was found that the IC_{50} values could be graded as: *Rauwolfia serpentina* > *Pterocarpus marsupium* > *Podophyllum hexandrum* > *Aconitum heterophyllum* in MCF-7 cell lines. Each of these crude extracts were comparable to the positive control resveratrol, cisplatin or 5-FU but their cytotoxicity was less than the actidione. A number of polyphenols in crude form have anti-inflammatory (Metzger et al., 2008), anti-tumor (Afaq et al., 2005) and anti-platelet effects (Shanmuganayagam et al., 2002). As several studies have reported pure herbal compounds are active against the cancer cell lines when IC_{50} values are in the range < 150 µg/ml (Afaq et al., 2005; Metzger et al., 2008). Therefore, the methanolic extracts of *Rauwolfia, Podophyllum, Aconitum and Pterocarpus* could consider being active against MCF-7 cells.

Although the crude extracts of phytochemicals have higher effective concentration, it is observed that even at these concentrations they are generally physiologically non-toxic. They mostly lack side effects which points towards their safe use (Virk-Baker et al., 2010). Hence, the efficacy of phytochemical extracts cannot be compared to that of the series of copper complexes.

The cytotoxicity assay was further validated by the acridine orange staining. The cell and nuclear morphology revealed that although *Rauwolfia* extract had been the most potent in causing cancer cell death but the process was predominantly necrosis rather than apoptosis. Hence, it can be concluded that the extract could not distinctly differentiate between the cancer cells and normal cells. On the other hand, the next potent extract (*Pterocarpus sp.*) was apoptotic to the breast cancer cells. Therefore, the cytological analysis of the acridine orange staining revealed that the methanolic extract of *Pterocarpus marsupium* needs to be further studied on the breast cancer cells to understand its mode of action as a potent anti-tumor agent.

It can be concluded from the present data that the efficacy of CuP1 and *Pterocarpus* are the most prominent ones among the series of chemicals tested at least in the primary screening. CuP1 in addition to causing development of characteristic apoptotic nucleus, causes DNA binding in vitro. Any damage to DNA is susceptible to apoptosis with the release of apoptotic signals (O'Connor and Fan, 1996). Therefore, it is quite interesting to see the efficacy of both CuP1 molecule and the lead molecule in the *Pterocarpus* extract in (i) causing DNA strand

breaks in breast cancer cells, (ii) the mechanism of apoptosis and (iii) its effect in physiological conditions. The experimental analysis on these aspects is described in the following chapters. Since these two molecules showed potent anti-cancer properties, they were taken up for further detailed analysis at the cellular levels in the subsequent chapters of this thesis.

CHAPTER 5

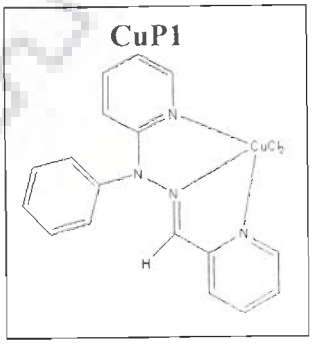
CuP1 AS ANTI-CANCER COMPOUND

5 CuP1 as anti-cancer compound

5.1 Introduction

Copper, zinc and iron are present in the metallo-proteins having different biological activities. Most prominent of these biologically essential molecule is the copper which is present in Zn-Cu superoxide dismutase (Petrovic et al., 1996), tyrosinase (Petris et al., 2000), dopamine β hydroxylase (Blain et al., 1998), cytochrome oxidase (Gennis et al., 1995) and blue copper proteins (Sedlák et al., 2008a,b). Copper complexes were studied extensively mainly because of its biologically relevant redox properties and its high affinity for nucleobases (Humphreys et al., 2002; Koichiro et al., 2004; Chen et al., 2007). They have been reported to show oxidative, hydrolytic and photolytic nuclease activities (García-Giménez et al., 2009; Dhar et al., 2003; Zhang et al., 2004). Synergistic action with antioxidant molecules like curcumin diketimines has also been reported for bis(salicylate) copper (II) complexes showing cytotoxic and antiviral activities (Annaraj et al., 2005). Further, glycine conjugated to copper has been shown to reverse drug resistance in T-cell acute lymphoblastic CEM leukemia cells (Majumdar et al., 2006). Recently, Schiff base copper quinoline complexes have been found to be proteosome inhibitors of human prostate cancer cell lines (Adsule et al., 2006). In summary, all these reports suggests that complex of this element not only targets nucleic acids but also affect the functions of proteins. Thus there has been a substantial interest in the development of copper based metal complexes which have antitumor activities.

In the previous chapter, a series of copper complex compounds were screened for cytotoxic activities using MTT assay finally out of these series of compounds [Cu(Pyimpy)Cl_2] (CuP1) was picked up based on its lowest IC_{50} value. The ligand frame consists of pyrimidine-imine-pyrimidine which stabilizes the oxidation states of copper (I&II). Moreover the strong electronegative monovalent chloride (Cl^-) attached to copper can easily detach from the complex in the ionic cellular environment. It was designed in a similar way to the structure of cisplatin and its molecular mechanism of action was expected to occur by the displacement of the Cl^- with OH^-. This might be the cause of the binding to the nucleic acid to form the metal-DNA adducts. Irreparable DNA damage finally triggers apoptosis. The biological characteristics were analyzed further for its anticancer activities initially using MCF-7 cell line and then finally tested on the tumor bearing rat models.



5.2 Brief experimental protocols

5.2.1 Cell lines

In this study the MCF-7 cells were taken as the representative of the breast cancer cell line for all *in vitro* experiments. Apart from MFC-7 cells, PC3 and SiHa cells were used only in caspase 3 assay. The culture conditions of the cell lines have been described in section 3.4.1.

5.2.2 In vitro experiments

Single cell gel electrophoresis assay and ***DNA fragmentation assay*** were performed according to the methods detailed in Chapter 3 (Material & Methods)

5.2.2.1 Caspase assay

The assay clearly confirms if there is an activation of caspase 3 in cancerous cells. The effect of CuP1 to induce apoptosis was therefore checked by this assay. Detailed description of the protocol has been given in section 3.12 (Material & Methods). Due to the fact that MCF-7 cells are not a good model to test caspase 3 activity, the enzyme activity was also checked in PC3 and SiHa cell lines.

5.2.3 In vivo experiments

The detailed description for the ***Determination of LD_{50}*** and ***Development of mammary tumor models*** are given in the Materials & Methods (section 3.5).

5.2.3.1 Treatment of mammary tumor models

The tumor bearing female rats were divided into following groups for further treatments. Treatment was started when the tumors developed in the animals reached a size of 12 mm^3. The animals were otherwise housed with proper food and water *ad libitum*.

Group I : Healthy female, non-tumor model (n=10)
Group II : Breast tumor model (n=11)
Group III : CuP1 treated breast tumor model (n=11)
Group IV : Cisplatin treated breast tumor model (n=11)
where n was the total number of individuals in a group.

The animals were injected with CuP1 and cisplatin each at a dose of 1 mg/kg body weight for 1 month. The animals of Group I & II were similarly treated with vehicle (normal saline).

The effect of CuP1 on the development of breast tumor was analyzed further by histology of tumor tissues, analysis of antioxidant profiles, RT-PCR of some marker genes and immunoblot analysis of some marker proteins from these tissues.

5.3 Result

5.3.1 In vitro

5.3.1.1 CuP1 induces DNA fragmentation in MCF-7 cells

The *in vitro* DNA binding properties of CuP1 was checked on the intracellular DNA in MCF-7 cells. The cells were incubated with 5μM CuP1 for 24 h. Alkaline lysis of single cell suspension showed marked DNA fragmentation. As shown in Fig. 5.1 the comet tailing of the individual nucleus as stained by ethidium bromide directly support that CuP1 causes DNA breaks in cancerous cells. The intensified fragmentations of DNA in MCF-7 cells are marked by arrows. The results were again comparable to cisplatin which also caused DNA tailing. However, vehicle treated cells did not show any DNA fragmentation (Fig. 5.1).

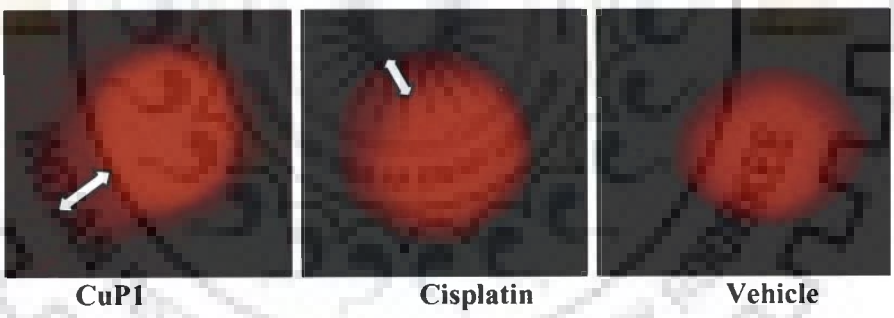


Fig. 5.1: *Induction of DNA fragmentation by CuP1 as detected by single cell alkaline gel electrophoresis. MCF-7 cells were grown in 9 cm plates and treated with CuP1 (5 μM), cisplatin (100 μM) or vehicle for 24h. Thereafter the cells were collected and analyzed by alkaline gel electrophoresis. Nuclear staining was visualized under 20X objective in fluorescent microscope. The DNA breaks from individual cells are well visualized as comet tails (marked by arrows). The experiment was performed in triplicates and a representative experiment is presented.*

Further the DNA in each comet tail was determined by the formula given in Chapter 3. As shown in Figure 5.2, CuP1 induces 1.4 fold more tailing ($P<0.05$) than the cisplatin treated MCF-7 cells.

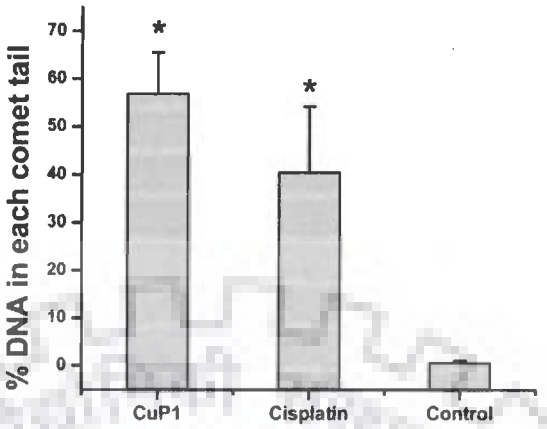


Fig. 5.2: *Percentage of DNA in comet tails following treatment with test compounds. Comet tail area of 100 cells (means ± S.E.M.) were calculated with the help of image analyzer software. * indicates statistically significant (P<0.05) levels of DNA in comet tail in drug treated cells with respect to control MCF-7 cells.*

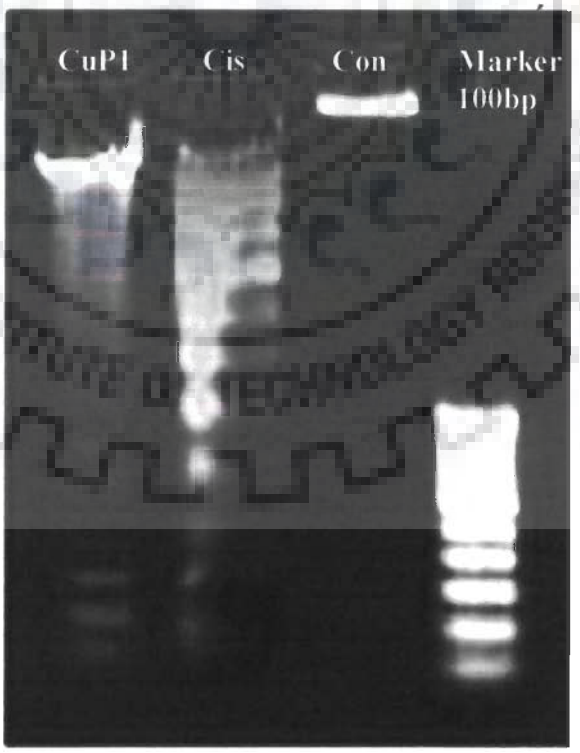


Fig. 5.3: *Induction of DNA fragmentation by CuP1. MCF-7 cell lines were treated with the respective 5 μM of CuP1 and an equivalent dose of cisplatin (100 μM) for 24 h. The experiment was performed in triplicate and a representative experiment is presented.*

The results of comet assay were further verified by DNA fragmentation assay where the DNA of the apoptotic cells were extracted and analyzed on 2% agarose gel electrophoresis.

CuP1 caused a significant DNA damage as compared to cisplatin (Fig. 5.3). The genomic DNA isolated shows smearing along with smaller DNA fragments in CuP1 and cisplatin treatments whereas the control cells did not show such DNA breaks.

5.3.1.2 Caspase activity

As it is already known that any damage to DNA is susceptible to apoptosis with the release of apoptotic signals (O'Connor and Fan, 1996), it was worth to check the effect of CuP1 on the marked apoptosis. Following 24 h CuP1 treatment, caspase 3 activity did not show any significant difference in case of MCF-7, whereas, caspase 3 activity increased by almost 7.4- and 9- folds in PC3 and SiHa respectively as compared to respective untreated control cells (Fig. 5.4). This discrepancy could be attributed to an earlier finding that MCF-7 cells lack exon-3 which prevents the caspase 3 activity in this cell line but the other caspases are sufficient to cause apoptosis in a similar manner (Kagawa et al., 2001).

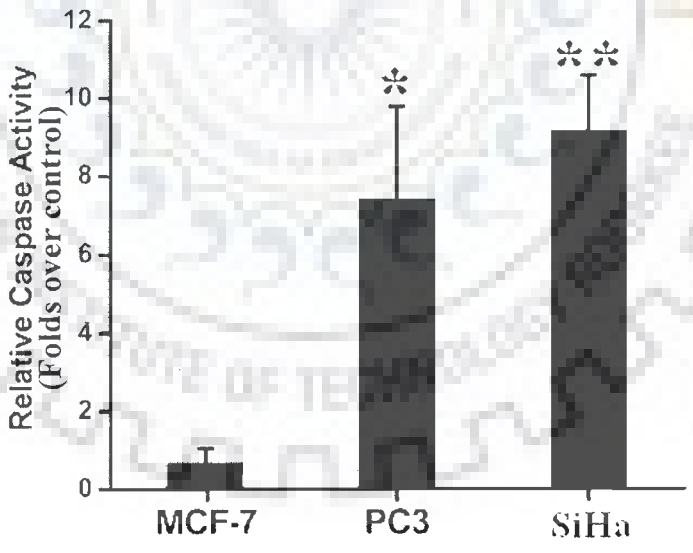


Fig. 5.4: *CuP1 induced caspase activity in cancer cells. The data are represented as folds over control of the caspase activity of respective control (vehicle treated) cell lines. The experiment was performed in triplicate and mean ± S.E.M. of three independent experiments is shown here. *, ** indicates statistically significant ($P<0.05$) increase in activity with respect of control PC3 and SiHa cells, respectively.*

5.3.2 . In vivo

5.3.2.1 Determination of sub-acute toxicity of CuP1 in normal rats

In order to determine the test dose of the novel compound on *in vivo* animal model the LD_{50} was determined. The effect on the rat physiology was also monitored as the mean body weight change and serum SGPT : SGOT as shown in table 5.1. LD_{50} value of CuP1 was found to be 24 mg/kg body weight/ week.

Table 5.1: Effects of CuP1 on some of the physiological parameters and mortality rates in rats (Determination of LD_{50})

Animal groups (n=10)	Dose (*i.p.*) (mg/kg body weight)	Number of deaths after 21 days	Initial mean body weight (g)	Final mean body weight (g)	Mean SGPT:SGOT
I	0.5	0	120	125	1.1
II	1	0	120	125	1.3
III	10	0	120	125	1.67
IV	20	4	120	115	1.9
V	30	7	120	90	2.0
VI	40	10	120	-	-
VII	50	10	120	-	-

The non-tumor bearing healthy female rats when injected with 1 mg/kg body weight twice a week did not show any sub-acute toxicological parameters like lethargy or weight loss even after 21 days of treatment. Further, the animals gradually gained weights at par to vehicle treated control group throughout the treatment period. The ratio of SGOT : SGPT was found to be around 1.3 in case of CuP1 treated group and vehicle treated groups had a value of 1.1. Histological analysis of liver, kidney and spleen also did not show any significant signs of toxicity (Fig. 5.5). Based on these results, 1mg/kg CuP1 was injected in normal and tumor bearing rats of group III (as discussed in 5.2.3.1) twice a week to determine the antitumor efficacy of this compound.

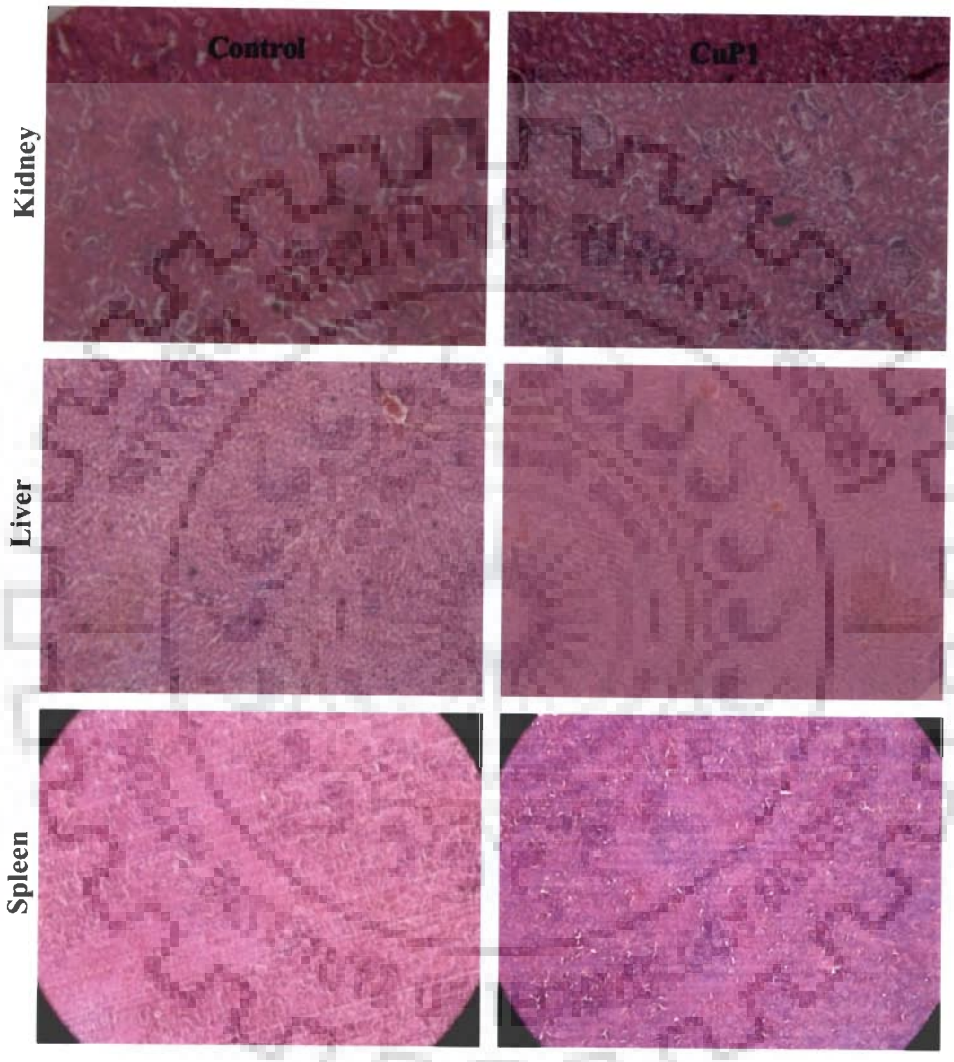


Fig. 5.5: *Histology of kidney, liver and spleen of normal rats treated with with CuP1. Magnification (20X objective).*

5.3.2.2 Effect of CuP1 on breast tumor models

Tumor bearing rats were developed and grouped as described in Chapter 3 and section 5.2.3.1 of the present chapter. The treatment was initiated when the tumor volume reached about 12 mm^3. Specific tumor growth rate was decreased by 56% after 30 days of CuP1 treatment (group III) as compared to vehicle treated tumor rats (Group II) (Fig. 5.6). In case of cisplatin treatment, specific growth rate was decreased only 21%, which was significantly different from the group II animals ($P < 0.05$) (Fig. 5.7 A).

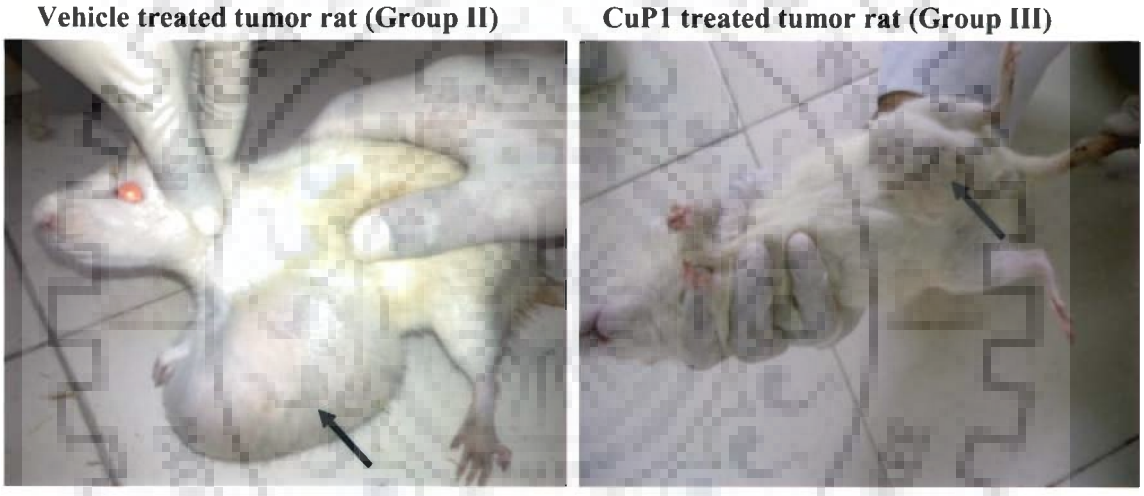


Fig. 5.6: *CuP1 inhibits the development of MNU induced mammary tumors in rats. The image represents one of the representatives of the group after completion of 30 days of treatment. The breast tumor are shown by arrows.*

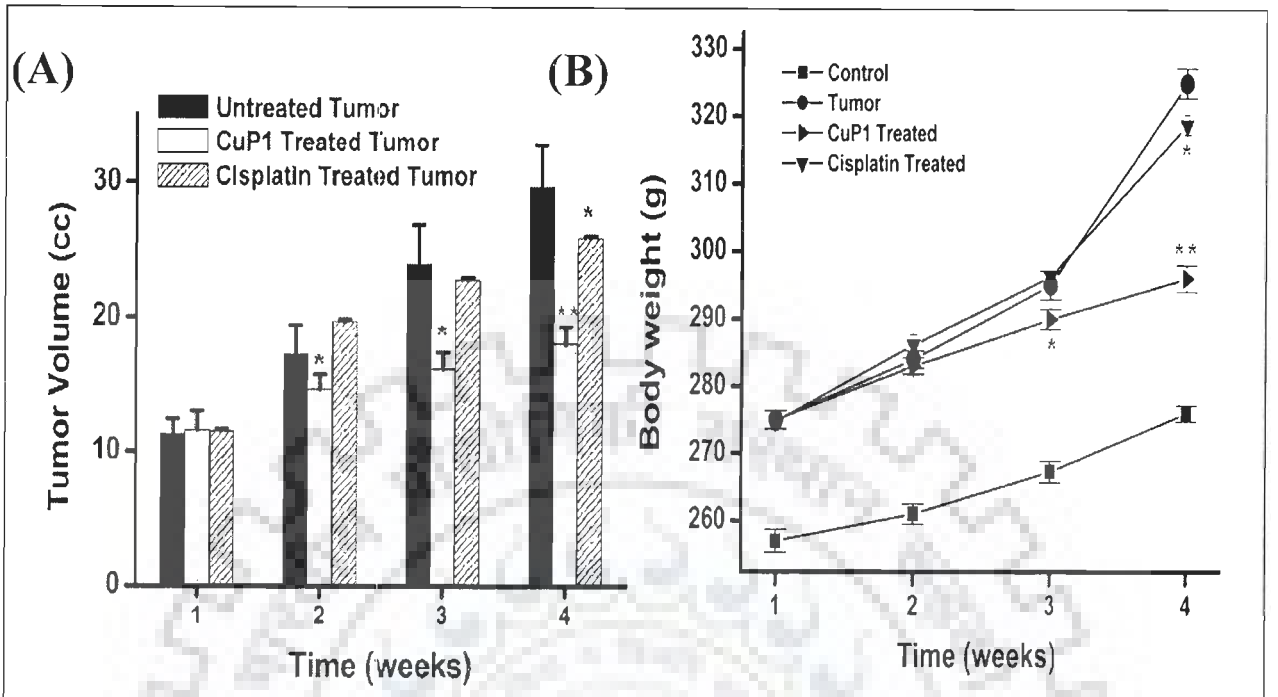


*Fig. 5.7: (A) Changes in the tumor volume in CuP1 and cisplatin treated animals. (B) Change in body weight in various groups of animals throughout the treatment period. Values represent mean ± S.E.M and n = 11. * and ** indicates significantly different at P<0.05 and P<0.01 respectively, as compared to tumor untreated group at respective time points.*

There was a significant ($P < 0.05$) inhibition in tumor growth rate after two weeks of treatment with CuP1 (Fig. 5.7A). The drastic increase in the body weights of the untreated tumor bearing animals could be linked to the synergistic increase in the tumor volume and was significantly inhibited by CuP1 ($P < 0.01$) after 4th week (Fig. 5.7B). Marginal reduction in the body weights were also observed in the cisplatin treated tumor group. Even the tumor doubling time increased by almost two times after CuP1 treatment (Table 5.2) which was at par with the findings of S´anchez et al. (2006), where the prostate tumor xenograft is inhibited by capsaicin.

Table 5.2: Breast tumor growth inhibition by CuP1

Treatment	Initial tumor volume (cc)	Final tumor volume (cc) after 30 d	Specific growth rate of tumor	Tumor doubling time (in days)
Group I (vehicle treated)	ND	ND	ND	ND
Group II (vehicle treated, n=11)	11.2 ± 1.5	29.645 ± 2.5	0.032	21.65
Group III (CuP1 treated, n=11)	11.6 ± 1.4	18.022 ± 1.3*	0.014	49.5
Group IV (Cisplatin treated, n=11)	11.8 ± 1.1	25.8 ± 1.9*	0.026	26.57

ND, not detected. * respectively indicates statistically significant as compared to 30 days vehicle treated Group II animals ($P < 0.05$). Values represent mean ± S.E.M. of the animals of individual groups.

5.3.2.3 Histopathological studies of rat tumor after treatment with CuP1

Histopathological analysis showed that 30 days of treatment with CuP1 resulted in the formation of a number of apoptotic bodies (Fig. 5.8 C & D) which was further magnified and viewed in Fig. 5.8 D. On the contrary, in case of untreated animals highly proliferating cells of ductal carcinoma covered the full microscopic field (Fig. 5.8 B). However, no such proliferating cells or apoptotic bodies were visible in the histological section of the normal breast tissue (Fig. 5.8 A). Number of apoptotic bodies was not very promising in the breast tumor histological sections treated with cisplatin (Fig. 5.8 E, F).

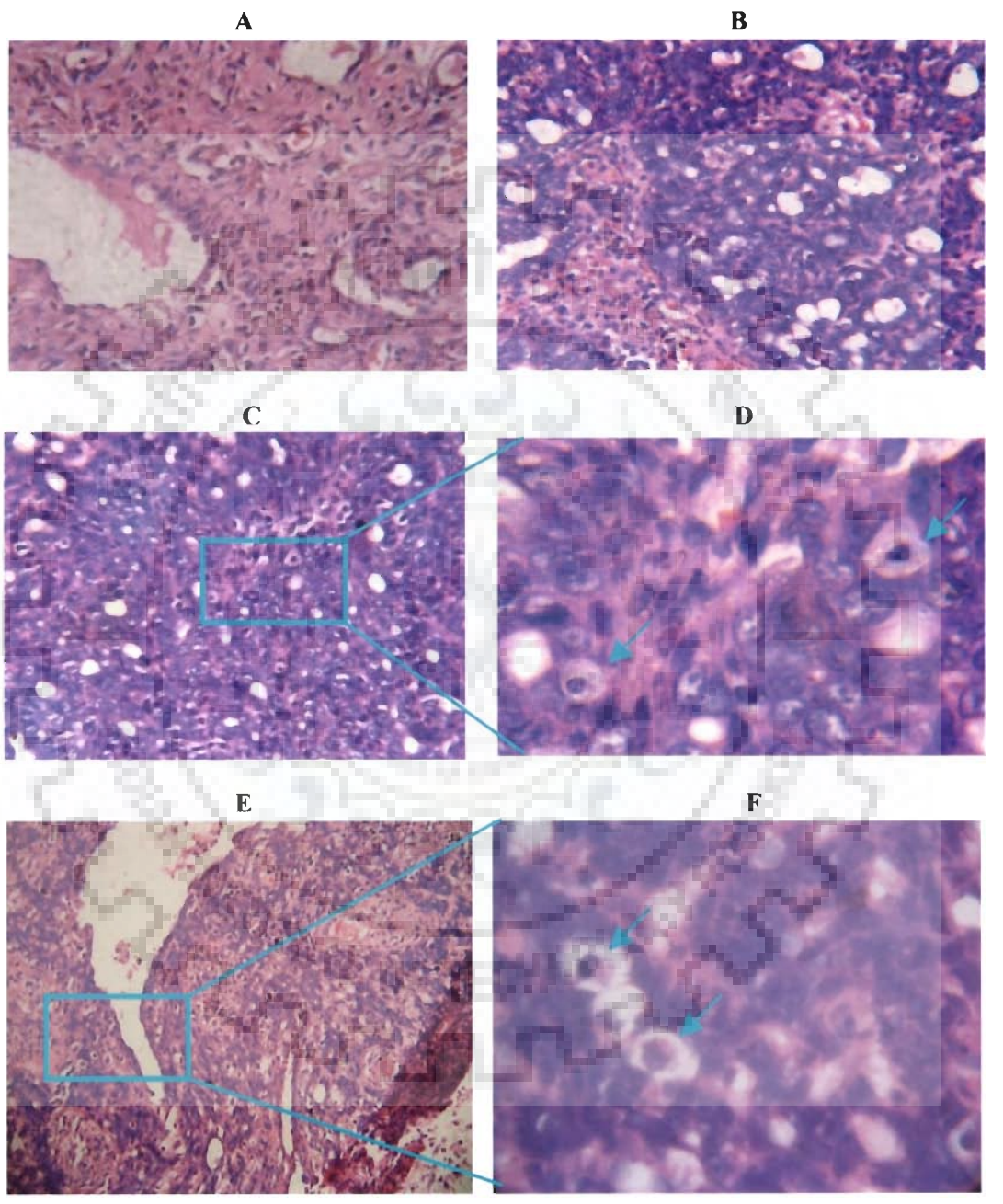


Fig. 5.8: *Histopathology of breast tissues stained with H& E from various groups of animals. (A) control, Group I (20X objective); (B) tumor untreated, Group II (20X); (C) tumor treated with CuP1, Group III (20X); (D) the inset of apoptotic cells showing chromatin condensation (marked by arrows) (100X); (E) tumor treated with cisplatin, Group IV (20X) and (F) the inset of apoptotic cells showing chromatin condensation (marked by arrows) (100X).*

As shown in Fig. 5.9, the mitotic index decreased sharply after treatment, in contrast to the increase in the apoptotic index in the tumor treatment group with CuP1, establishing that CuP1 was comparatively more effective than cisplatin (Fig. 5.9).

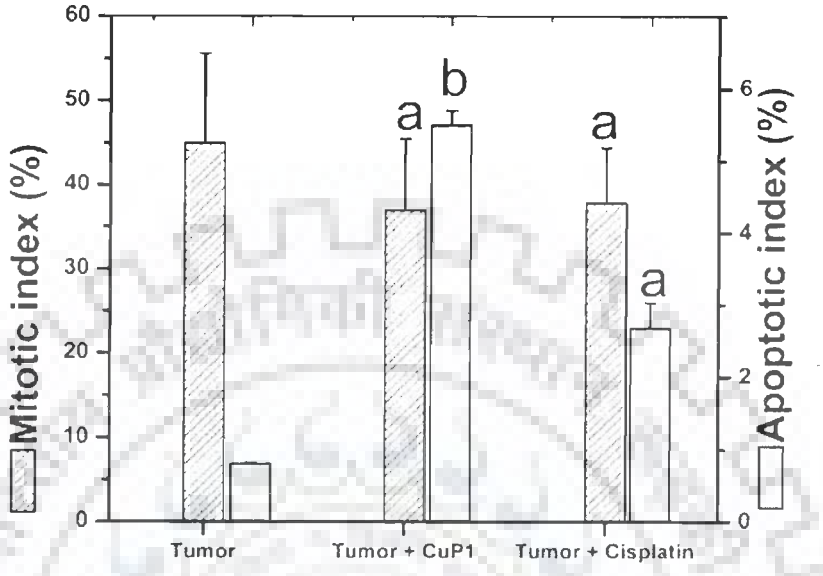


Fig. 5.9: *The mitotic index and apoptotic index as calculated from the H & E stained sections of vehicle and drug treated groups of animals. Values represent mean ± S.E.M. of 10 different microscopic fields from each group of animals. a and b indicates significantly different at P<0.05 and P<0.01 respectively, with respect to respective vehicle treated tumor group.*

5.3.2.4 Haematological parameters after treatment with CuP1

In general, most of the anticancer drugs resulted in systemic toxicity which is marked by severe RBC toxicity thereby causing anemia. Therefore, it was obvious to check the general haematological parameters in the animal groups after the completion of treatment. The general haematological parameters of tumor bearing rats after treatment with CuP1 (Group III) showed statistically significant increase in the RBCs (3.3 to 4.5 × 10^6/cu mm) and hemoglobin (9.2 to 10.8 g/dl) as compared to tumor bearing rats (Group II) (Table 5.3) ($P < 0.001$) thereby showing no anemic symptoms. Therefore, this data confirmed that CuP1 does not aid the general anemic condition in tumor. Further, in the tumor model total WBC counts decreased significantly ($P < 0.001$) which after treatment was found to be more close to normal. Also, some of the major blood parameters like MCV (mean corpuscular volume), MCHC (mean corpuscular

haemoglobin concentration) which were related to the condition of RBC were nearly same in the treatment group (Group III) to that of the healthy animal's blood parameters (Group I) (Table 5.3).

Table 5.3: Haematological parameters of animals from various groups

Parameters	Normal (n=10)	Tumor (n=11)	CuP1 treated (n=11)
WBC 10^3 /mm^3	7.683 ± 0.13	18.576 ± 0.16 *	12.476 ± 0.78 *, **
Grnulocytes %	30.886 ± 0.94	76.474 ± 0.57*	34.97 ± 0.57 **
Lymphocytes %	67.134 ± 1.41	18.54 ± 0.28 *	61.317 ± 0.88 **
Monocytes %	1.01 ± 0.01	2.23 ± 0.09*	1.2 ± 0.06**
Haemoglobin g/dl	13.551 ± 0.10	9.231 ± 0.17 *	10.845 ± 0.23*, **
RBC 10^6/mm^3	4.475 ± 0.07	3.304 ± 0.05*	4.58 ± 0.10**
MCV fL	54.96 ± 0.4	79.11 ± 0.46*	54.42 ± 0.94**
MCH pg	16.7 ± 0.19	46.311 ± 0.6*	19.43 ± 0.43*, **
MCHC g/dl	33.359 ± 0.53	58.096 ± 0.77*	35.90 ± 0.86**
Hct % (haematocrit %)	45.67 ± 0.69	18.33 ± 1.6*	34.61 ± 0.66*, **
Platelets 10^6/mm^3	846 ± 6.94	415.72 ± 18.4*	942.45 ± 27.9**
Urea mg%	47.23 ± 0.75	55.06 ± 0.83 *	55.23 ± 0.82 *
Creatinin %	0.81 ± 0.01	0.82 ± 0.02	0.81 ± 0.01
SGOT : SGPT	1.11 ± 0.04	1.56 ± 0.09*	1.54 ± 0.1 *

Data are Mean ± S.E.M. of the respective group of animals. *, ** indicates statistically significant difference at P<0.001 as compared to normal group and tumor group respectively.

Despite the significant improvement ($P<0.001$) in the level of mean corpuscular haemoglobin (MCH) as compared to untreated tumor animals, it failed to reach the normal range. This could be attributed to the decrease in the overall haematocrit value. Interestingly, the platelet count which dropped significantly in tumor bearing animals, increased even more than the normal animals upon treatment with CuP1 ($P<0.001$). Although, at this point it is difficult to speculate exact reasons for this response, it can be interpreted as a secondary inflammatory response and it needs further validation. However, no significant hepato-toxicity was observed after treatment as the serum levels of urea and creatine remained almost unaltered in all the three groups of animals (Table 5.3). SGOT : SGPT ratio increased as compared to the normal rats but the range was within the sub-acute toxicity levels and there was no significant difference between the tumor (Group II) and CuP1 treated (Group III) animals. Haematological parameters of cisplatin treated rats have been well studied which also matches with our studies, hence these results are not included in our results.

5.3.2.5 Antioxidant enzyme analysis

The levels of various antioxidant enzymes were analyzed to evaluate the effect of CuP1 on tumor tissues. As shown in Fig. 5.10, catalase and glutathione peroxidase activities were reduced approximately 15 and 1.5-folds, respectively, after 30 days of treatment with CuP1 as compared to untreated tumor while the level of glutathione reductase was increased about 3-folds as compared to untreated tumor animal groups ($P<0.05$). The catalase and glutathione peroxidase enzyme activities in the CuP1 treated animals were even reduced as compared to the normal rats ($P<0.05$) while the glutathione reductase remained almost same (no significant difference) in both normal and CuP1 treated groups.

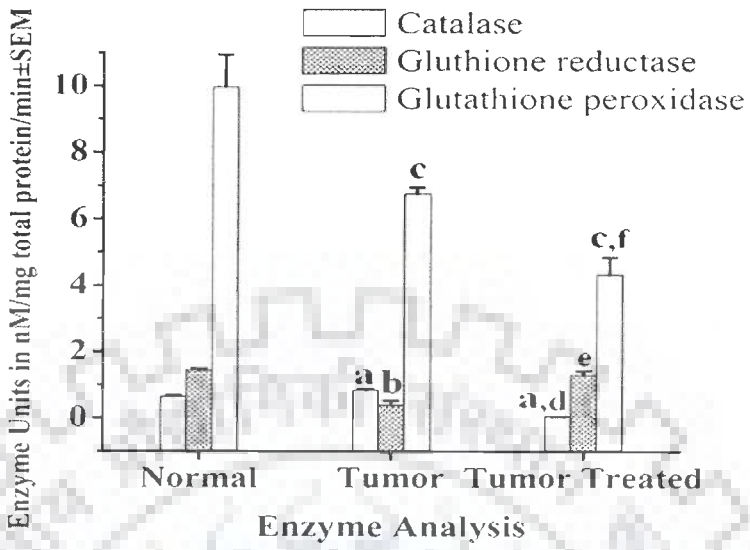


Fig. 5.10: *Effect of CuP1 on the antioxidant enzyme status in breast tissues from various groups of animals. The data are expressed as Mean ± S.E.M. of four independent experiments. a, b and c represents statistically significant at P<0.05 differences for catalase, glutathione reductase and glutathione peroxidase, respectively, with respect to normal (Group I) animals. d, e and f represent respectively the significant difference at P<0.05 between catalase, glutathione reductase and glutathione peroxidase activities with respect to untreated tumor tissues (Group II).*

5.3.2.6 Gene expression by semi-quantitative RT-PCR

Rats treated with CuP1 showed a significant down-regulation of tumor marker genes such as α- methyl-acyl CoA racemose (AMACR), Akt, Wnt, Bcl-2 and the metastasis marker MMP9 (matrix metalloproteinase 9) as compared to control) (Fig. 5.11 A, B). At the same time there was up-regulation of essential apoptotic genes, Bax (1.6-folds) and caspase 3 (14-folds) while TNF-α increased by 1.2-folds (Fig. 5.11 C). The relative intensity of the signals were quantified by densitometer and normalized against the internal control GAPDH (Fig. 5.11 C,D; where 'a' represents statistically significant with respect to tumor untreated group II at P<0.05).

5.3.2.7 Western blot analysis

Western blot analysis demonstrated a reduced expression of phosphorylated Akt and p38 in treated breast tumor tissues as compared to untreated tumor group (Figure 5.12A). On the other hand, there was a significant up-regulation in the expression of caspase 3. Further,

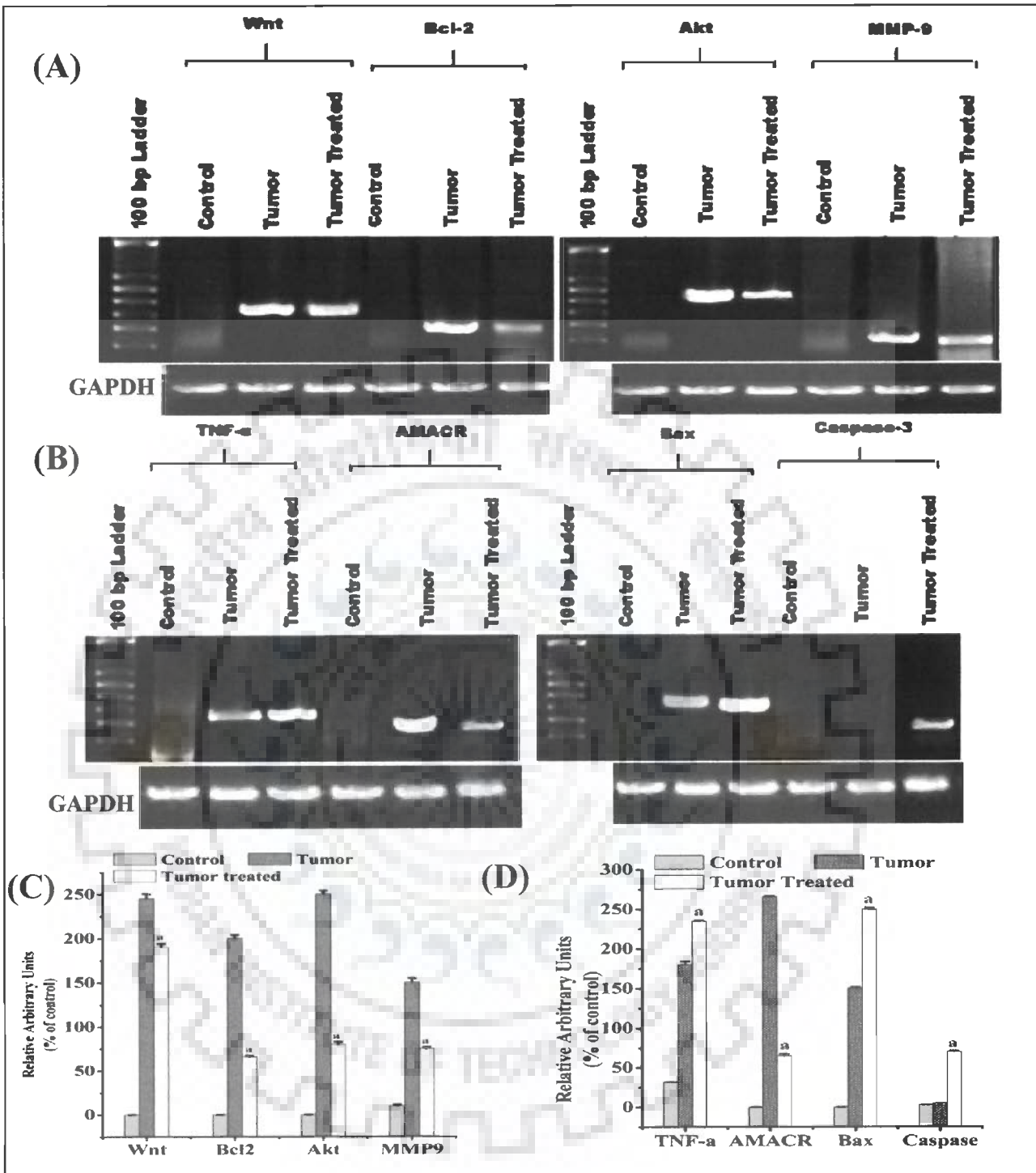


Fig. 5.11: *Transcriptional analysis of various marker genes in in vivo animals in response to CuP1. (A) Represents expression of Wnt, Bcl-2, Akt and MMP9 and (B) represents expression of TNF-α, AMACR, Bax and Caspase 3; (C) & (D) the relative intensities of the RT-PCR products were quantified by densitometer and quantified against internal control GAPDH. The data are expressed as Mean ± S.E.M. of 3 independent experiments. a represent statistically significant with respect to tumor untreated group II ($P < 0.05$).*

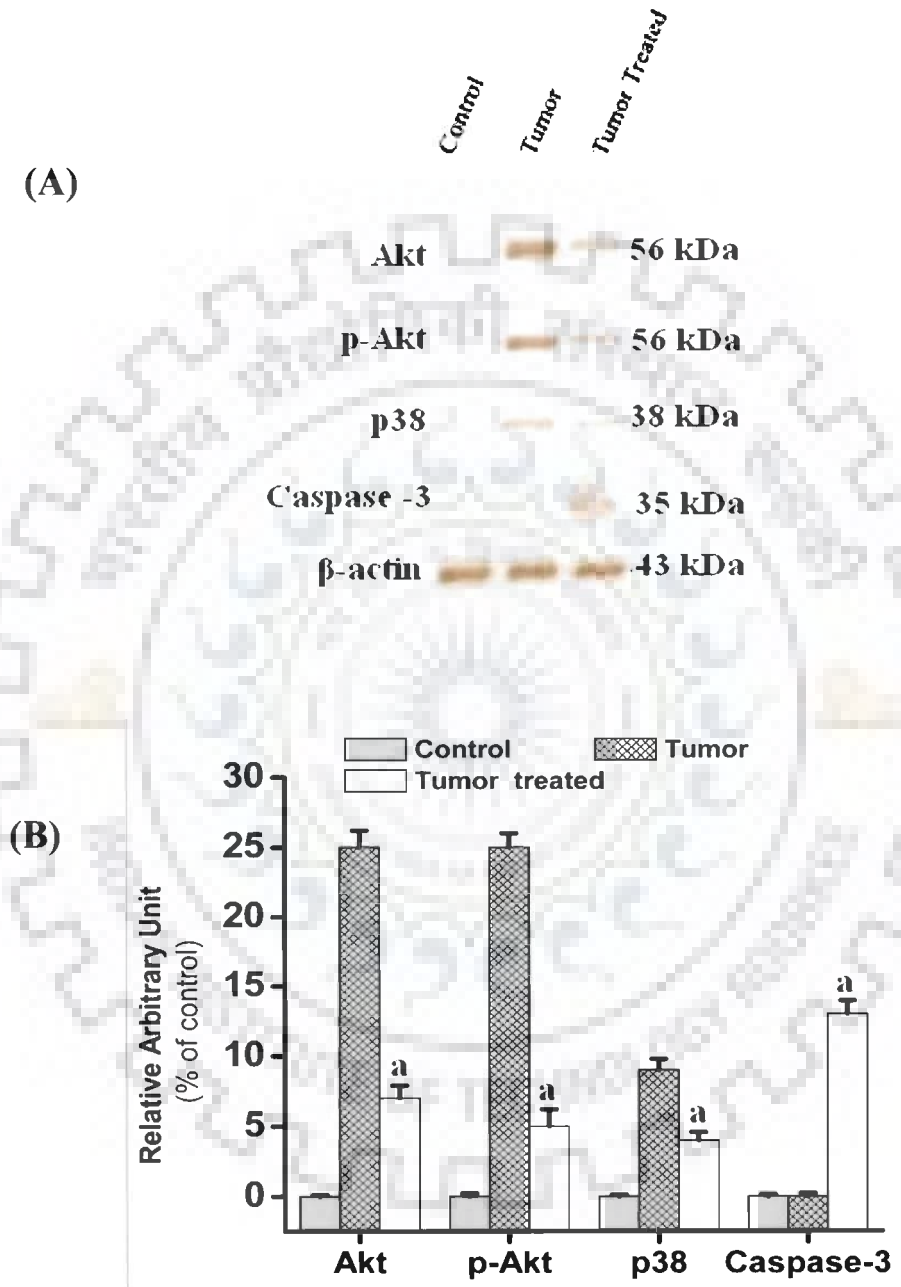


Fig. 5.12: *Immunoblot analysis of differential marker genes for tumor and apoptosis in breast tissues in response to CuP1. (A) Represents expression of non-phosphorylated Akt, phosphorylated Akt, p38 and caspase 3. The blots were developed with freshly prepared solution of 0.05% DAB and 0.015% H_2O_2. (B) The histogram showing the mean of arbitrary pixel intensity ± S.E.M. of 3 immunoblots. 'a' represents statistically significant difference with respect to tumor untreated group ($P<0.05$).*

the total non-phosporylated Akt level was also significantly down-regulated in the CuP1 treated group as compared to untreated group which was in line with our RT-PCR data as shown earlier (section 5.3.2.6).

5.4 Discussion

Transitional metal chemistry is evolving for its potency to target biological molecules and there is considerable interest for the discovery of new metal based drugs for anticancer activities. In this study we have tested the anticancer activities of CuP1 *in vitro* and *in vivo*. In metal based compounds the solubility in normal saline or their accumulation at the site of injection at physiological pH are some of the limitations associated with these drugs (Hartinger et al., 2006). However, CuP1 had an added advantage over all these compounds since it was soluble in normal saline even at a high concentration thus alleviating its delivery problem. It is known that cisplatin acts through N7 site of guanine base in the DNA where it forms DNA-adduct and activates hMSH2, which is a member of the mismatch repair complex (Donahue et al., 1990; Fink et al., 1998; Chaney and Vaisman, 1999). Thus the irreparable damage to DNA leads the cells to accumulate at the G2 phase (Demarcq et al., 1994) and p53 molecules initiates apoptosis in a caspase dependent manner (Bullock and Ferst, 2001; Morgan and Kastan, 1997).

The present data confirms that CuP1 causes DNA cleavage and DNA fragmentation. This property of CuP1 is in concurrence with the earlier report that copper complexes interacts with DNA leading to strand breakage (Adsule et al., 2006; Rajendiran et al., 2007). The CuP1 induced DNA damage to the cancerous cells is evident from the single cell DNA strand breaks after illuminating the cells (EB stained) with UV light (fluorescent microscopy). CuP1 was also found to induce caspase 3 activity *in vitro* in two cancerous cell lines tested. Although caspase 3 is absent in MCF-7, the apoptosis is known to take place by means of caspase 7 and 9 (Kagawa et al., 2001). The substrate used for the assay had some reactivity with caspase 6 and 7 and hence might be responsible for the mild caspase activity in MCF-7 as observed in Fig. 5.4. Caspases are the final machinery which leads to apoptosis.

Although many anticancer compounds were found to be effective in *in vitro* assays, they failed to be a potent drug due to their toxic effects *in vivo*. Many of the above discussed copper complexes have not yet been studied in animal models. On the other hand, in order to designate a compound to be an effective anticancer molecule the *in vitro* and *in vivo* effects should be comparable. Hence, in the next phase of study the present compound (CuP1) was tested on

MNU treated rat breast tumor model to check its efficacy in the *in vivo* system. Serum biochemical analysis essentially provides an assessment for the pathological and toxicological changes in the animals (Venkatesan et al., 2006). In our study, CuP1 did not show any haemotoxic effect which is generally marked by the anaemic condition due to RBC toxicity by the drug. A similar pattern of non-haematotoxic effect was reported recently from another copper-glycinate complex, which is under investigation showing a closely related value of haemoglobin content in the ehrlich ascite bearing mice before and after treatment (Mazumdar et al., 2006). When tested for the antitumor effect of the compound, it was found that the increment in the breast tumor volume decreased by 56% after the treatment with CuP1 for 30 days. This was further confirmed by histopathological findings on tumor tissues, where the apoptotic body formation was significantly increased in the CuP1 treated animals. CuP series were designed with complex ligand modification of pyimpy and it was more effective in the breast cancer model than its reference compound cisplatin which is effective mainly against ovarian cancers (Helm et al., 2009).The results of CuP1 on the *in vivo* breast tumor were also consistent with the data reported by John et al. (2002) on copper chelates of curcuminoids, where a decrease of 39% in the breast tumor volume was obtained after treatment.

In order to understand the molecular events leading to CuP1 induced apoptosis in a highly metastatic ductal carcinoma model, we further investigated major cell proliferating and apoptotic gene expressions by RT-PCR and immunoblot analysis. Our results showed that the growth promoting factor like Akt, Wnt and Bcl-2 were down-regulated after the treatment with CuP1. These results clearly supported the antitumor action of CuP1 when it is already known that the MNU treated tumor models have an increased phosphorylated Akt (Liao et al., 2005). One issue which we could not justify at this point is the overall reduction in the expression of Akt in the treatment group. Hence, further studies are warranted to solve this puzzle. Reports show that breast cancer becomes metastatic due to the overexpression of some metabolic enzymes like α- methyl-acyl CoA racemose (Witkiewicz et al., 2005, Goss and Strasser 2004) or the proteinases like matrix metalloproteinase (Martin et al., 2008) which was very clearly down-regulated after treatment with CuP1. This report is also supported by the fact that other copper Schiff bases have also been found to inhibit the metastatic markers like VEGF and inhibits cyclooxygenases and Bcl-2 in pancreatic cell lines (Ambike et al., 2007). Another interesting feature of CuP1 molecule is that it mainly acted through caspases which was validated by testing both caspase 3 (caspase 3 enzyme activity in PC3 and SiHa cells and caspase 3 RT-PCR in rat

breast tumor tissues) and caspase 9 in immunoblot analysis and inhibited both Akt and MAPK pathway in rat breast tumor models (via inhibition of p38).

Antioxidant enzymes play an important role in the drug metabolism leading to drug resistance (Goto et al., 1999; Sakamoto et al., 2008). In general, copper is a redox active metal and the copper based metallo-complexes react to DNA leading to production of reactive oxygen species (ROS) (Gaetke and Chow, 2003; Liochev and Fridovich, 1992). However, the present data showed that CuP1 caused reduction in the overall load of antioxidant enzymes after treatment thus nullifying the future resistance of the drug. Studies on drug resistance have shown that intracellular glutathione depletion increases the cellular accumulation and retention of drugs (Mazumdar et al., 2006). Thus glutathione depletion reduces the efflux of drugs from the tumor tissue (Mazumdar et al., 2006) which is again in concurrence with our present findings. It is also evident from reports that increase in ROS production also accompanies the activation of caspases leading to apoptosis (Kim and Park, 2003). Moreover, a link is previously established between ROS and TNFα by Kim et al. (2010). This study also showed increased expression of TNFα by CuP1 treatment. Thereby CuP1 provides an indirect and supportive evidence of the increase in ROS status in the breast tumor tissues.

In conclusion, CuP1 possesses prominent anticancer activities *in vitro* as well as *in vivo*. CuP1 has a very low inhibitory concentration (IC_{50}~ 4.2μM) *in vitro* in MCF-7 cell line among the family of copper complexes containing Schiff base ligands (Table 4.2). *In vitro* it has shown prominent DNA strand breaks and chromatin condensation. Out of all these, the most promising result which was revealed from the present study is that the drug inhibits tumor growth by an increase in the tumor doubling time. Moreover the mitosis is also inhibited with the prominence of apoptosis at the site of tumor in breast tissue. Further this compound induces apoptosis in breast tumors mainly through the involvement of the caspase pathway. The most promising result from the above study is that the overall load of antioxidant enzymes in tumor tissue is markedly reduced, which can make the compound an useful candidate along with the other existing anticancer drugs like doxorubicin where its application becomes limited due to drug resistance. The compound although showed marginal toxicity related to inflammation but there is still scope to modify it further. In summary, the properties of this compound have opened scopes to investigate more on the members of the same ligand frame to find a better anticancer drug. Further, its higher efficacy as compared to the known potent anticancer drug, cisplatin, makes the compound a promising anticancer candidate molecule in future.

CHAPTER 6

PURIFICATION & CHARACTERIZATION OF PTEROSTILBENE

6 Purification & Characterization of Pterostilbene

6.1 Introduction

It has been reported that pterostilbene is the major constituent of some plant sources like the heartwood of *Pterocarpus marsupium* (Maurya et al., 1984), *Pterocarpus santalinus* (Sehadri, 1972). This stilbene is also secreted in response to fungal infected grape berries of varieties Chardonnay and Gamay (Adrian et al., 2000) and is also present in healthy and immature berries of varieties Pinot Noir and Gamay (Pezet et al., 1988), *Vitis vinifera* (Langcake et al., 1979). These are basically the secondary metabolites in plants synthesized in response to environmental stress or fungal infections (Pont et al., 1990). Pterostilbene has also been reported to inhibit the lipopolysaccharide induced activation of PI3K/Akt and NFκB thus found to bear anti-inflammatory effect on murine macrophages (Pan et al., 2008a). It caused apoptosis in human gastric cancer cells due to the induction of mitochondrial genes (Pan et al., 2007). The anti-oxidative activity of this phytochemical was first demonstrated *in vitro* by the inhibition of methyl linoleate oxidation (Charvet-Faury et al., 1998). Pterostilbene was also reported to inhibit metastatic activity in B16 melanoma cells (Ferrer et al., 2005), causing apoptosis in multi-drug resistant leukemia cells (Tolomeo et al., 2005) and effective against cervical and colon carcinogenesis (McDougall et al., 2008; Suh et al., 2007). Further, pterostilbene was also found to activate PPARα, a transcription factor, which is involved in lowering cholesterol and other blood fats (Rimando et al., 2005; Mizuno et al., 2008).

In chapter 4, the methanolic extract of *Pterocarpus* showed potent cytotoxic effect in different cancer cell lines. It also showed induction of characteristic apoptotic nuclei in MCF-7 cells. The present study was thus conducted to purify the active stilbene component from *Pterocarpus* extract. Thereafter the anti-proliferative effects of natural pterostilbene were checked in MCF-7 breast cancer cell line. The next aim was to characterize the various targets of pterostilbene and understand its mode of action in breast cancer cell line. Finally, the effect of pterostilbene was checked on *in vivo* breast cancer models.

6.2 Brief experimental protocols

6.2.1 Purification of pterostilbene from Pterocarpus marsupium extract

The heartwood was washed thoroughly with water and then air dried. The stem was powdered and was then extracted with chloroform to remove lipids. The residue was dried and then 100 g of it was mixed with 3 volumes of methanol : acetone : water : formic acid (40:40:20:0.1) according to the protocol described previously (Rimando et al., 2004). After the prescribed processing for 6 h continuously, the clear supernatant was collected and the residue was again mixed with 250 ml of extraction solvent and processed for another 2 h before filtering. The filtrates were combined into a round bottom flask and the organic solvents were removed under vacuum. The remaining aqueous portion was freeze dried. The freeze dried extract was then suspended in approximately 10 ml of deionised water and loaded on a 10 g silica column which was previously conditioned with 70 ml of methanol and 150 ml of water. After washing the column with 150 ml of water, the stilbene fraction was eluted with 50 ml of methanol : acetone : water : formic acid (40:40:20:0.1). The eluted fraction was vacuum dried and dissolved in methanol and fluorescence was measured against synthetic standard where the excitation wavelength was set at 330 nm and the emission spectra were scanned from 600 to 400 nm.

6.2.2 Thin layer chromatography (TLC)

The presence of pterostilbene in the eluted extract was checked on a TLC plate using the mobile phase (as described in section 3.22) and compared with the standard pterostilbene (Sigma, St. Louis, MO, USA).

6.2.3 HPLC purification

Due to the presence of at least 4 different fractions in the TLC analysis, the stilbene was further purified with HPLC. For HPLC purification, both the standard and the extracted stilbene fraction were dissolved in methanol. The samples were injected in duplicate onto a HPLC (Knauer, Berlin, Germany) equipped with 10 ml pump head 1000, manual injection valve, UV detector 2500, photodiode array (PDA) detector 2600 and Eurochrom operating software. The system was equipped with reverse-phase C18 column (250 mm × 4.6 mm I.D., 5 μm). Before loading all the samples were filtered with 0.2 μm filter and 20 μl of the sample was injected. The HPLC program was as follows: 0 min 83% A, 17% B; 13 min 82% A , 18% B; 15 min 82% A ,

18% B; 17 min 77% A , 23% B; 21 min 75% A, 25% B; 27 min 68% A, 32% B; 37 min 53% A, 47% B; 39 min 15% A, 85% B; 40-48 min 0% A, 100% B, where solvent A was glacial acetic acid in water (52.6:900 v/v) and solvent B was 20% phase A and 80% acetonitrile, according to the method described earlier (Romero-Perez et al., 2001). The identification of the pterostilbene was carried out by comparing the retention time of the extracted fraction with the standard. They were also characterized by their UV spectra from 250 to 400 nm using PDA (Knauer, Berlin, Germany) and by spectral comparison with the standard.

6.2.4 Gas chromatography – Mass spectroscopy (GC-MS)

The pure fraction isolated from the HPLC was vacuum dried and further analyzed by GC-MS. The elute was dissolved in 10 µl of methanol and 2 µl of the sample was injected into Perkin-Elmer GC- MS (Perkin-Elmer Clarus 500 GC and Perkin-Elmer Clarus 500 MS, Massachusetts, USA) fitted with GC capillary column of 30 m length, 0.32 mm i.d., 0.1 µm, df (Elite-1, Perkin-Elmer, Massachusetts, USA) and the mass detector was operated at 70 eV. The GC temperature program was as follows : initial temperature 80 °C with hold for 1 min, increased to 260 °C at the rate of 7 °C per minute and a final increase to 285 °C at the rate of 2 °C per minute with a final hold for 2 min. The carrier gas was ultrahigh purified helium at a 1 ml/min flow rate. The analysis of the compound was performed by comparing the chromatogram and calculating the mass spectra with the standard pterostilbene (Sigma, St. Louis, MO, USA).

6.2.5 Cell lines

In this study, MCF-7 (ER positive) cell lines were taken as the representatives of the breast cancer cells for all *in vitro* experiments. Purified pterostilbene was also tested on T47D cells (PR positive) for some of its anticancer properties. The culture conditions of the cell line are described in section 3.4.1.

6.2.6 In vitro assays

Cytotoxicity assay, ***Clonogenic assay***, ***Soft agar assay***, ***Acridine orange/Ethidium bromide staining assay***, ***DNA fragmentation assay***, ***Comet assay***, ***Caspase assay*** and ***Scanning electron microscopy*** were carried to prove the anticancer properties of purified pterostilbene in breast cancer cell lines. Further, ***Measurement of intracellular ROS***, ***Antioxidant enzyme assays***, ***Immunofluorescence study***, ***RT-PCR*** and ***Immunoblot analysis*** were carried out to

understand the mechanism of action of purified pterostilbene, as an anticancer agent, in breast cancer cell lines. The detailed protocols of all the assays have been described in Chapter 3 (Materials & Methods).

6.2.7 In vivo experiments

The detailed description for the ***Development of mammary tumor models*** has been given in the *Materials & Methods* section.

6.2.7.1 Treatment of mammary tumor models

The tumor bearing female rats were divided into following groups for further treatments. Treatment was started when the tumors developed in the animals reached a size of 12 mm^3. The animals were otherwise housed with proper food and water *ad libitum*.

Group I : Healthy female, non-tumor model (n=10)
Group II : Breast tumor model (n=11)
Group III : Pterostilbene treated breast tumor model (n=11)
Group IV : Cisplatin treated breast tumor model (n=11), where n was the total number of individuals in a group.

The animals were injected (i.p.) with pterostilbene at a dose of 20 mg/kg body weight for 1 month. The dose was determined according to the earlier reports by Ferrer and his colleagues (2005). The animals of Group IV were treated with 1 mg/kg body weight of cisplatin. The animals of Group I & II were similarly treated with vehicle (normal saline).

The effect of purified pterostilbene on the development of breast tumors in rats, were further checked by measuring the tumor growth rate and analyzing the ***Histology of tumor tissues***.

6.3 Results

6.3.1 TLC

The eluted fraction from the silica column was checked on a TLC plate. Four different components were detected in the chromatogram. Of these, the top most component corresponded to pterostilbene which was identified by the same resolving position as the pure pterostilbene standard in parallel (Fig. 6.1).

Fig. 6.1:*Analysis of the partially purified extract from Pterocarpus marsupium as determined by thin layer chromatography. The detailed protocol has been described in the methodology section.*

6.3.2 HPLC purification

The partial purified fraction was further purified by HPLC. The elute showed single major peak (Fig. 6.2B) at 306 nm which matched exactly with the standard (Fig. 6.2A).

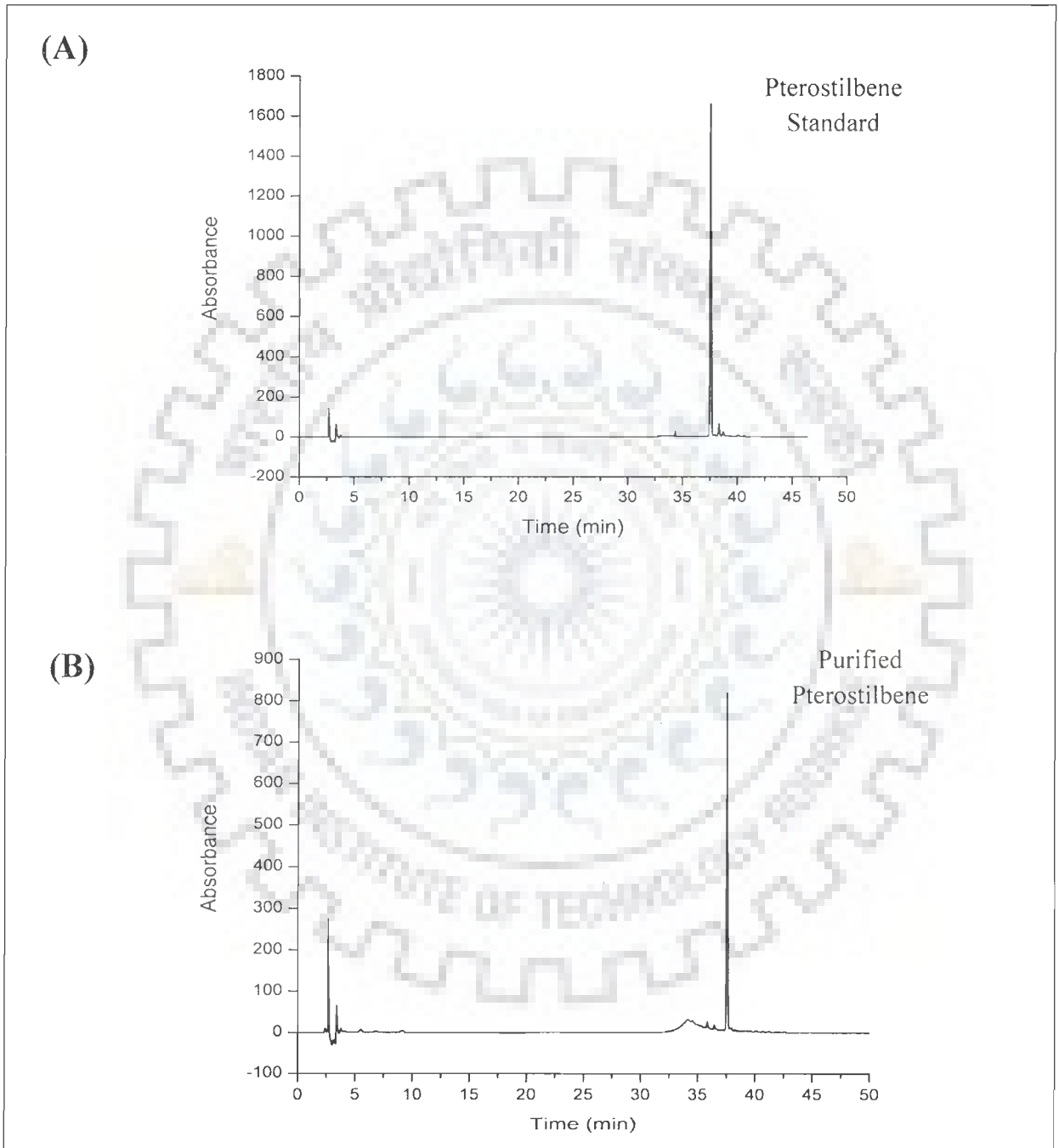


Fig. 6.2: *Purification of pterostilbene from Pterocarpus marsupium by HPLC. The figure represents (A) chromatogram of standard pterostilbene and (B) that of purified pterostilbene.*

The yield of pure pterostilbene from HPLC was 0.25 mg from an initial of 100g of the heartwood powder. The purity of pterostilbene thus isolated was further analyzed by GC-MS.

6.3.3 GC-MS

The purity of pterostilbene was further established by GC-MS analysis and comparing the mass spectra of the standard and the isolated compound. The chromatogram showed a broad peak area within 11.4 to 16.0 min (inset) which had a uniform mass spectrum throughout (Fig. 6.3). This spectrum was solved and compared with standard synthetic pterostilbene. The compound was found to be 98% pure.

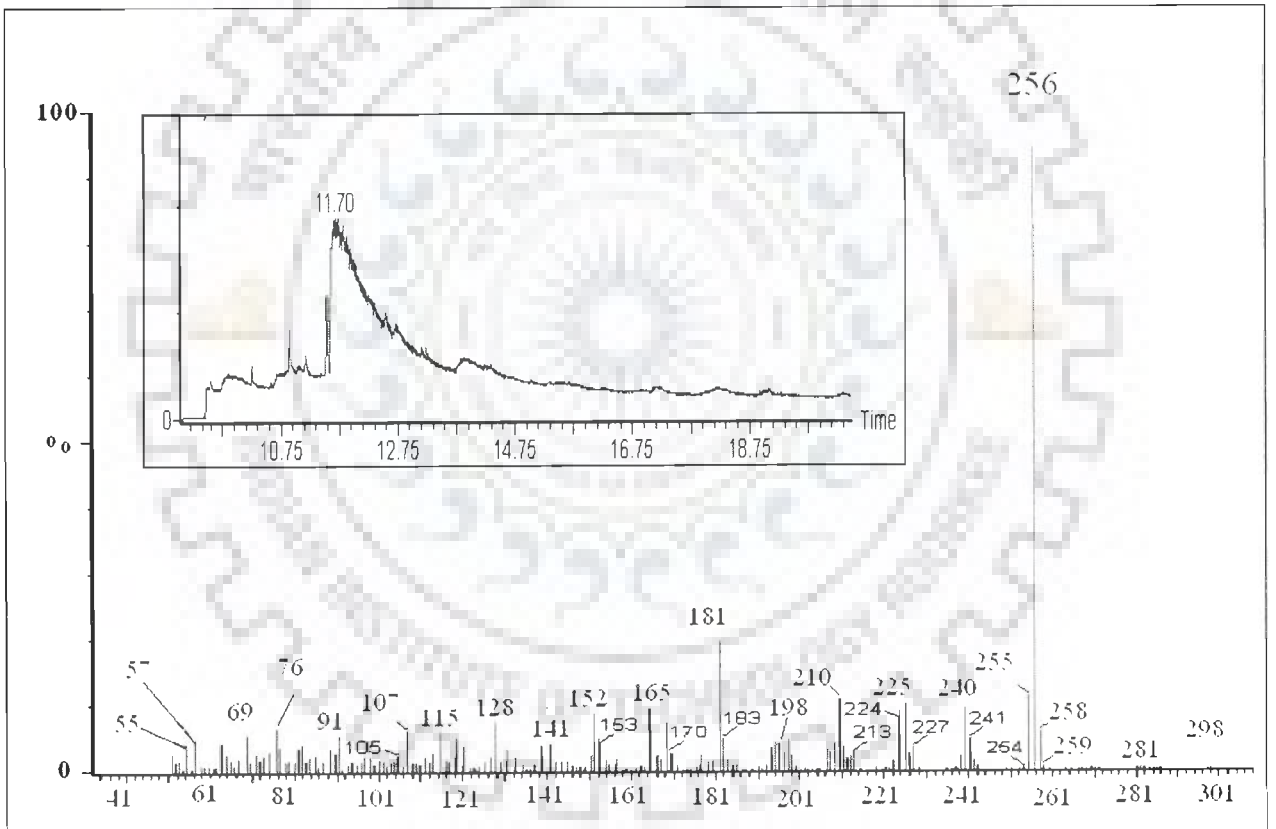


Fig. 6.3: *Analysis of mass spectra of the major peak obtained from the gas chromatogram (inset) of pure pterostilbene isolated from Pterocarpus marsupium heartwood.*

6.3.4 Preparation of the final stock of the pterostilbene for biological studies

From the mass spectral analysis, the molecular mass of isolated pterostilbene was found to be 256. The compound is further dissolved in ethanol at a concentration of 100 mM. Serial

dilutions from the mother stock were made in order to check the biological activities of the compound.

6.3.5 Measurement of cytotoxicity

The effects of purified pterositlbene were checked on breast cancer cell lines at different time points. After an initial screening of the concentration gradient ranging from 0.5-150 μM on the exponentially growing cells, the range was further narrowed down to 10-80 μM where a clear inhibition was observed particularly at this range. The IC_{50} values of pterostilbene in MCF-7 and T47D cell lines are given in Table 6.1.

Table 6.1: Cytotoxicity of purified pterostilbene at different time points.

Duration of exposure	IC_{50} value (μM)	
	T47D	MCF-7
24h	62.3±1.23	68.8±3.12
48 h	39.56±3.65	44.06±2.19
72 h	19.78±9.2	35.56±1.53

The data are the average from independent triplicate experiments (±S.E.M.).

This data showed that purified pterostilbene was a potent cytotoxic agent on both the breast cancer cell lines. The efficacy of the compound increases with the increase in the duration of drug treatment.

6.3.6 Pterostilbene inhibits the colony formation of breast cancer cells

In order to check the effect of pterostilbene on the mitotic potential of the breast cancer cell lines, MCF-7 and T47D cell lines were exposed to pterostilbene for various time intervals. Number of colonies reduced by almost 80% after 24 h which declined further to 99% after 72 h of exposure as compared to the vehicle treated cells in MCF-7 (Table 6.2 A & B). On the other hand, number of newly formed colonies was reduced by almost 77% to 99% in T47D for the same time points. Each colony is considered to be a cluster of at least 50 or more cells.

Table 6.2: Colony Formation Assay for measuring mitotic potential after pterostilbene treatment for the respective time points in (A) MCF-7 cells and (B) T47 D cells.

(A)

MCF-7			
Duration of pterostilbene exposure	Negative control	Treatment	Colony formation with respect to negative control
12 h	1296	725	53.75 ± 1.4%
24 h	3250	628	19.41 ± 1.1%
48 h	6391	234	3.75 ± 0.12%
72 h	12653	50	0.37 ± 0.04%

(B)

T47D			
Duration of pterostilbene Exposure	Negative control	Treatment	Colony formation with respect to control
12 h	1134	456	40 ± 2.18%
24 h	2351	561	23 ± 1.34%
48 h	3897	367	9.4 ± 0.92%
72 h	5410	30	0.5 ± 0.04%

The data are the average from independent triplicate experiments (±S.E.M.).

6.3.7 Pterostilbene inhibits the metastatic capacity of MCF-7 breast cancer cells

In the next stage of experiments, the effect of pterostilbene on the metastatic potential was also assessed by its capacity to prevent the invasiveness of MCF-7 cells in soft agar matrix. After 72 h of exposure to pterostilbene, the MCF-7 cells almost lost their invasive capacity to form colonies (> 0.5 mm at 10X objective magnification) against the control MCF-7 cells

(Figure 6.4). In the vehicle treated control cells large numbers of colonies were found. Although the pterostilbene treated cells were still alive (as examined by trypan blue staining, Figure 6.4) these cells lost their capacities to migrate in the anchorage independent soft agar base and form colonies.

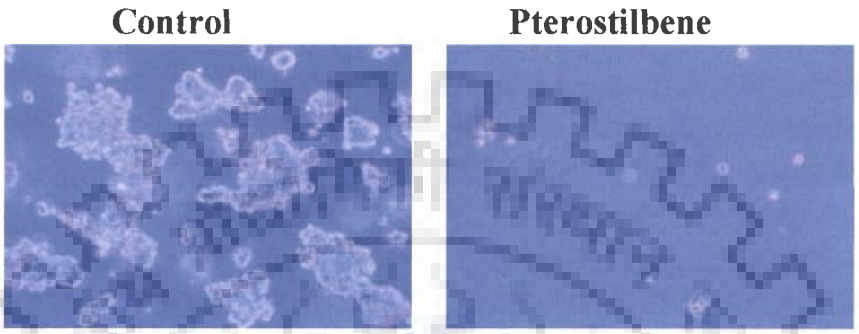


Fig. 6.4: *Pterostilbene induced inhibition of the metastatic potential of MCF-7 cells. MCF-7 cells were mixed with 0.3% soft agar and plated on top of 0.6% bottom agar in each of a 6 cm plates. On the fifth day the cells were exposed to pterostilbene (30 μM) for 72 h. The migration potential of the cells was determined by soft agar migration assay as described in materials and methods section. Each picture shows one representative experiment of three independent experiments (10X objective magnification)*

6.3.8 Pterostilbene induced apoptosis

When MCF-7 cells were treated with IC_{50} equivalent dose of pterostilbene, clear apoptosis was observed after 24 h of incubation. Live cells under the stress of early and late apoptosis could be distinguished by the clear chromatin condensation and the percentage uptake of AO : EB. Since AO permeated all cells, the nuclei appeared green (without chromatin condensation) in vehicle treated MCF-7 cells (Fig. 6.5 control cells). EB was taken up by the cells only when the cytoplasmic membrane integrity is lost in late apoptosis or in necrosis and stains the nucleus red. In the present study, early apoptotic cells had bright green nucleus with condensed or fragmented chromatin as seen in the pterostilbene and cisplatin treated cells marked by '1'. On the other hand the late apoptotic cells displayed condensed and fragmented orange chromatin '2', where EB dominates over AO. In addition, the cells which underwent direct necrosis, had a structurally normal orange colored nucleus found in both cisplatin and pterostilbene treated cells '3' (Fig. 6.5). These results were analyzed in accordance with the earlier report by Renvoize et al. (1998).

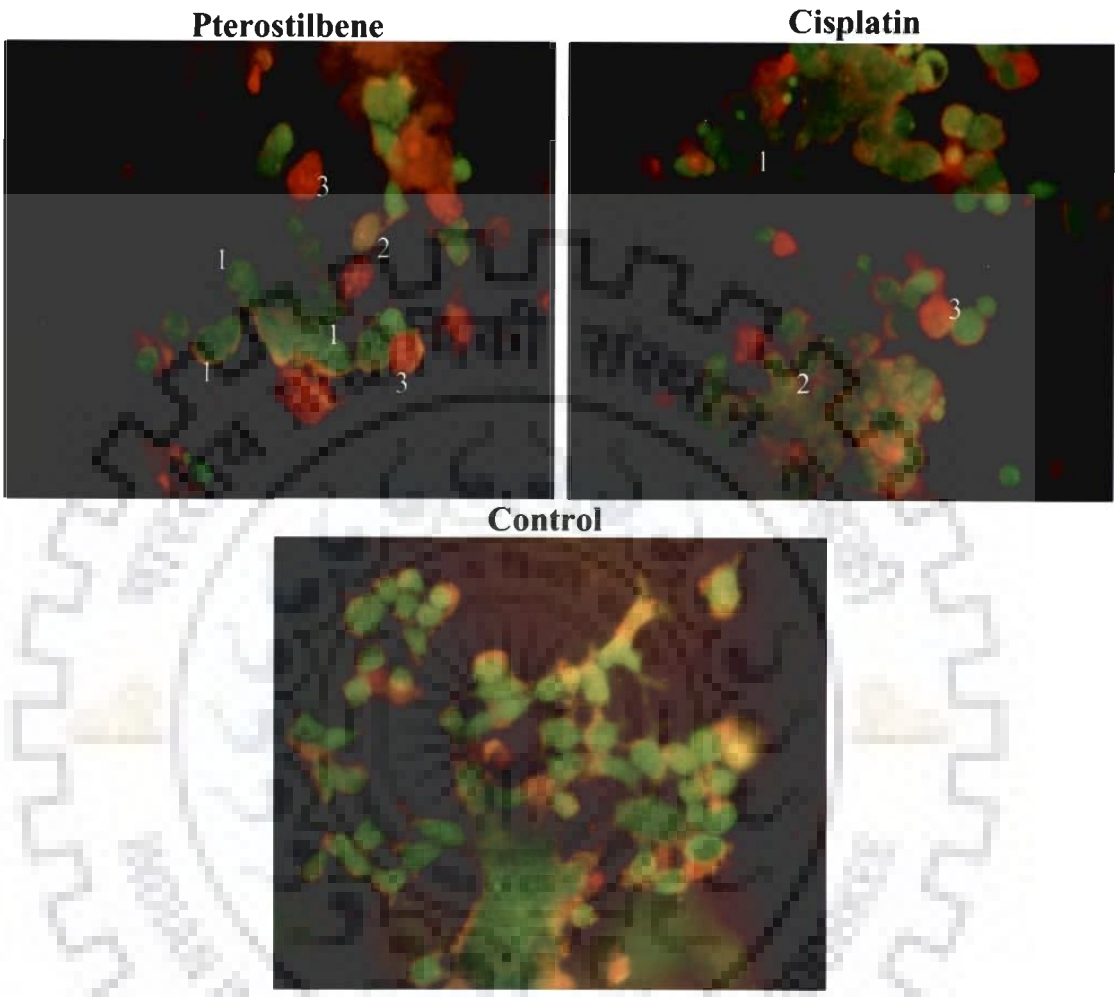


Fig. 6.5: *Induction of apoptosis followed by membrane blebbing and chromatin condensation by pterostilbene in MCF-7 cells. 1, 2, 3 represent early and late apoptosis and necrotic/dead cells respectively. The experiment was performed in triplicate and a representative experiment is presented.*

6.3.9 Induction of DNA fragmentation

As shown in Fig. 6.6, pterostilbene caused an intensified fragmentation of genomic DNA isolated from cancer cells, after 24 h of drug exposure. This was further validated by comet assay where the genomic DNA of each of single cells was visualized under fluorescent microscope (Fig. 6.7). The comet assay showed the formation of tailing in individual cells

resulting from DNA breaks (shown by arrow). However, vehicle treated cells did not show any DNA fragmentation in both the assays (Fig. 6.6 and 6.7). The comet tail area of 100 cells

Fig. 6.6: *Induction of DNA fragmentation by pterostilbene in MCF-7 cells. The experiment was performed in triplicate and a representative experiment is presented. Con, Ptero and Cis represents cells treated with vehicle, pterostilbene (68 μM) and cisplatin (100 μM) respectively.*

showed a very high percentage of DNA tailing in these breast cancer cells following treatment with pterostilbene as compared to cisplatin. Control untreated cells showed no prominent tailing (Fig. 6.7). The percentage of DNA damaged is represented as bar diagram

(Fig. 6.7 B) according to the formula given in the methodology section of chapter 3 (section 3.9).

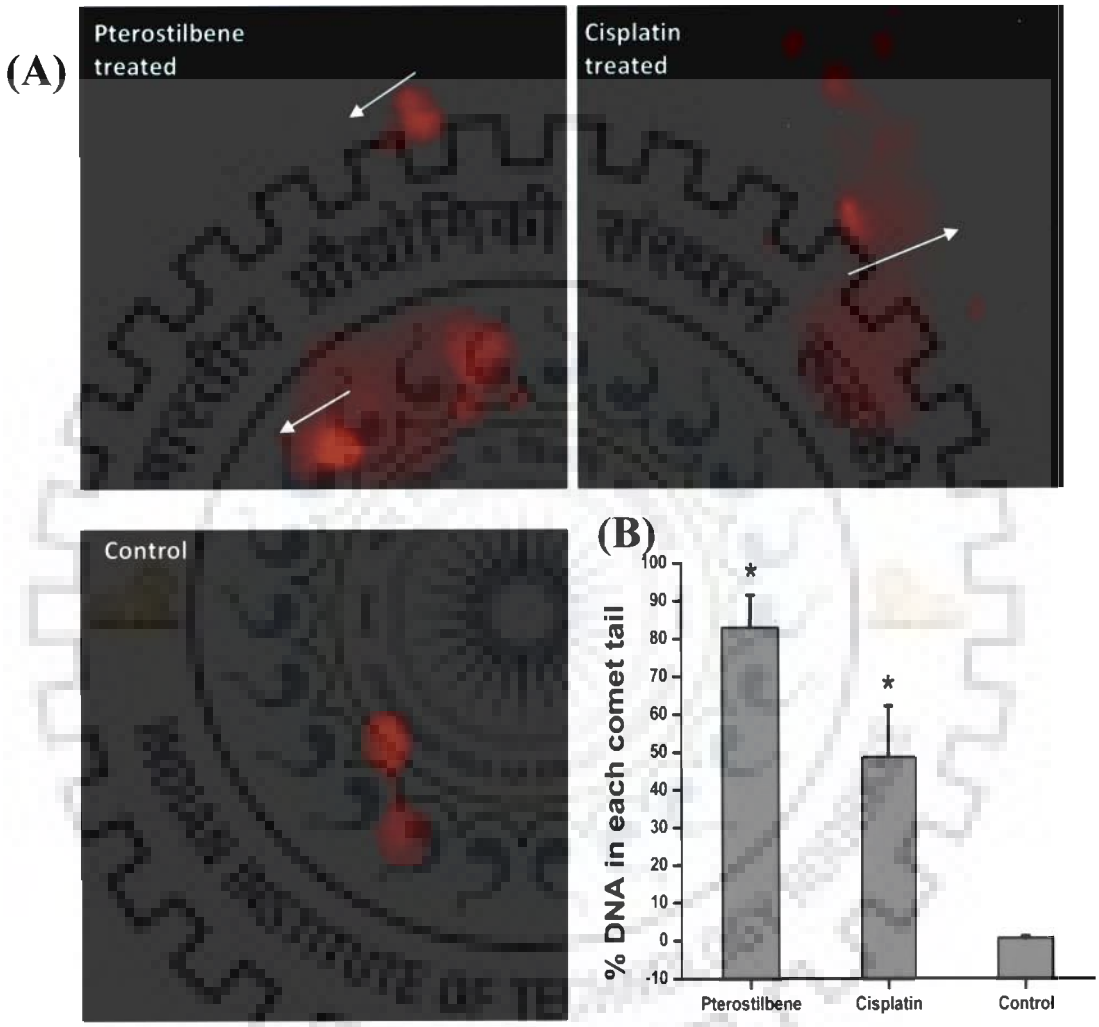


Fig. 6.7: *(A) Single cell alkaline gel electrophoresis showing DNA fragmentation. Nuclear staining was visualized under 20X objective of fluorescent microscope. The DNA strand breaks from individual cells are well visualized as comet tails (marked by arrows). The experiment was performed in triplicate and a representative experiment is presented. (B) Percentage of DNA in comet tail following treatment with test compounds. Comet tail area of 100 cells (mean ± S.E.M.) were calculated with the help of image analyzer software. * indicates statistically significant (P<0.05) levels of DNA in comet tail in drug treated cells with respect to control MCF-7 cells.*

6.3.10 Caspase assay

As shown in Fig. 6.8, the caspase 3 activity in T47D cells was found to increase by 3.5-folds as compared to control untreated T47D cells following 24 h pterostilbene exposure. On the other hand, pterostilbene could not induce caspase 3 activity in MCF-7 cells probably due to lack exon-3 (Kagawa et al., 2001). But the high induction of caspase activity in T47D breast cancer cell lines clearly showed that pterostilbene caused apoptosis due to the activation of caspases in breast cancer cells.

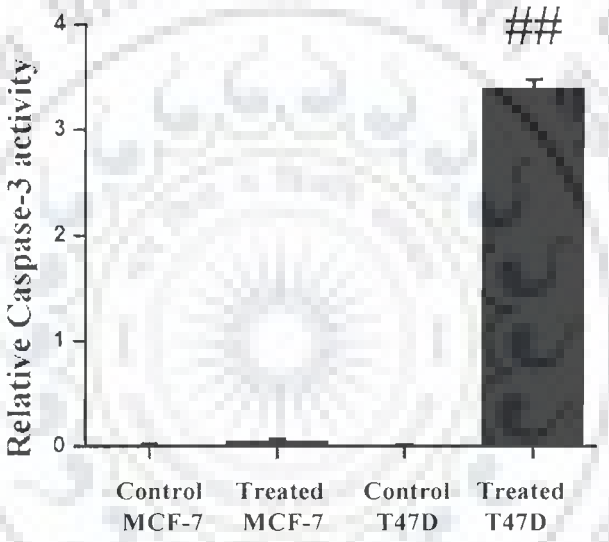


Fig. 6.8: *Pterostilbene induced relative caspase 3 activity in breast cancer cells. The experiment was performed in triplicate and mean ± S.E.M. of three independent experiments are shown here. ## indicates statistically significant (P<0.05) increase in activity with respect of control T47D cells.*

6.3.11 Scanning electron microscopy

The induction of apoptosis, leading to formation of membrane blebbings, was visualized by scanning electron microscopy of the MCF-7 cells exposed to pterostilbene for 24 h. As shown in Fig. 6.9, the cell surface morphology was found to be distorted after treatment with pterostilbene and cisplatin (marked by thin arrows). Also the cell line was found to lose intercellular attachments, a characteristic specific to either metastatic cells or cells undergoing

apoptosis. Membrane blebbings were also seen in response to both pterostilbene and cisplatin treatments (Fig. 6.9 marked by thick arrows).

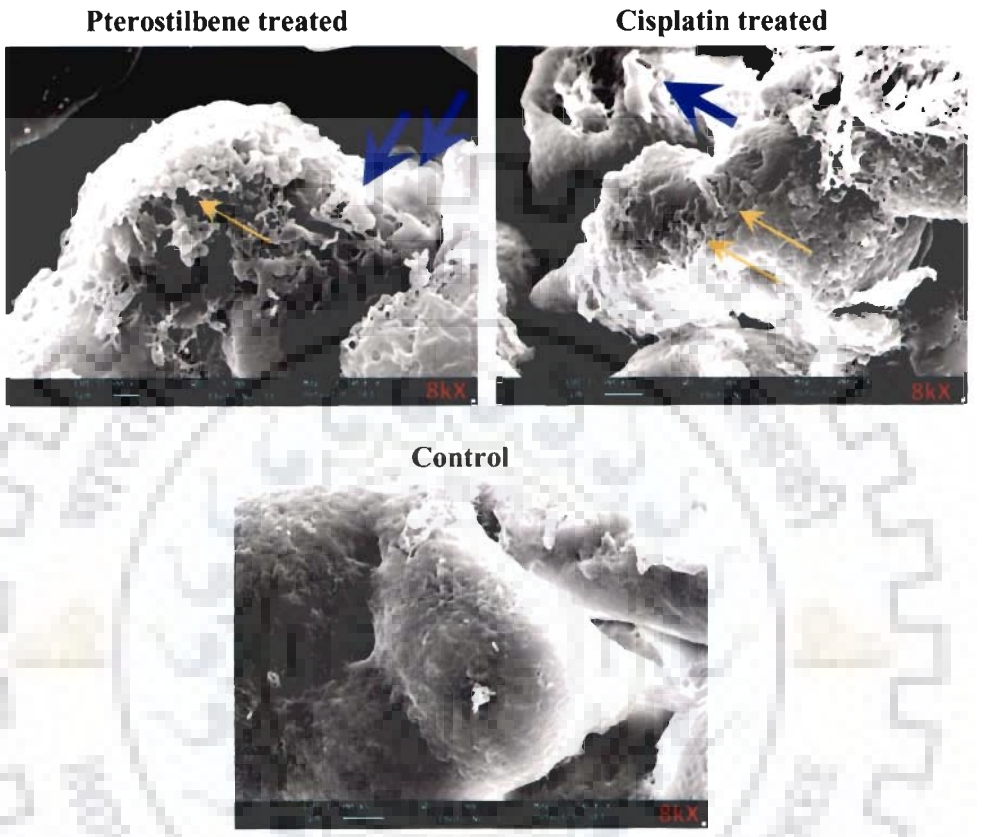


***Fig. 6.9**: Scanning electron microscopy of MCF-7 cells in presence of pterostilbene. The cells were incubated for 24 h with IC_{50} equivalent values of pterostilbene (68 μM) and cisplatin, positive control (100 μM) prior to processing. Preparation for scanning electron microscopy was as described under Materials & Methods. Blue arrows show membrane blebbings and yellow arrows represent surface distortions. Each picture is 8kx magnification. The experiment was performed in triplicate and a representative experiment is presented.*

6.3.12 Intracellular ROS levels

In order to check the probable reason for apoptosis in cancer cells on treating with pterostilbene, the intracellular reactive oxygen species was measured. Figure 6.10A shows that the production of reduced formazan increased significantly ($P<0.05$) by 8.5 folds in contrast with vehicle treated cancer cell lines which showed a low level of intracellular ROS. In order to check which type of reactive oxygen species is generated during apoptosis caused by

pterostilbene, we used free radical scavangers to quench the produced free radicals specifically. For this, 1 mM of sodium azide (scavenger of singlet oxygen) and 1 μM of catalase (H_2O_2 scavengers) could reduce the formazane production by about 2 folds (Fig. 6.10B) ($P<0.05$) but 1 mM potassium iodide (scavenger of $[OH]^-$) did not produce any significant change ($p<0.05$) in the total ROS production. H_2O_2 (1 μM) on the other hand acts as a positive control for the assay thereby increasing the total intracellular ROS production.

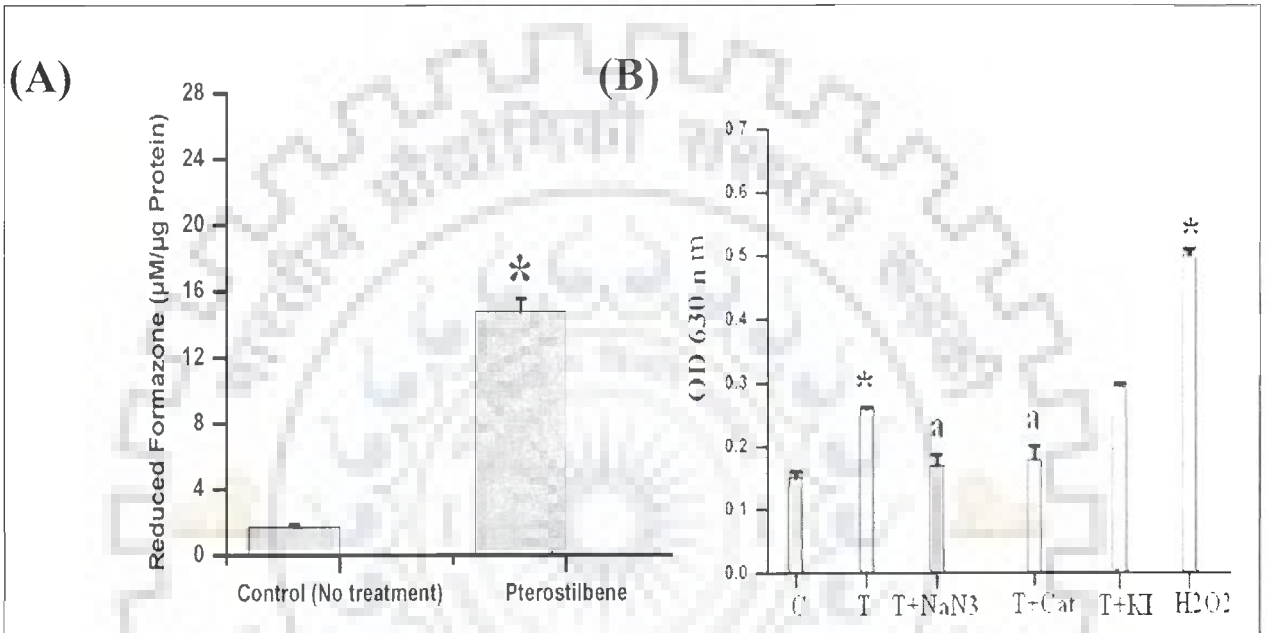


Fig. 6.10: *Production of ROS in MCF-7 cells due to treatments with pterostilbene for 24h. (A) Comparison of the production of reduced formazan between control and treated cells. (B) Inhibition of the ROS production in presence of free radical scavengers (where, C= vehicle treated control, T Treated cells with pterostilbene and 'Cat' represents treatment with catalase along with pterostilbene). * represent statistically significant difference with respect to vehicle treated control (C) and 'a' represents statistically significant difference with respect to pterostilbene treatments without scavengers (T), respectively at $P<0.05$. Values represent Mean ± S.E.M. of three independent experiments.*

6.3.13 Antioxidant enzyme profiles

When tested for the glutathione related antioxidant enzymes, the glutathione peroxidase was found to increase 1.2-folds (Fig. 6.11A) whereas there was no significant change in glutathione reductase activity. However, glutathione-S-transferase activity decreased marginally which was otherwise not significant at $P<0.05$. There was no significant change in the total glutathione level in this cell line (Fig. 6.11 B) ($P<0.05$).

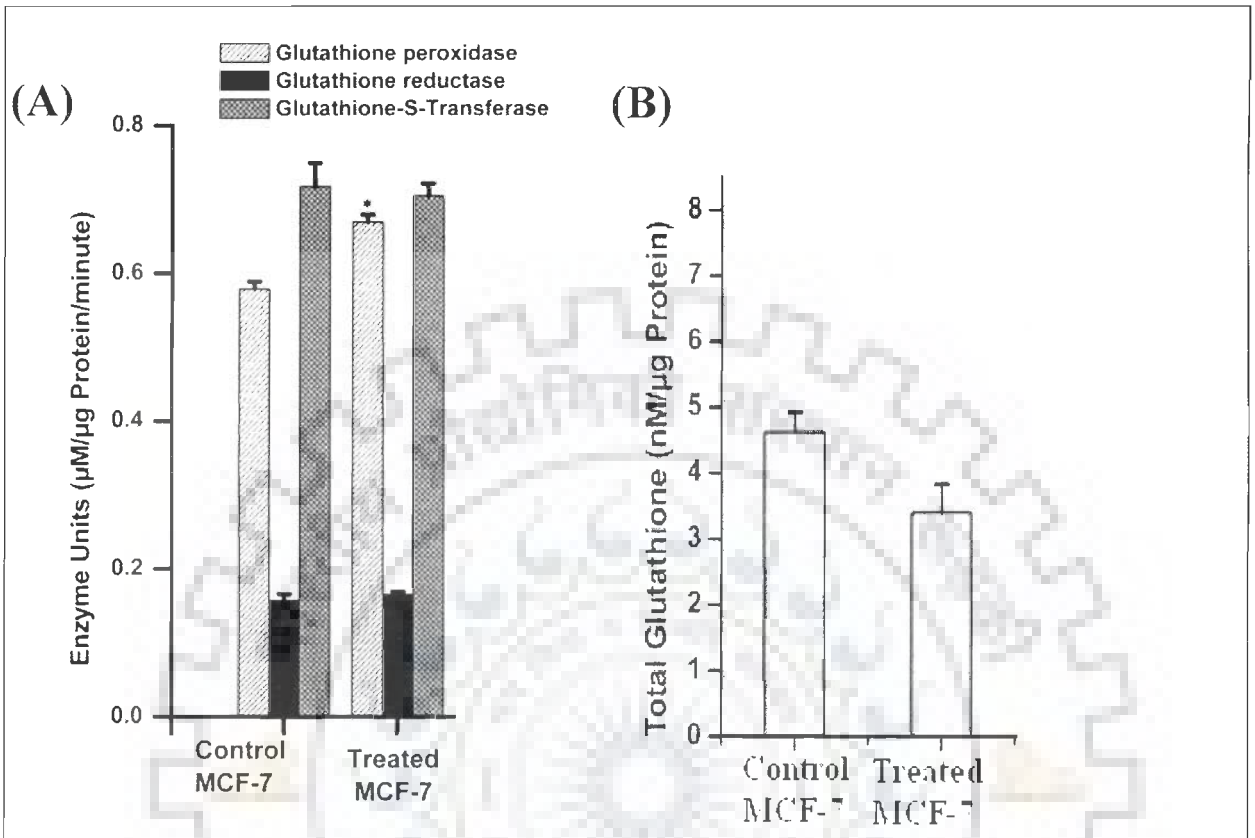


Fig. 6.11: *Antioxidant enzyme status of MCF-7 breast cancer cells treated with pterostilbene for 24h. * represents statistically significant difference in glutathione peroxidase activity in pterostilbene treated cells with respect of control ($P<0.05$). Values represent Mean ± S.E.M. of three independent experiments.*

6.3.14 Immunofluorescence of cytochrome c

The ROS status was further verified by the immunofluorescence analysis of the cytoplasmic content of cytochrome c. As shown in Fig 6.12, there was an appreciable increase in the intracellular cytochrome c due to pterostilbene treatments as compared to control.

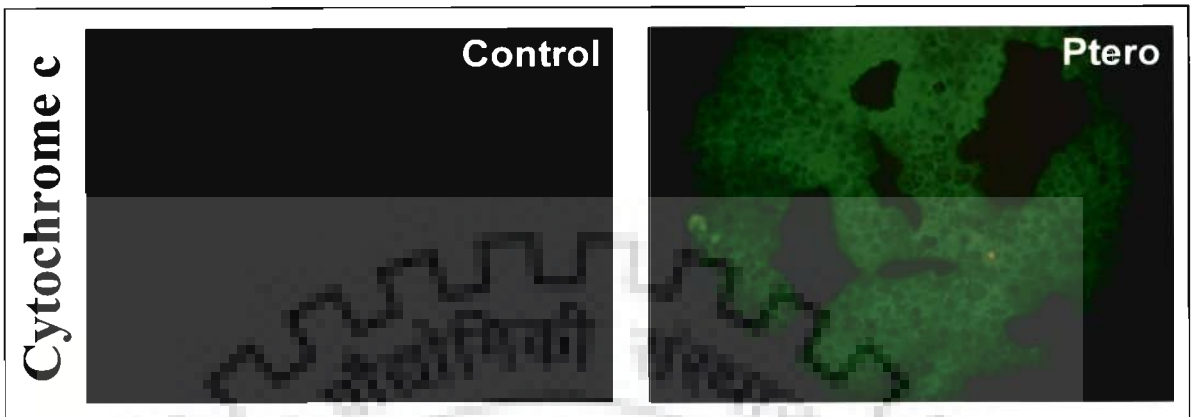


Fig. 6.12: *Pterostilbene induced increase in the cytoplasmic expression of cytochrome c. The cells are fixed and analysed according to the protocol supplied in chapter 3. The images were at 20X magnification of the objective (Ptero represents pterostilbene treated cells). The image is a representative of 3 independent experiments.*

6.3.15 Reverse transcriptase polymerase chain reaction

In order to check the effect of pterostilbene on the transcriptional status of various genes controlling cell proliferation and apoptosis, the mRNA expression was checked by semi-quantitative RT-PCR. As shown in Fig 6.13, there were approximately 2 and 2.5-folds increase in the expression of Bax and p53 respectively whereas, caspase 3 increased by only 1.7- folds. The primers were designed such that we could measure the mRNA transcript of caspase-3 downstream of exon 3 that was in general deleted in MCF-7 cells (Kagawa et al., 2001). This was further supported by 2-2.5-fold decrease in the expression of each of Bcl-2, Akt and MMP9 (Fig. 6.13). Again pterostilbene was found to decrease the expression of metastatic marker genes α -methylacyl-CoA racemase and Nkx3.1 by 1.4 -folds and 3-folds respectively in the breast cancer cell line (Fig. 6.13). The right panel (Fig. 6.13) summarizes the densitometric scanning data for the expression profiles of various genes. This result was further validated by the fact that there was an over-expression of tissue inhibitor of matrix metalloproteinase (TIMP) which is a known inhibitor of matrix metalloproteinase (MMP). Figure 6.14 shows intense cytoplasmic fluorescence of TIMP-3 in pterostilbene treated cells as compared to the control cancerous cells.

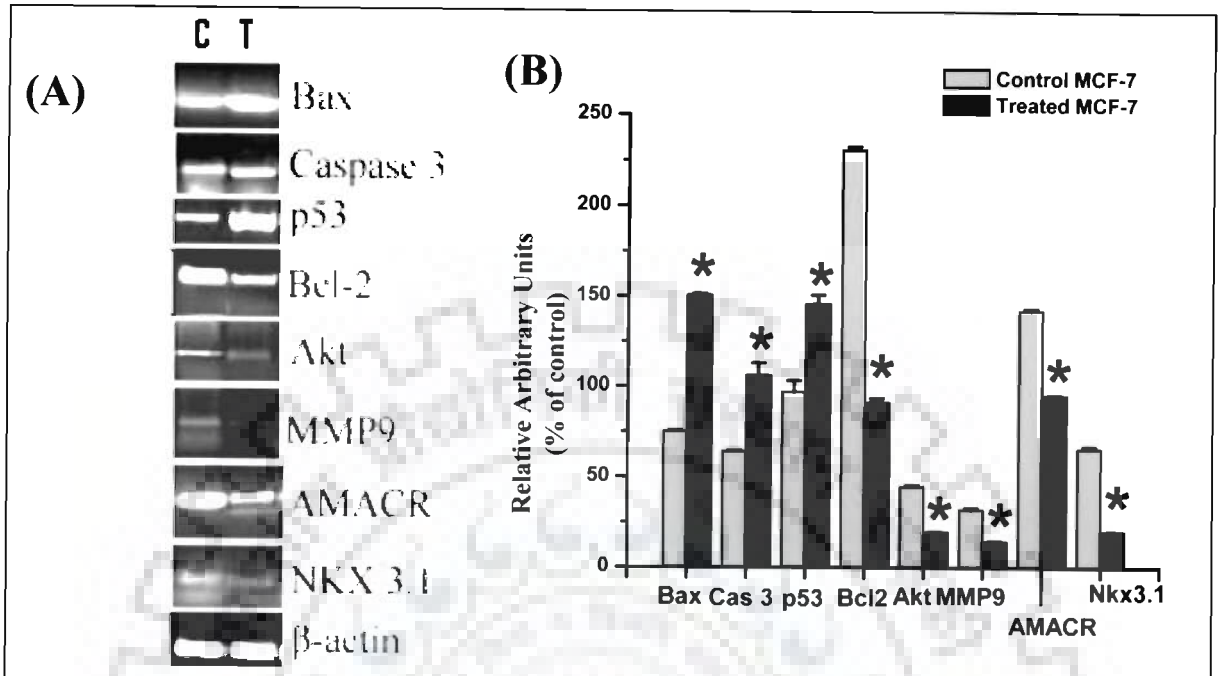


Fig. 6.13: *Transcriptional analysis of various genes in the apoptotic or the cell survival pathway in MCF-7 cells in response to pterostilbene (A). C and T represents, control and cells incubated with pterostilbene for 24 h respectively. (B) Represents relative intensities of the RT-PCR products that were quantified by densitometer and normalized against internal control β-actin. The data are expressed as Mean ± S.E.M. of 3 independent experiments. * indicates statistically significant (P<0.05) levels of gene expression in pterostilbene treated cells with respect to control.*

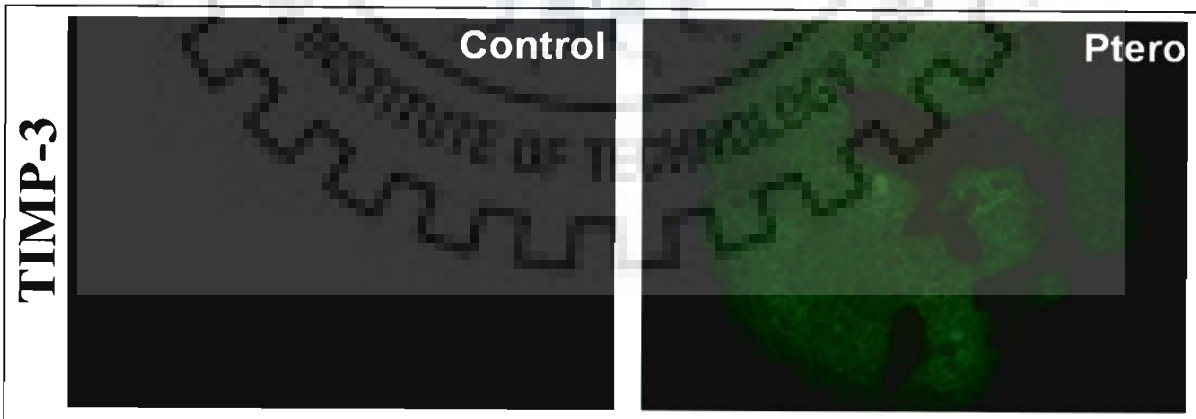


Fig. 6.14: *Effect of pterostilbene on the cytoplasmic expression of TIMP-3 proteins. The cells are fixed and analyzed according to the protocol described in chapter 3. (Ptero represents pterostilbene treated cells). The images are represented at 20X magnification of the objective. The present image is a representative of 3 individual experiments.*

6.3.17 Western Blot

The changes in the transcriptional profiles of various apoptotic genes were further reconfirmed by western blot analysis. Potent apoptosis marker genes like Bcl-2, Bax and caspase 9 were all found to be significantly altered in response to the pure phytochemical. As shown in Fig. 6.15 A, B, pterostilbene increased the expression of Bax and caspase 9 but inhibited Bcl-2 in the breast cancer cell line.

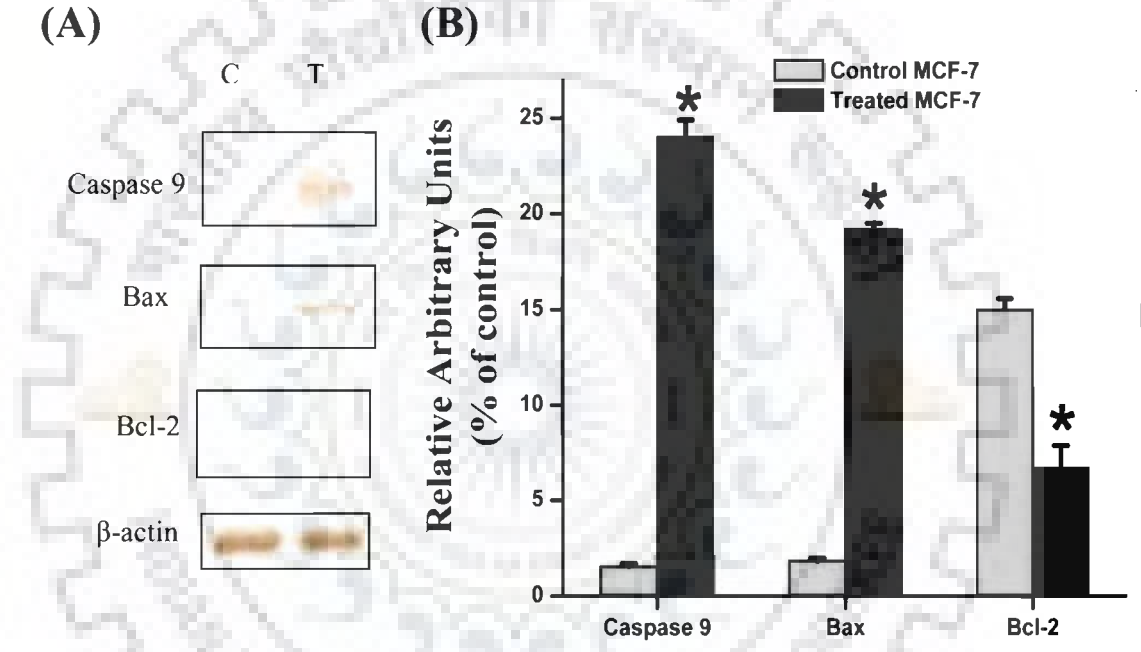


Fig. 6.15: *Effects of pterostilbene on the expression of some marker genes for tumor and apoptosis. (A) Represents the immunoblot showing the leves of Caspase 9, Bax and Bcl-2; (B) the histogram showing the mean of arbitrary pixel intensities of 3 individual experiments ± S.E.M. C and T represents, control cells and cells incubated with pterostilbene (68 μM for 24 h) respectively. * indicates statistically significant (P<0.05) levels of gene expression in pterostilbene treated cells with respect to control MCF-7 cells.*

6.3.17 Effect of pterostilbene on in vivo animal model

6.3.17.1 Pterostilbene inhibits mammary breast tumor development

Finally, pure pterostilbene was tested on *in vivo* rat breast cancer models in order to find whether the phytochemical had the potency to inhibit growth of breast cancer. When the size of breast tumor reached approximately 12 cc, pterostilbene was injected at a dose of 20 mg/kg body

weight thrice per week for 1 month. Thereafter, tumor volume at the beginning and the end of the treatment period was compared with the vehicle (negative control, Group II) and cisplatin treated (positive control, Group IV) animals. As shown in figure 6.16 the histological sections of vehicle treated breast tumors had more of highly proliferating cancer cells, whereas in pterostilbene and cisplatin treatments, the reduction in ductal carcinoma structures were observed. Under lower magnification, the light microscopic images showed that the overall ductal carcinoma cells were reduced with simultaneous increase of the apoptotic cells (Fig. 6.16) in pterostilbene treatment (group III). These apoptotic cells were further magnified in figure 6.16 and showed in the lower most panels. Moreover, pterostilbene reduced the specific growth rate of MNU induced breast tumor by ~ 50% as compared to the vehicle treated tumor and the tumor doubling time increases significantly by ~ 2 folds ($P<0.5$) (Table 6.3).

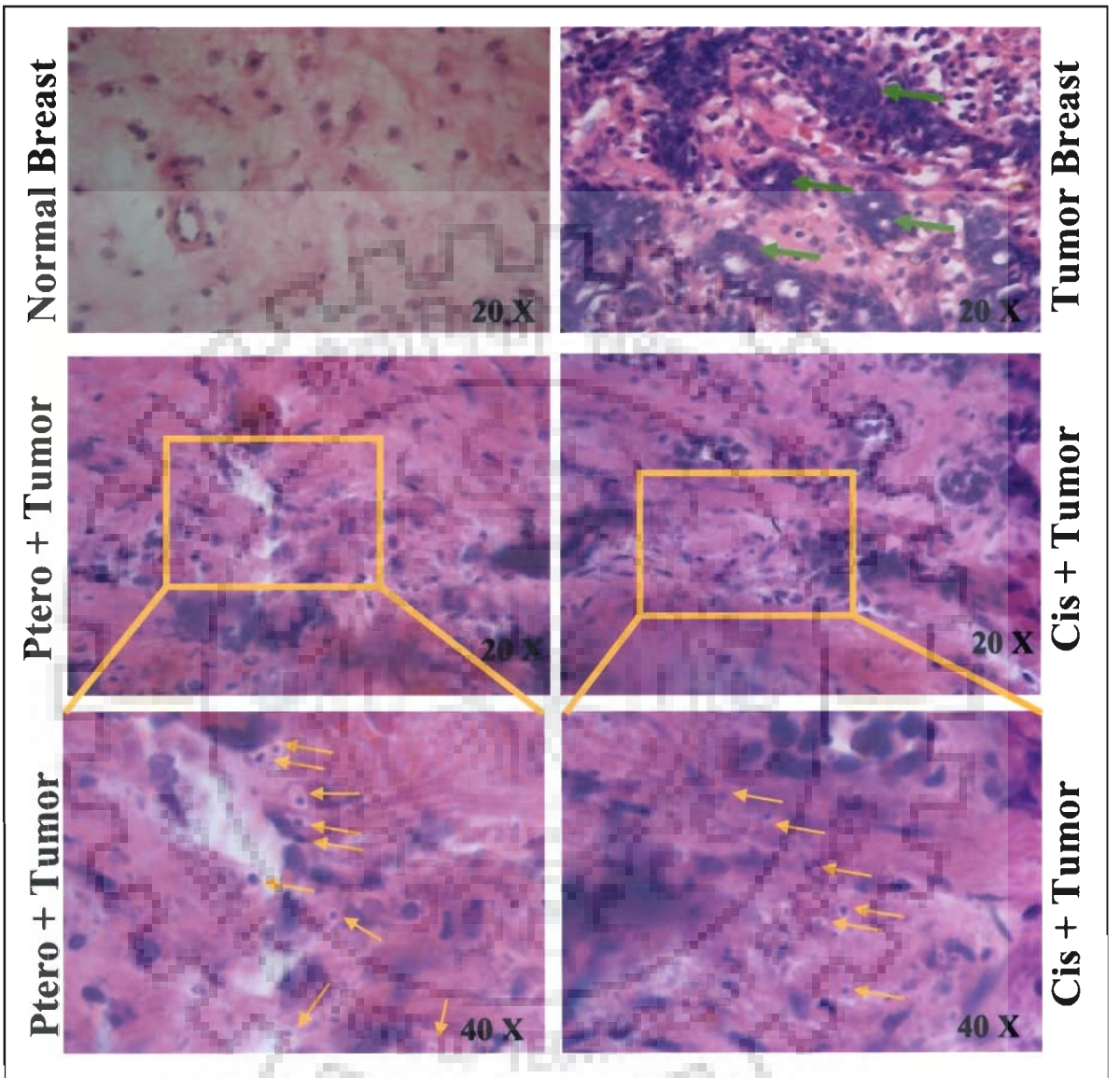


Fig. 6.16: *Histopathology of rat breast tissues stained with H & E from various groups of animals. Yellow boxes highlights the apoptotic areas in the section.' Ptero' and 'Cis' represents pterostilbene and cisplatin treated breast tumor groups respectively. Apoptotic cells showing chromatin condensation are marked by yellow arrows (40X) and the highly proliferating ductal carcinoma cells from vehicle treated groups are marked by green arrows.*

Table 6.3: Breast tumor growth inhibition by pterostilbene

Treatment	Initial tumor volume (cc)	Final tumor volume (cc) after 30 days	Specific growth rate of tumor	Tumor doubling time (in days)
Group I (vehicle treated)	ND	ND	ND	ND
Group II (vehicle treated, n=11)	11.2 ± 1.5	35.45 ± 4.5	0.046	24.45
Group III (Ptero treated, n=11)	12.6 ± 1.4	18.2 ± 2.4*	0.023	52.6
Group IV (Cis treated, n=11)	11.8 ± 1.1	25.8 ± 1.9*	0.026	26.57

ND, not detected. (n, represents number of animals/group) * respectively indicates statistically significant as compared to vehicle treated Group II animals ($P < 0.05$) after 30 days of pterostilbene treatments. Data represented as mean ±S.E.M.

6.4 Discussion

Epidemiological studies have linked multi-dimensional benefit of the use of wooden containers made up of heartwood of *Pterocarpus marsupium* as described in the Ayurvedic system of medicine (Dhanabal et al., 2006; Grover et al., 2002). Earlier, pterostilbene had been reported to be anti-proliferative against cervical and colon cancer cell lines (Suh et al., 2007) and anti-metastatic against the melanoma cells (Ferrer et al., 2005). The 3,5-dimethoxy motif at the A-phenyl ring of the general resveratrol backbone provides potential pro-apoptotic properties (Roberti et al., 2003). Due to this structural characteristic, they are even found to be more effective against the multidrug resistant leukemia cells as compared to resveratrol (Tolomeo et al., 2005). The present data showed that the purified pterostilbene was effective on MCF-7 cells even more than that of resveratrol as reported earlier (Sgambato et al., 2001). The IC_{50} value of pterostilbene as found in this study was 68.5 µM in comparison with 98 µM for pure resveratrol in MCF-7 cells. In summary, our study showed that pterostilbene was 1.5 folds more potent in MCF-7 cells as compared to resveratrol following 24 h of incubation. Longer exposure of pterostilbene had been very effective in inhibiting both the mitotic and metastatic potential of either/both ER- and PR- postitive breast cancer cell lines. A recent study on PC3 cells using synthetic pterostilbene showed that the said chemical induced apoptosis by DNA fragmentation in cancer cell lines (Pan et al., 2008b) which is in concurrent with our study with

purified pterostilbene. Phytochemicals like resveratrol is also protective against the estrogen metabolism induced DNA adduct formation (Zahid et al., 2008). Differing from resveratrol in having dimethoxy group, pterostilbene, might have enhanced efficacy than its analogue resveratrol due to its structure. It is worth mentioning here that a synthetic tetramethoxy stilbene moiety is also very effective in blocking the cancer risk in murine mammary gland (Kim et al., 2011).

Cell surface morphology of apoptotic cells changes from that of cancer cells. Though actin modulation occurs in both the cases, the membrane becomes distorted and ruptured along with the formation of apoptotic bodies (Meira et al., 2005). Scanning electron microscopy data in our study clearly showed that cancer cells in the presence of pterostilbene not only developed membrane distortions but also lost actin modulation before undergoing apoptosis. A similar effect has been reported in MCF-7cells treated with clotrimazol where the cell cycle was arrested and the cells lost their protrusions (Meira et al., 2005).

The change in the mitochondrial membrane potential was found to be an essential factor responsible for causing apoptosis (Bernardi et al., 1999). Synthetic drugs like doxorubicin (Kotamraju et al., 2000) and herbal apoptotic drugs like gingerol (Nigam et al., 2009), curcumin (Thayyullathil et al., 2008) and resveratrol (Aziz et al., 2006) are known to act by the production of reactive oxygen species which affects the intracellular mitochondrial membrane potential. There was a prominent production of reactive oxygen species which increased by 8.5 folds after incubation with pterostilbene. It was also found that pterostilbene produced more of singlet oxygen species and hydrogen peroxide molecules in the intracellular environment which further stimulated apoptosis. This finding was further validated by the enhanced immunofluorescence of cytochrome c following exposure to pterostilbene. ROS have been reported to be responsible for the bioactivity of other compounds like genistein and capsaicin (Dou et al., 2011; Ullah et al., 2011) which finally leads to apoptosis via ubiquitin-proteasome system dysfunction (Maity et al., 2010). Further, it has also been reported that the ROS scavengers inhibit genistein induced cell death in breast cancer cells (Ullah et al., 2011). The experimental data proved that singlet oxygen and/or hydrogen peroxide were the major product of ROS generation in MCF-7 cells which in turn caused apoptosis. This shows that in case of MCF-7 cells the stilbene distorts the ionic balance maintained by the mitochondria which is needed for cancer cell growth progression.

Apart from this, it was found that the antioxidant molecules, which are also responsible for stabilizing the ionic imbalances in the cells thus allowing their growth, were almost inactive in our study. Antioxidant enzymes like peroxiredoxin II, protects the cancer cells from death (Zhang et al., 1997). It has been found that although the glutathione related antioxidant enzymes are active in MCF-7 cells but these antioxidant enzymes could not protect the cells from the enhanced ROS production induced by pterostilbene. Although glutathione peroxidase increased following pterostilbene exposure in MCF-7 cells, the other antioxidant enzymatic activities were only basal.

The present data also showed an involvement of p53 as a downstream effector signal molecule. The production of ROS was directly linked to the higher expression of p53 (Liu et al., 2008). Thus it can be presumed that pterostilbene inhibited the cellular proliferation and induces apoptosis by the production of ROS in MCF-7 cells.

Based on the present data in relation to the content of reactive oxygen species and the cytoplasmic release of cytochrome c, it may be hypothesized that the apoptosis caused by this phytochemical is through mitochondrial apoptotic signal transduction pathway. This was proved in this study by the inhibition of expression of Bcl-2 and induction of expression of Bax at the transcriptional and translational level by pterostilbene. Similar reports were also found in human gastric carcinoma cells (Pan et al., 2007). The anti-fungal effect of pterostilbene was also linked to its effect on the mitochondrial genes (Pan et al., 2008a). Thus it can be concluded that the generation of ROS and the differential expression of Bcl-2 family proteins are indirectly linked to the release of cytochrome c from the mitochondrial membrane. This further leads to activation of caspase and thus results in apoptosis. This apoptotic pathway is also reported in cardiomyocytes as a result of ROS and the various antioxidant molecules are known to alter this effect (Zhao et al., 2009).

It is already known that inhibitors of Akt can be used for the prevention of cancer. Moreover, hergulin a ligand of HER-2 (breast cancer signature proteins) and the TGF-β selectively increases expression of MMP-9 (Kwok et al., 2009; Xu et al., 1997), and also urinary plasminogen activator, uPA and its receptor, uPAR (Chambers et al., 2001).The latter was shown to be mediated via p38, MAPK and MEK to enhance both cell motility and invasiveness. Increased MMP-2 expression and decreased TIMP-2 were found in human mammary epithelial cells (MCF10A) transfected with H-ras, c-erbB-2, or both (Giunciuglio et al. 1995). TIMP and MMP are antagonistic in their effects (Roy et al., 2007). Our study showed that pterostilbene

causes transcriptional down-regulation of both Akt and MMP9. In support of this finding, a marked increase in the expression of TIMP-3 in the cytoplasm was also found. Resveratrol, a near homologue of pterostilbene is also known to cause apoptosis by inhibiting the cancer cell metastatic markers (Banerjee et al., 2002). Further reports suggest that intake of high amount of branched chain fatty acids in beef and dairy products have led to high expression of α - methylacyl-CoA racemase (AMACR) in 62% of hormone refractory and non-hormone refractory metastatic prostate and 44% of breast cancer lesions (Luo et al., 2002; Zhou et al., 2002). On the other hand, overexpression of p53 has been known to downregulate this invasive breast cancer marker AMACR (Chen et al., 2009a). To the best of our knowledge, the present study for the first time showed that pterostilbene inhibits the expression of (AMACR) α-methylacyl-CoA racemase with the simultaneous up-regulation of p53 in MCF-7 cells. Further, it was also found that pterostilbene inhibited another breast metastatic marker NKX3.1 which was in accordance of an earlier report on curcumin (Zhang et al., 2007). However, the result is contrary to the fact that anti-tumor genes should increase the expression of tumor suppressor genes like NKX3.1, as was shown by Bowen et al. (2000) and that the loss of expression of this gene leads to tumor progression. The present study is supported by the fact that the over expression of this protein has also been reported in prostate cancer (Xu et al., 2000; Irer et al., 2009). However, further studies are thus needed to draw any conclusive evidence for this controversy in relation to the NKX3.1 expression in MCF-7 cells.

The anticancer properties of pterostilbene were finally verified and confirmed by the *in vivo* reduction in the mammary tumor. The drastic cessation of the ductal carcinoma cells by pterostilbene and the arrest of the tumor growth by 2-folds, proved pterostilbene to be highly effective against breast cancer.

In conclusion, the present study demonstrates that pterostilbene plays an important role in the apoptosis of breast cancer cell lines by increasing the reactive oxygen species and release of cytoplasmic cytochrome c. Pterostilbene induces the expression of apoptotic genes and at the same time down-regulates few major metastatic target molecules MCF-7 cancer cell lines. It induces apoptosis mainly via the mitochondrial pathway. Apoptosis induced by mitochondrial pathway sometimes lead to the inhibition of ubiquitin-proteasome system as in case of curcumin or aspirin (Dikshit et al., 2006; Jana et al., 2004). The present study therefore opens up new opportunities to investigate more on the link of ROS, mitochondrial and the proteasome system in future. Further, pterostilbene probably affects the branched chain fatty acid metabolism in

cancer cells through the decreased expression of (AMACR) α-methylacyl-CoA racemase. Pterostilbene also inhibits the cancer progression in *in vivo* rat models. All these data thus warrants further studies to understand the exact cross-talk among various apoptotic pathways to make this phytochemical as an extremely valuable anti-cancer drug.

CHAPTER 7

PTEROSTILBENE INDUCED AUTOPHAGY AND CELLULAR DIFFERENTIATION

7 Pterostilbene induced autophagy and cellular differentiation

7.1 Introduction

Our previous studies have shown that pterostilbene causes cytotoxic effects in MCF-7 breast cancer cells due to its capacity to generate reactive oxygen species (ROS) (Chakraborty et al., 2010). ROS have been mainly responsible for the bioactivity of other compounds like genistein and capsaicin (Dou et al., 2011; Ullah et al., 2011). Among these ROS, hydrogen peroxide and singlet oxygen species were subsequently produced which activated the death signaling pathways. In normal cells ROS is generated as a natural byproduct of cellular metabolism inside the mitochondrial matrix. If the balance of ROS increases more than the scavenging capacity of the intracellular antioxidant system, the cell undergoes a state of oxidative stress with significant impairment of cellular structures (Stadtman, 1992). Oxidative stress can lead to the auto-oxidation of sterols (Murphy and Johnson, 2008; Payre et al., 2008) thereby affecting the cholesterol biosynthetic pathway mainly the postlanosterol derivatives (Medina et al., 2009). The intracellular accumulation of oxysterols directs the cell to its autophagic fate and may also induce it to differentiate (Medina et al., 2009; Payre et al., 2008). This phenomenon was reported earlier by two different groups where tamoxifen and N-pyrrolidino-(phenylmethyphenoxy)-ethanamine-HCl (PBPE), a tamoxifen metabolite, have been reported to show differentiation of the MCF-7 human breast cancer cells to more of normal epithelial cell like morphology with accumulation of large human milk globulin proteins. The differentiated breast cancer cells tend to lose their clonal growth potential (Elstner et al., 1998). In a mutual way, it was found that in the presence of fatty acids like docosahexaenoic acid and eicosapentaenoic acid, the synthesis of lipid increases within the breast cancer cells (Chamras et al., 2002). Although the cholesterol lipid rafts are involved in cell proliferation (Simons and Toomre et al., 2000), the membrane lipids phosphatidylinositol-3-phosphate (PtdIns3P), generated by class III PI3K, functions in tumor suppression (Stenmark, 2010). Lipids thus have differential effect on cancer development and cell death. The PPARγ ligands (troglitazone and all-trans retinoic acid) alter the intracellular lipid machinery and inhibit cell proliferation in breast cancer cells due to dramatic decrease of the bcl-2 protein levels only in carcinoma tissues (Elstner et al., 1998). The role of lipids in the process of differentiation in breast cancer cells has been further supported by the studies of O'Rourke et al, (1997) which showed that growth arrest induces the lipogenic markers, CCAAT/enhancer binding protein (C/EBP) isoforms, in mouse mammary epithelial cells.

The ABC transporters are important ATPases which are present in different organisms starting from Mycobacterium (Banerjee et al., 1998; Chakraborti et al., 1999; Sarin et al., 2003) to human. In human they are responsible for efflux of oxysterols extracellularly (Baldan et al., 2006; Rudolph et al., 2007). Not only the growth in MCF-7 cancer cells is inhibited by differentiation, the ROS producers like tamoxifen and its metabolite PBPE have been found to inhibit MCF-7 proliferation by inducing self-catastrophic phenomenon of autophagy (Medina et al., 2009).

In the present chapter of this thesis, an attempt was made to link the ROS mediated effect of pterostilbene to control the growth of MCF-7 breast cancer cells through the differentiation and autophagy. The specific aim of this study was to determine the role of ROS in the production of oxysterol and the effects on MCF-7 cell proliferation. The data showed that pterostilbene induced ROS participates in the intracellular lipid accumulation and autophagy.

7.2 Brief experimental protocols

7.2.1 Cell lines

In the present study MCF-7 breast cancer cells were used as the in vitro model to check the autophagy and differentiation inducing properties of pterostilbene. The culture conditions have been described in the 'materials and method' section. The cell culture medium was changed every day in this study to avoid nutrient deprivation to the cells which is otherwise responsible for autophagy.

7.2.2 Dose and duration of exposure of test compounds

MCF-7 cells were constantly exposed to pterostilbene for 72 h at a concentration of 30 μM which was lower than its IC_{50}. Tamoxifen (2.5 μM) and estradiole (1 nM) served as the positive and negative control for the following experiments.

7.2.3 Extraction of intracellular sterols

In order to characterize intracellular sterol molecules accumulated due to pterostilbene, the sterols were extracted followed by their analysis by TLC. Detailed protocols have been given in the methodology section (chapter 3).

7.2.4 Gas chromatography-mass spectroscopy

The sterols thereby isolated were analyzed by GC-MS according to the protocol described by Kedjouar et al. (2004) and Keller and Jahreis (2004) with slight modifications. The lipids were then derivatized by addition of 50 µl of pyridine and 50 µl of BSTFA at 60 °C for 30 min and analyzed by GC-MS. The sterols extracted were dissolved in 10 µl of methanol and 2 µl of the sample was injected into GC- MS (Perkin-Elmer Clarus 500 GC and Perkin-Elmer Clarus 500 MS, Massachusetts, USA) fitted with GC capillary column of 30 m length, 0.32 mm i.d., 0.1 µm, df (Elite-1, Perkin-Elmer, Massachusetts, USA) and the mass detector was operated at 70 eV. The GC temperature program was as follows : oven temperature was about 60 °C during the injection, after 3 min it was rapidly increased to 200 °C, and was then programmed from 200 to 250 °C at a rate of 3 °C/min and from 250 to 300 °C at a rate of 6 °C/min. Compound identifications were confirmed by both GC coupled mass spectrometry (GC-MS) and the comparison of GC retention times with those of available standard sterols. Sterols not represented by a visible GC peak were considered to be of negligible importance.

7.3 Results

7.3.1 Effect of pterostilbene induced ROS production is inhibited by catalase

As seen in chapter 6, pterostilbene induced cytotoxic effects on MCF-7 cell lines which may be linked to the production ROS, it was therefore necessary to check whether the ROS scavengers played any protective role against the cytotoxicity caused by pterostilbene. Hence the effects of catalase, a potent ROS (H_2O_2) scavenger, were evaluated on the pterostilbene induced cytotoxicity at varying concentrations and time points. Pretreatment of MCF-7 cells with catalase (400 U/ ml) increased the cell survival ratio in comparison to that of only pterostilbene as determined by MTT assay (Table 7.1). A maximum increase of 60% cell viability was observed at higher concentrations (30 and 50 µM) and long exposure (72 h) ($P< 0.001$). From the present data it could be probably presumed that pterostilbene generates ROS which is in turn inhibited at least in part by catalase.

Table 7.1: Effect of catalase on the viability of MCF-7 cells after pterostilbene treatment at different doses (μM) time points (h).

Dose (μM) / Time of exposure (h)	**Mean % live cells** (*Pterostilbene*)	**Mean % live cells** (*Pterostilbene + Catalase 400U/ml*)	**Increase in % of viable live cells** (*Difference between Pterostilbene treatment and Pterostilbene + Catalase treatment*)
50 /24	61.46 ± 2.00	74.12 ± 3.34**	12.65 ± 5.31
30 /24	72.96 ± 0.86	79.43 ± 0.57*	6.47 ± 1.32
25 /24	81.64 ± 0.81	83.52 ± 0.49*	1.88 ± 1.29
15 /24	92.55 ± 1.33	94.82 ± 1.18*	2.27 ± 2.49
50 /48	9.00 ± 0.64	20.26 ± 0.44**	11.26 ± 0.81
30 /48	33.79 ± 1.34	73.06 ± 0.60*	39.27 ± 1.91
25 /48	39.06 ± 0.87	86.96 ± 0.92**	47.89 ± 1.50
15 /48	79.41 ± 2.57	95.97 ± 1.84**	16.55 ± 3.98
50 /72	9.77 ± 0.73	70.07 ± 0.30**	60.29 ± 1.03
30 /72	24.47 ± 1.71	82.71 ± 0.76**	58.24 ± 2.44
25 /72	68.86 ± 0.85	92.80 ± 0.85**	23.94 ± 1.51
15 /72	89.11 ± 0.70	105.34 ± 5.14*	6.22 ± 4.47

The data represent the average from independent triplicate experiments (±S.E.M.). * and ** represents statistically significant difference with respect to only pterostilbene treatments at $P<0.05$ and $P<0.01$ respectively for respective treatment groups.

7.3.2 Pterostilbene induced accumulation of intracellular neutral lipids and autophagic vacuoles

It has already been reported that low doses of various small molecules like tamoxifen that generates ROS, oxidize the sterol metabolites and accumulate neutral lipids, also induce milk fat globulins in MCF-7 cells (Payre et al., 2008). Based on this report, it was interesting to

to check the accumulation of intracellular neutral lipids or sterols at sub-acute concentrations in response to pterostilbene. It has been reported earlier that the neutral lipids are stained by ORO (Vosper et al., 2001). As shown in figure 7.1, both pterostilbene and tamoxifen increased the accumulation of neutral lipids within the cells after 72 h of continuous exposure of MCF-7 cells.

Fig. 7.1: *Accumulation of neutral lipids in MCF-7 cells treated with pterostilbene for various time points. Morphological changes of ORO-stained cells as observed by light microscopy (10X objective magnification). There is a gradual accumulation of the red coloured stain with time. The data is a representative of 4 individual experiments.*

The induction of these neutral lipids by pterostilbene was inhibited by a simultaneous exposure of 400 U/ml of catalase (Fig. 7.2) thereby proving that ROS plays a significant role in the induction of neutral lipids by pterostilbene.

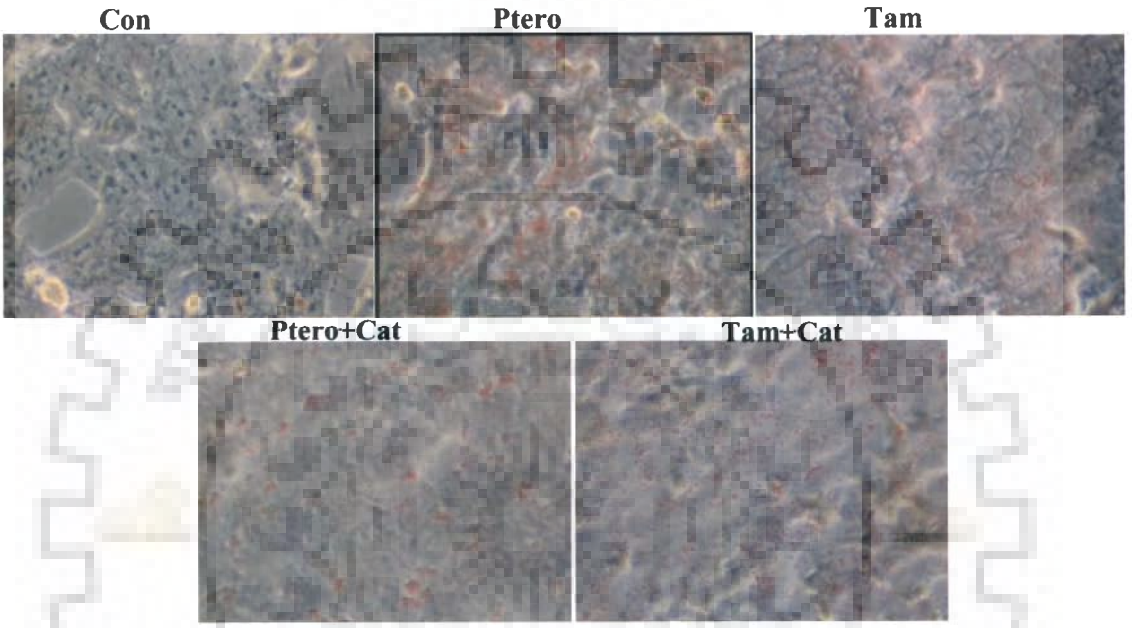


Fig. 7.2: *Accumulation of neutral lipids in MCF-7 cells in presence or absence of catalase. Cells were exposed to pterostilbene or tamoxifen for 72 h. Morphological changes of ORO-stained cells as observed by light microscopy (20X objective magnification). Con, control MCF-7; Ptero, pterostilbene treated; Tam, tamoxifen treated; Ptero + Cat represents pterostilbene treatment in presence of catalase; Tam + Cat denotes tamoxifen treatment with simultaneous addition of catalase to the culture media.*

The accumulation of the neutral lipid could be detected significantly ($P<0.05$) after 48 h of treatment (Fig. 7.3 A). The present data was further linked to the accumulated cellular triglyceride which increased to 2.3 -folds as compared to control after 72 h (Fig. 7.3 C) ($P<0.05$).

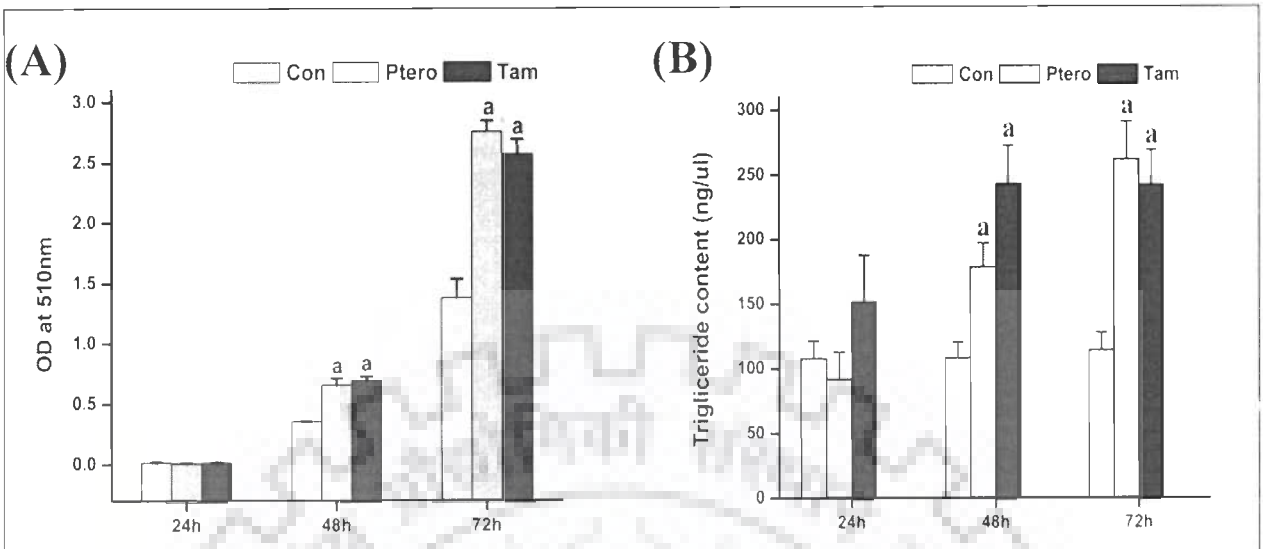


Fig. 7.3: *(A) Estimation of intracellular neutral lipids by quantifying ORO stain spectrophotometrically; (B) Estimation of cellular triglyceride content. a represents statistically significant difference with respect to control MCF-7 cells at respective time points ($P<0.05$). The data represent the average from independent triplicate experiments (±S.E.M.). Con, vehicle treated; Ptero, pterostilbene treated; Tam, tamoxifene treated.*

Since pterostilbene treatment resulted in the accumulation of triglycerides and lipid droplets maximally at 72 h (as shown earlier) it was obvious to examine if there were any changes in the cellular ultra-structures. Interestingly, transmission electron microscopy of the treated cells clearly showed accumulation of unilamellar and multilamellar autophagic vesicles after 72 h of treatment by tamoxifen and pterostilbene (Fig. 7.4). However, these vesicles were completely absent in untreated (control) and estrogen treated cells. These autophagic vesicles could not be detected before 72 h and hence it could be concluded that this is the optimum time required for the formation of these structures.

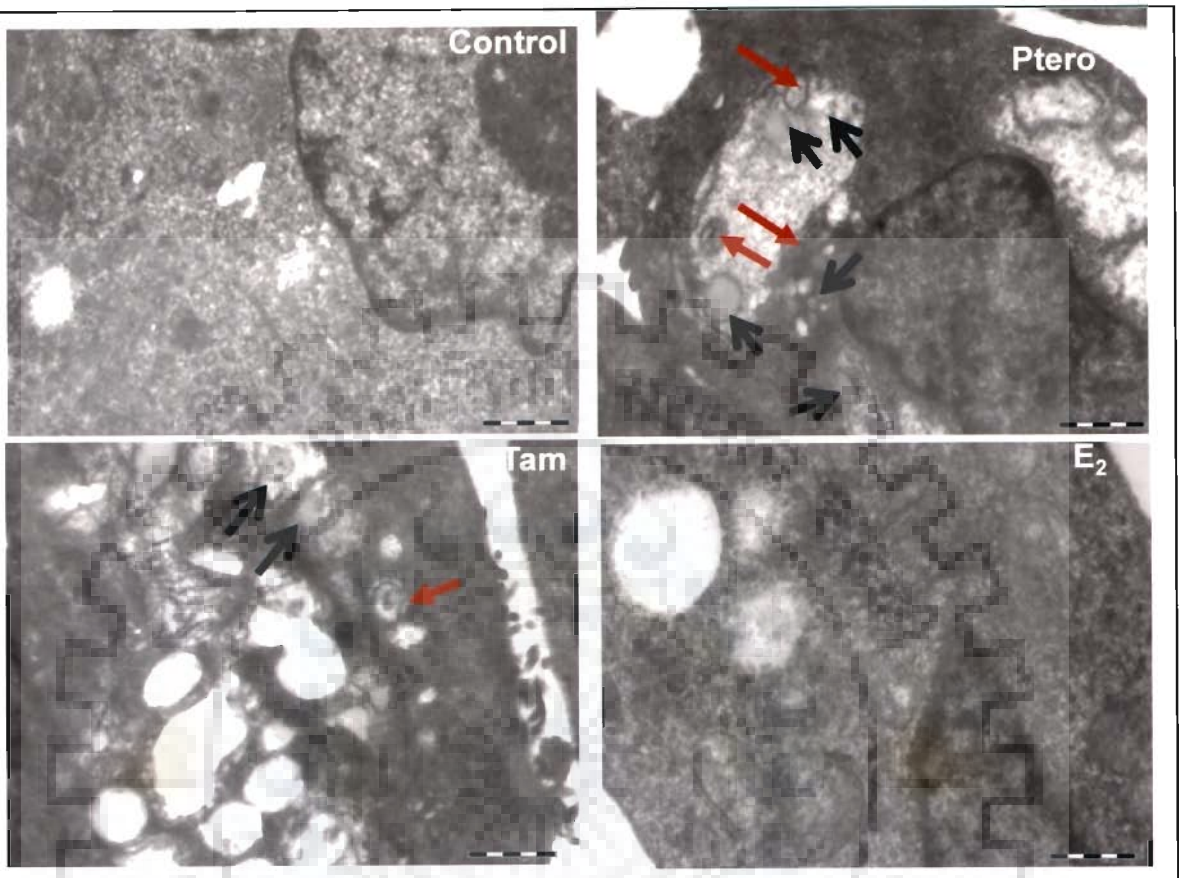


Fig. 7.4: *Transmission electron micrograph of MCF-7 cells showing accumulation of autophagic vacuoles on treatment with pterostilbene and tamoxifen. Black and red arrows mark the unilamellar and multilamellar vescicles respectively. The image is a representative of three independent experiments performed. Bar represents 1 μm. Ptero, pterostilbene treated; Tam, tamoxifen treated; E_2, estradiol treated.*

Given that pterostilbene and tamoxifen induced formation of autophagic vacoules in MCF-7 cells, it was interesting to evaluate whether the autophagic proteins, Beclin 1 and LC3, were also expressed simultaneously. As shown in figure 7.5 A, both Beclin 1 and LC3 accumulated within the MCF-7 cells after 72 h of incubation with pterostilbene and tamoxifen. Beclin increased by 2.2 and 2-folds in pterostilbene and tamoxifen treated cells respectively, whereas LC3 II increased by 1.8 and 2.4 folds respectively as compared to untreated cells (Fig. 7.5 B) ($P< 0.01$). In the next phase it was important to establish the time dependent initiation of the expression of autophagic proteins (Beclin and LC3) following continuous exposure with pterostilbene. As shown in figure 7.5 C, Beclin and LC3 proteins were expressed as early as 12h

with gradual progress in their expression with time and reaching maximal at 36 to 48 h after which it saturated with no further increase.

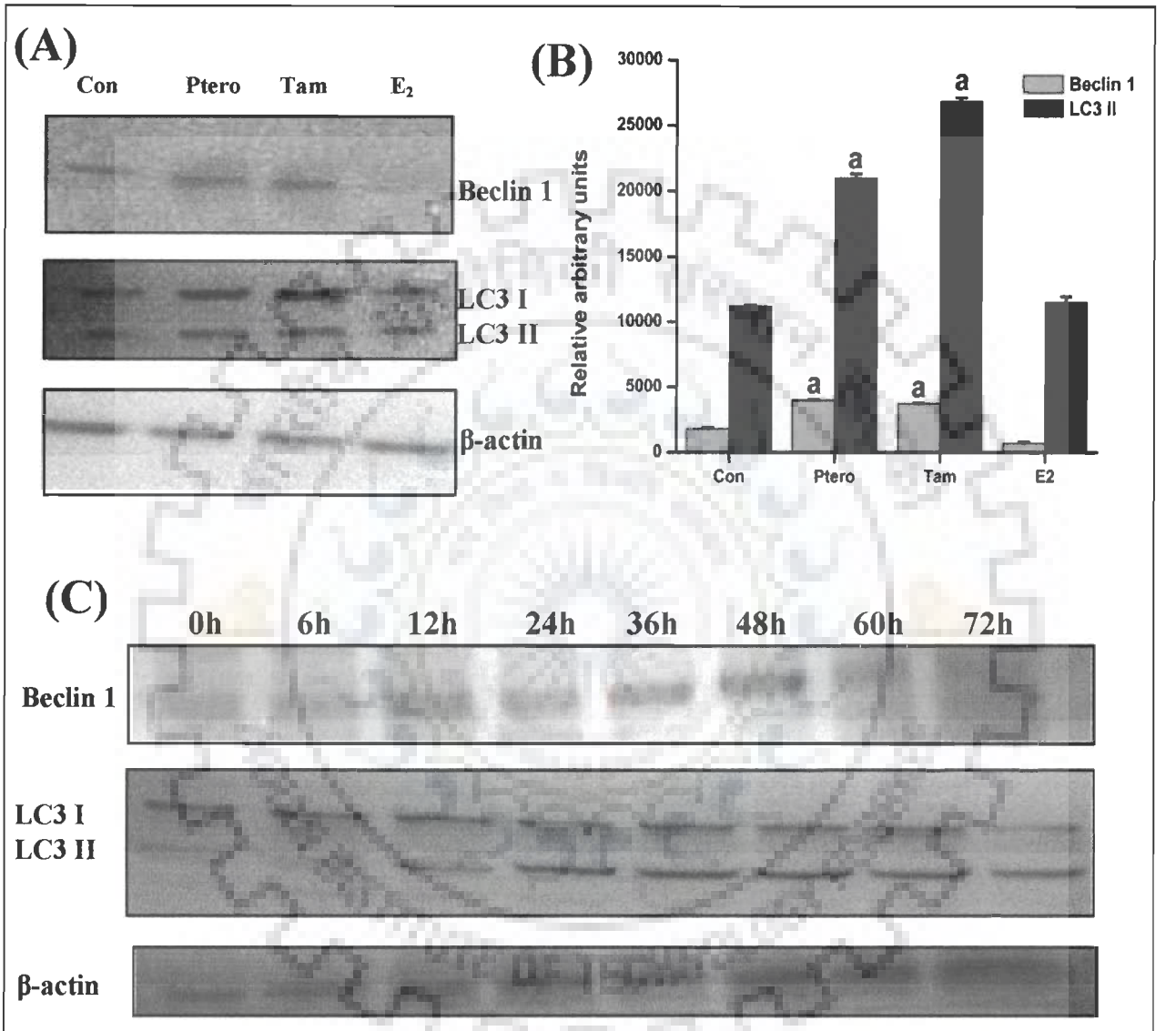


Fig. 7.5: *Effect of pterostilbene on the expression of two prominent autophagic marker proteins. (A) Western blot analysis of MCF-7 cells demonstrating pterostilbene induced autophagy at 72 h and (C) time dependent expression of the autophagic marker proteins. (B) Represents the histogram of the mean of arbitrary pixel intensities of the bands analyzed in (A) as measured by Image J software. a indicates statistically significant with respect to vehicle treated groups for respective marker proteins ($P<0.05$). The data represent the average from independent triplicate experiments (±S.E.M.). Con, vehicle treated; Ptero, pterostilbene treated; Tam, tamoxifene treated; E_2, estradiol treated.*

In order to check whether these autophagic proteins were neosynthesized, their expression pattern was checked in the presence of both transcriptional and translational

inhibitors (actinomycin D and cycloheximide respectively). It is worth mentioning here that both cycloheximide and actinomycin D completely abolished the pterostilbene induced accumulation of neutral lipids (Fig. 7.6 A) and the expression of autophagic proteins Beclin and LC3 (Fig. 7.6 B), thereby showing that new transcription and translation are required for induction of autophagy mediated by pterostilbene.

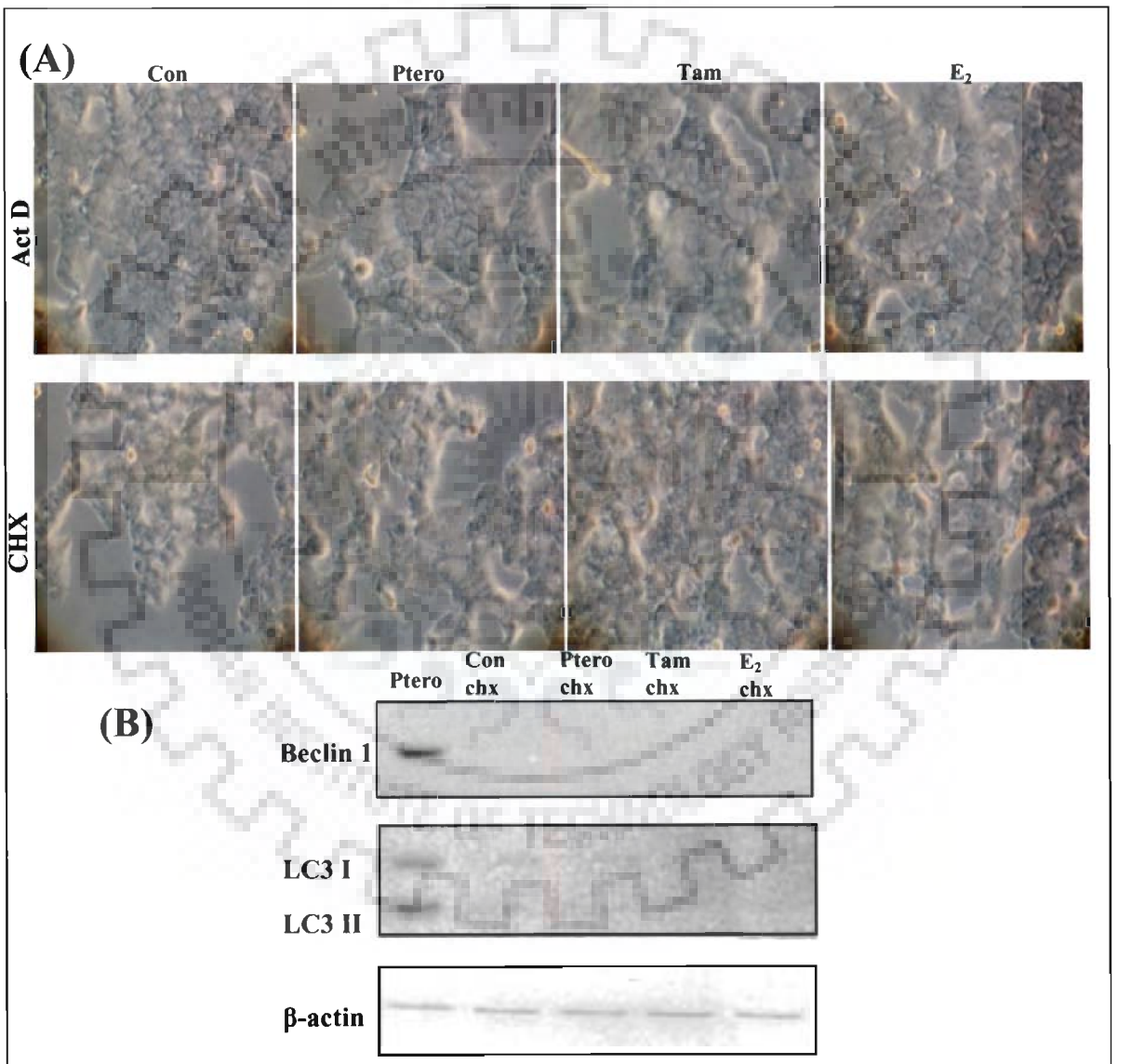


Fig. 7.6: *Effects of transcription and translation inhibitors on the autophagic process of MCF-7 cells in response to pterostilbene. Effects of actinomycin A (Act D) and cycloheximide (CHX) on the accumulation of (A) lipid droplets and (B) expression of autophagic proteins. Beclin 1 and LC3 in response to pterostilbene. Con, vehicle treated; Ptero, pterostilbene treated; Tam, tamoxifen treated; E_2, estradiol treated.*

7.3.3 Catalase induced inhibition of autophagy and differentiation of MCF-7 cells

When enzyme antioxidant, catalase, was added to the culture media 3h prior to the addition of pterostilbene, a significant reduction in both autophagy and differentiation was observed. Catalase inhibited the autophagosome formation as evidenced from the significantly reduced expression of Beclin and LC3 (Fig. 7.7 A). As shown in figure 7.7 B, when the concentrations of catalase was 100, 200, 300 and 400 U/ml in media the expression of Beclin reduced successively to 35, 22, 21and 8% with respect to pterostilbene treatments without catalase ($P<0.01$). In case of LC3, the expressions decreased to 19, 15, 13 and 7% with the gradual increase in the concentration of catalase in the culture media ($P<0.01$). Therefore, at a concentration of 400 U/ml catalase the autophagy was completely obliterated which is in accordance to the previous result where more than 60% viable cells were found in pterostilbene and catalase combined treatment as compared to the cells with only pterostilbene treatment (Table 7.1). Similar trend was observed in case of differentiation of the MCF-7 cells which was marked by the accumulation of neutral lipids. Accumulation of lipid droplets decreased by 48, 38, 21 and 3% respectively with increase in the concentrations of catalase (Table 7.2) ($P<0.01$). These results clearly showed that in the absence of neutral lipids due to the inhibition of ROS within the cells, the chronic effects of pterostilbene in breast cancer cells was abolished.

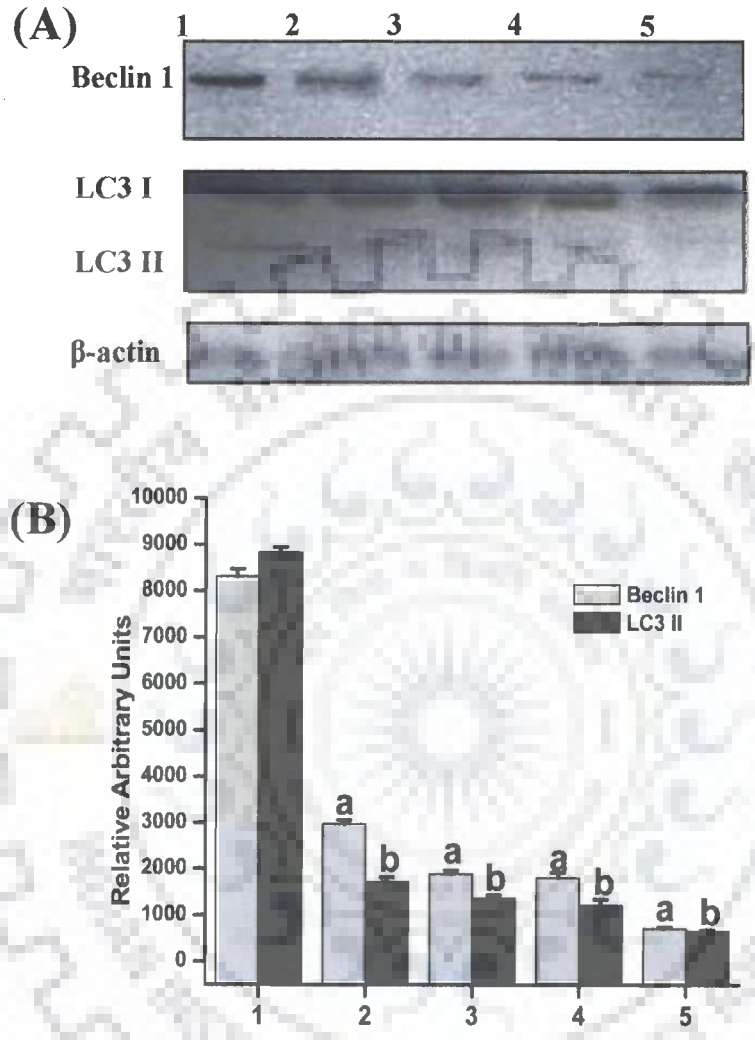


Fig. 7.7: *(A) Effect of catalase on pterostilbene induced autophagy in MCF-7 cells as shown by immunoblot analysis of autophagic marker proteins; (B) The histogram representing the mean of arbitrary pixel intensities of the bands as measured by Image J software. a and b indicates statistically significant ($P<0.01$) with respect to pterostilbene treated respective groups without catalase. The data represent the average from independent triplicate experiments (±S.E.M.). 1,2,3,4 and 5 represents 0, 100, 200, 300 and 400 U/ml of catalase respectively in media.*

Table 7.2: Inhibition of accumulation of lipid droplets by catalase in MCF-7 cells as determined by quantitative estimation of ORO stain.

Treatments	Mean OD_{510}nm	Percentage of decrease
Pterostilbene	1.929 ±0.12	--
Pterostilbene + 100 U/ml Catalase	0.937 ± 0.03*	48
Pterostilbene + 200 U/ml Catalase	0.744 ± 0.009*	21
Pterostilbene + 300 U/ml Catalase	0.423 ± 0.02*	21
Pterostilbene + 400 U/ml Catalase	0.0706 ± 0.001*	3

*The data represent the average from independent triplicate experiments (±S.E.M.). * represents statistical significant difference at (P<0.01) in comparison to pterostilbene treatment without catalase.*

7.3.4 Induction of morphological and biochemical differentiation by pterostilbene

We next evaluated the morphological changes in MCF-7 cells in response to pterostilbene at concentrations that caused complete growth arrest (30 μM). The untreated cells were round with scanty eosinophilic cytoplasm and indistinct cell margins, large basophilic nuclei and prominent nucleoli, characteristic of carcinoma cells (Fig. 7.8). While the cells treated with pterostilbene underwent significant changes with increase in size, reduced nucleus, flattened shapes and the originally round cells appeared more columnar and had distinct cellular boundaries (Fig. 7.8). The increase in cell size was predominantly attributable to an abundance of cytoplasm, which led to a decrease in the nuclear: cytoplasmic ratio.

7.3.5 Production of intracellular oxysterols and its effect on cholesterol metabolism

Transcriptional analysis showed that there was a significant variation in the expression of cEBPα (adipogenesis marker), oxysterol binding protein related homologue (ORP1L), the orphan receptor LXR, DHCR-7 (the cholesterol neosynthetic enzyme, Eacker et al., 2008), the ABC transporters and death effecter, CHOP. As shown in figure 7.9 A, pterostilbene induced significant increase in c/EBP and ORP1L respectively with simultaneous decrease in DHCR-7 levels. Similar effect is also found in case of tamoxifen. Previously it was reported by Wang et al. (2005) that oxysterols are involved in cholesterol metabolism due to its regulation of LXR.

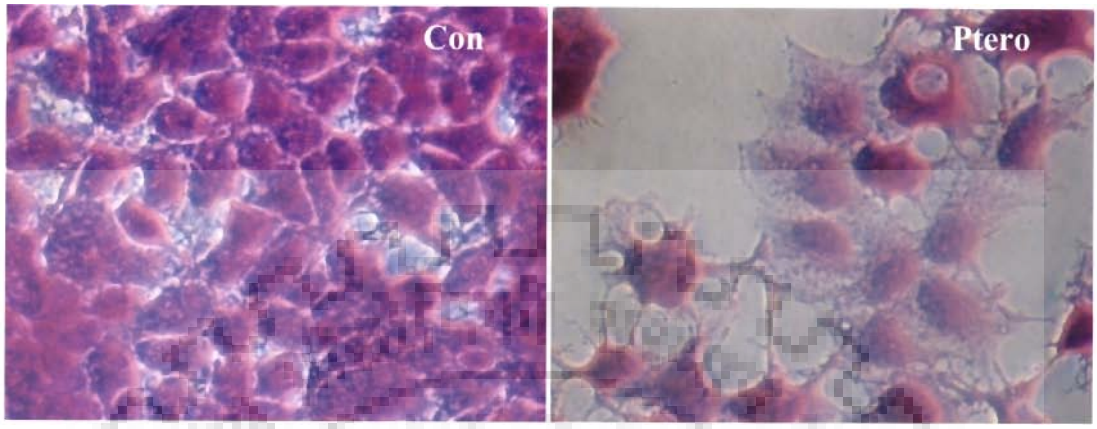


Fig. 7.8: *Morphological changes in pterostilbene treated cells as shown by haematoxylin staining (40X objective magnification). Con, control; Ptero, pterostilbene.*

We also found that the orphan receptor LXR was upregulated in the treated cells (both by pterostilbene and tamoxifen) as compared to the control (untreated) MCF-7 cells (Fig. 7.9 A). Further, it was found that the enzyme DHCR-7, which converts 7-dehydrocholesterol to cholesterol in the final step of cholesterol biosynthesis was also significantly inhibited by pterostilbene. Since the production of these oxysterols is linked to the expression of intracellular transporter proteins the expression patters of two prominent such transporters, ABCA1 and ABCG1 were also analyzed. As shown in figure 7.9 B, ABCA1 increased by about 2.2-folds and ABCG1 increased by 10-folds as compared to control after pterostilbene treatment ($P<0.05$).

Further a marked increase in death effecter protein i.e., CHOP, was found in the ptesrostilbene treated cells (Fig. 7.9 A). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum which in this case may be due to oxysterols.

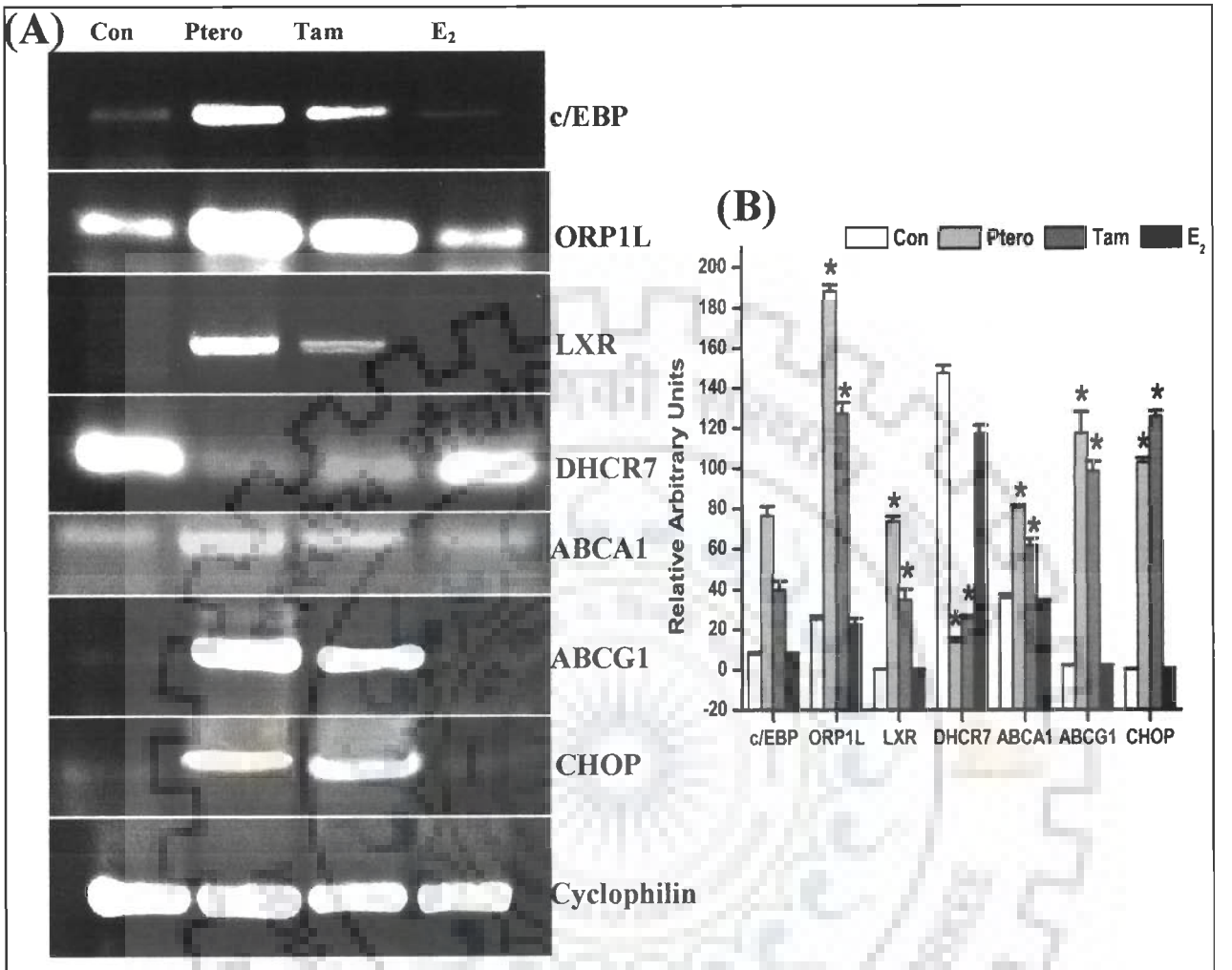


Fig. 7.9: *(A) Transcriptional analysis of the genes that are involved in cholesterol metabolism pathway, an adipogenic differentiation and a cell death marker as determined by reverse transcriptase PCR. (B) Histogram representing the mean of arbitrary pixel intensities of the bands as measured by gel documentation system. * indicates statistically significant ($P<0.05$). The data represent the average from independent triplicate experiments (±S.E.M.). Con, vehicle treated; Ptero, pterostilbene treated; Tam, tamoxifene treated; E_2, estradiol treated.*

7.3.6 Effect of PI3K and MEK inhibitors on pterostilbene induced autophagy and differentiation

In order to check the probable pathways which might be involved in the regulation of Beclin and LC3 in MCF-7 cells, they were pretreated with wortmanin (PI3K inhibitor, which also inhibits Beclin mediated autophagy) and PD98059 (MEK inhibitor) in the presence or absence of pterostilbene. MEK acts upstream of ERK which is responsible for the small molecule mediated autophagy. As shown in figure 7.10 A, pterostilbene resulted in significantly reduced expression for both Beclin 1 and LC3 II when they were pre-treated with wortmanin and PD98059 which showed that autophagy caused by pterostilbene is blocked. However, surprisingly, the expression of LC3 I was unaltered in the presence of both the inhibitors.

With the down regulation of the two prominent autophagic proteins (Beclin and LC3) the autophagy was also down-regulated even in the presence of pterostilbene. As shown in figure 7.10 B, the accumulation of autophagic vesicles were significantly reduced in both wortmanin and PD98059 treated cells. In spite of the fact that autophagy was inhibited, wortmanin and PD98059 increased the differentiation of the MCF-7 cells as evidenced by the estimation of ORO staining of the treated cells (Fig. 7.11). There was about 1.4-folds increase in the accumulation of lipid droplet in case of co-treatment with wortmanin and pterostilbene which was marginally higher than only pterostilbene treatment (Fig. 7.11). However, PD98059 did not induce any significant accumulation of neutral lipids in presence of pterostilbene.

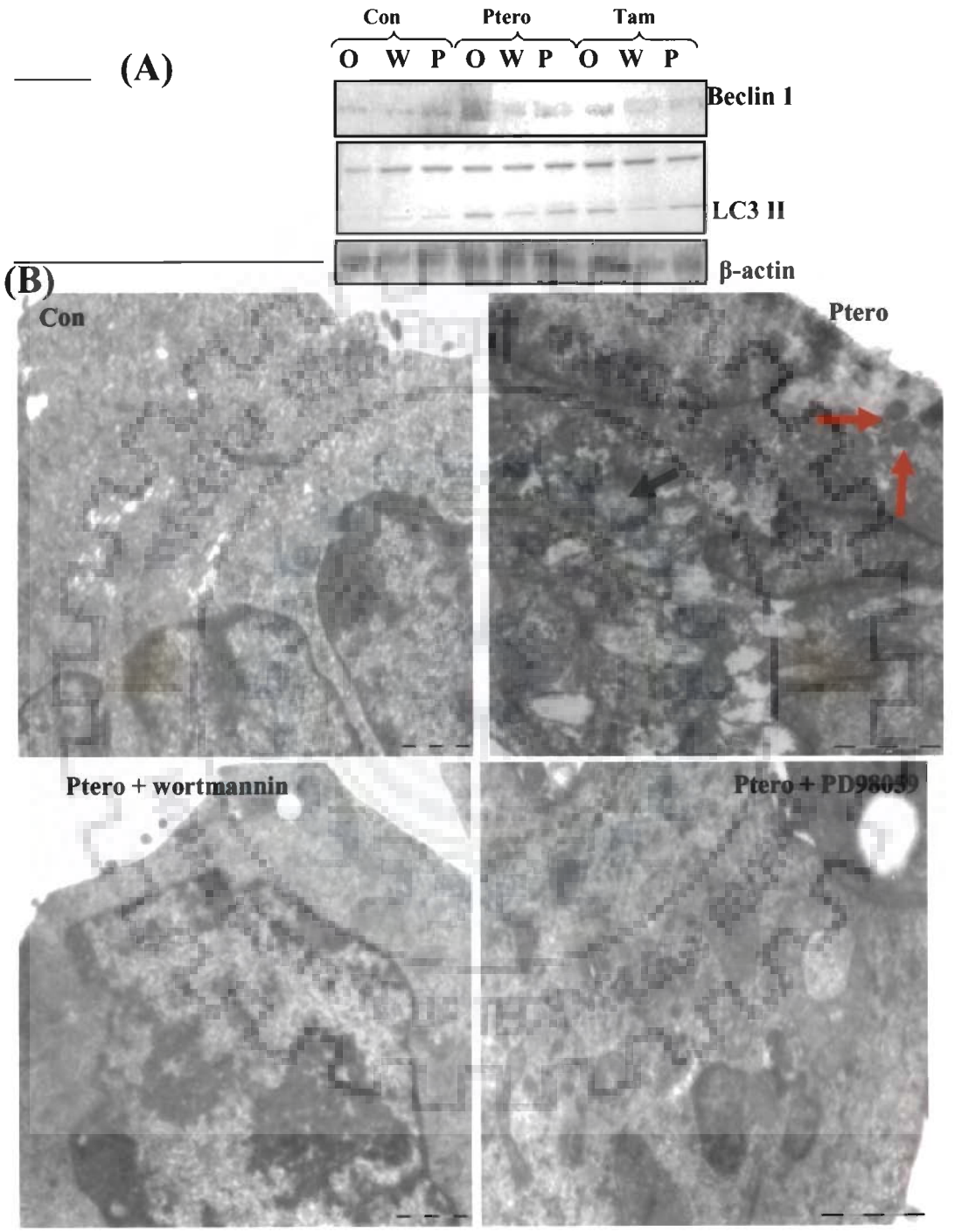


Fig. 7.10: *Effects of PI3K (wortmanin) and MEK (PD98059) inhibitors on pterostilbene treated MCF-7 cells. (A) Immunoblot analysis for Beclin 1 and LC3. (B) Transmission electron micropraphy showing autophagic vacuoles. 0, W, P represents vehicle, wortmanin and PD98059, respectively in the respective drug treatment group. Black and red arrows mark unilamellar and multilamellar vesicles respectively. The data is a representative of 3 independent experiments. Bar 1 μm.*

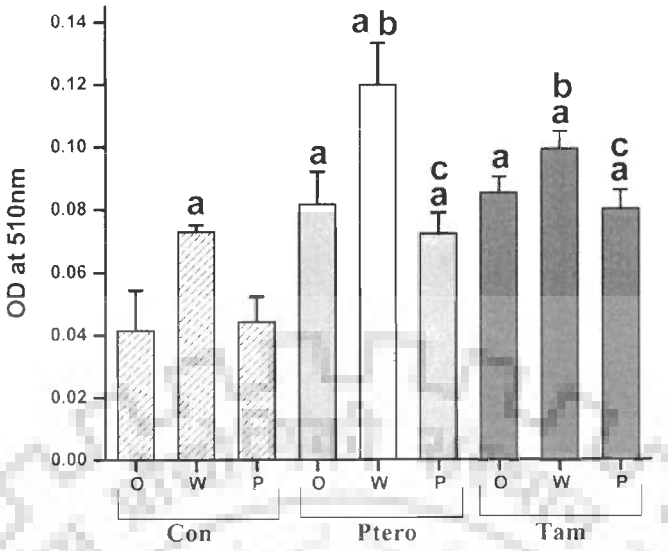


Fig. *7.11: Effects of PI3 kinase (wartmanin) and MEK (PD98059) inhibitors on neutral lipid accumulation induced by pterostilbene. Intracellular lipids were extraction of ORO stain. O, W, P represents vehicle, wortmannin and PD98059 treatments in the control (Con), pterostilbene (Ptero) and tamoxifen (Tam) treated cells respectively. a, b and c represents statistically significant difference with respect to control MCF-7 cells, wortmannin and PD98059 treated control cells respectively at $P<0.05$. The data represent the average from independent triplicate experiments (±SE).*

7.3.7 7-dehydrocholesterol is the main oxysterol metabolite produced in MCF-7 cells by pterostilbene

In order to identify the probable sterols generated inside the cells, the total sterols were isolated from pterostilbene treated MCF-7 cells and analyzed by thin layer chromatography (TLC) and then by GC-MS. From the TLC analysis it was found that the sterol content was rich in 7-dehydrocholesterol and desmosterol both in case of pterostilbene and tamoxifen treatments. A small portion of lathosterol was also found in pterostilbene treatments. But the control MCF-7 cells and the E_2 treated cells showed more of the presence of cholesterol (shown in Fig. 7.12). When the same extracted samples were analyzed by GC-MS, 4 distinct peaks were obtained in each of the gas chromatogram (Fig. 7.13 A) which matched with cholesterol, 7-dehydrocholesterol, lathosterol and marginally zymosterol. The sterols were identified by their retention times and respective mass spectra. Quantification was performed in the selected ion monitoring mode (SIM) and the ions were monitored at m/z 329, 353 and 368 for cholesterol;

m/z 325, 351 and 366 for 7-DHC; m/z 343,327 and 456 for desmosterol; m/z 255, 443 and 458 for lathosterol; and at m/z 384 , 369, 297, 271, and 213for zymosterol. Further analysis of mass spectra of the highest sterol peak (no. 2) from both control and pterostilbene treated cells showed the pattern which was similar as reported earlier for 7-dehydrocholesterol (Amaral et al., 2010) (Fig 7.13 B). The peak area was almost 2-folds higher in pterostilbene treated cell lysates as compared to the vehicle treated MCF-7 control cells (Table 7.3).

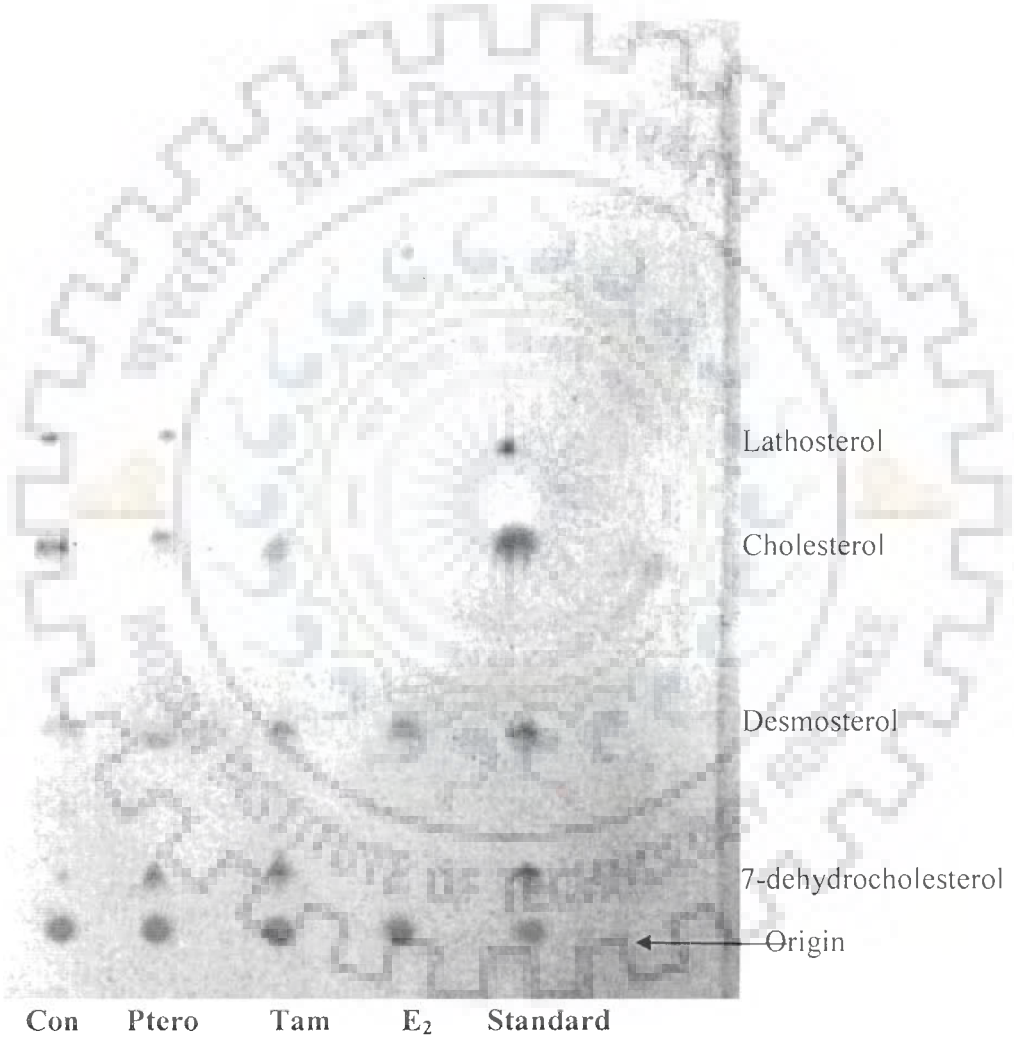


Fig. 7.12: A representative analysis of intracellular sterols by thin layer chromatography in response to various treatments. The details have been described in materials and methods section.

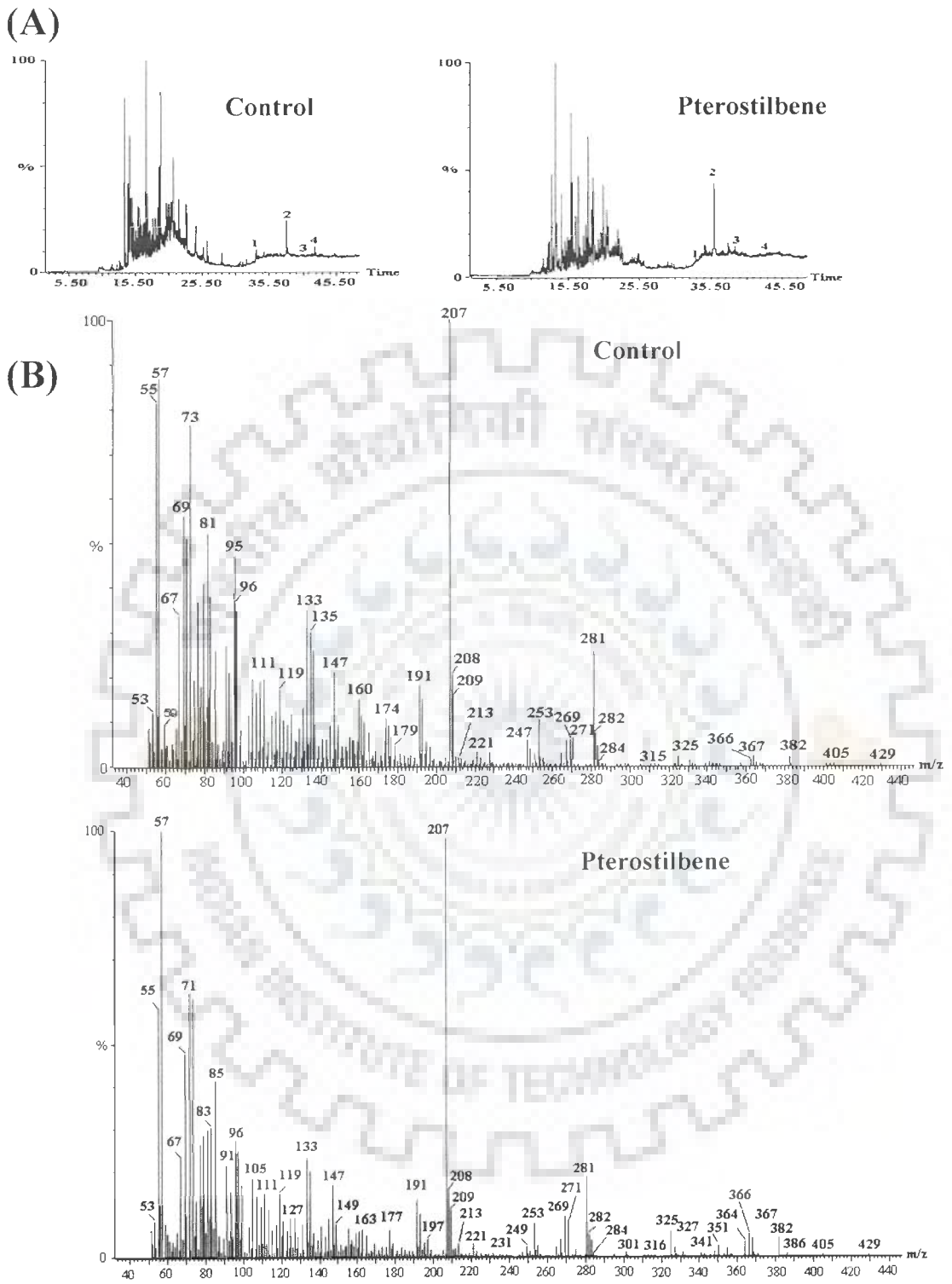


Fig. 7.13: *(A) Gas chromatogram of the sterols isolated from respective cell lysates after 72 h of treatment. Peaks: 1: Cholesterol, 2: 7-dehydrocholesterol, 3: Zymosterol, 4:Lathosterol. (B) Mass spectra of peak 2 of each of the samples.*

Table 7.3: The analysis of the respective peaks of gas chromatogram.

Peaks numbers	Compounds identified by MS	Percentage Peak Area Pterostilbene	Control	Ratio [Ptero/Con]
1	Cholesterol	8815071±38530	26058434±23892	0.33828092
2	7-dehydrocholesterol	2513300±19202	1291812±7189	1.94556174*
3	Zymosterol	111104±9781	100267±3718	1.10808142
4	Lathosterol	797±23.01	856±48.2	0.93107477

*Ptero, represents sterols extracted after pterostilbene treatments and Con, represents sterols extracted from vehicle treated MCF-7 control cells. The data represent the average from independent triplicate experiments (±S.E.M.). * represents statistical significant difference at (P< 0.05) in comparison to control MCF-7 cells.*

7.3.8 Role of LXR in pterostilbene induced differentiation and autophagy

LXR is an orphan receptor which can bind oxysterol and up regulates the transcription of a number of genes involving lipid metabolism and lipid transport. Further in our previous result it was shown to be up regulated by pterostilbene. In order to explore whether LXR has any role in differentiation, total neutral lipids were quantified after they were transfected with a full length cDNA construct of LXRβ (pCMX-LXRβ). As shown in figure 7.14 A, mock-transfected cells treated with pterostilbene and tamoxifen showed significant ($P<0.05$) accumulation of lipid droplets in comparison to control mock-transfected cells as was obtained in earlier non-transfected conditions. However, over expression of LXR significantly down regulated this pterostilbene or tamoxifen induced lipid accumulation (Fig. 7.14 A). Although the lipid accumulation was significantly higher in LXR transfected pterostilbene treated cells as compared to the mock transfected vehicle treated MCF-7 cells there was no significant difference with that of the LXR transfected vehicle treated control MCF-7 cells. This shows that pterostilbene could not significantly increase lipid accumulation in MCF-7 cells in presence of LXR ($P<0.05$). On the other hand when the extracted lipids were compared to the mock transfected pterostilbene treated cells there was a significant decrease in the lipid content in LXR transfected pterostilbene treated cells ($P<0.05$). This phenomena could be attributed to the efflux of sterols from the cells due to over-expression of LXR in MCF-7 cells as was reported earlier (Baldan et al., 2006; Rudolph et al., 2007; Schroepfer, 2000; Tall, 2008). Further, to check if there was any interaction of LXR with the autophagic pathway through Beclin and LC3, the

there was any interaction of LXR with the autophagic pathway through Beclin and LC3, the immunoblot analysis of these two autophagic proteins were performed using the MCF-7 cells over expressing LXR cDNA constructs in the presence or absence of pterostilbene. We did not find any significant change in the levels of expression of those two proteins even in the presence of pterostilbene (Fig. 7.14 B).

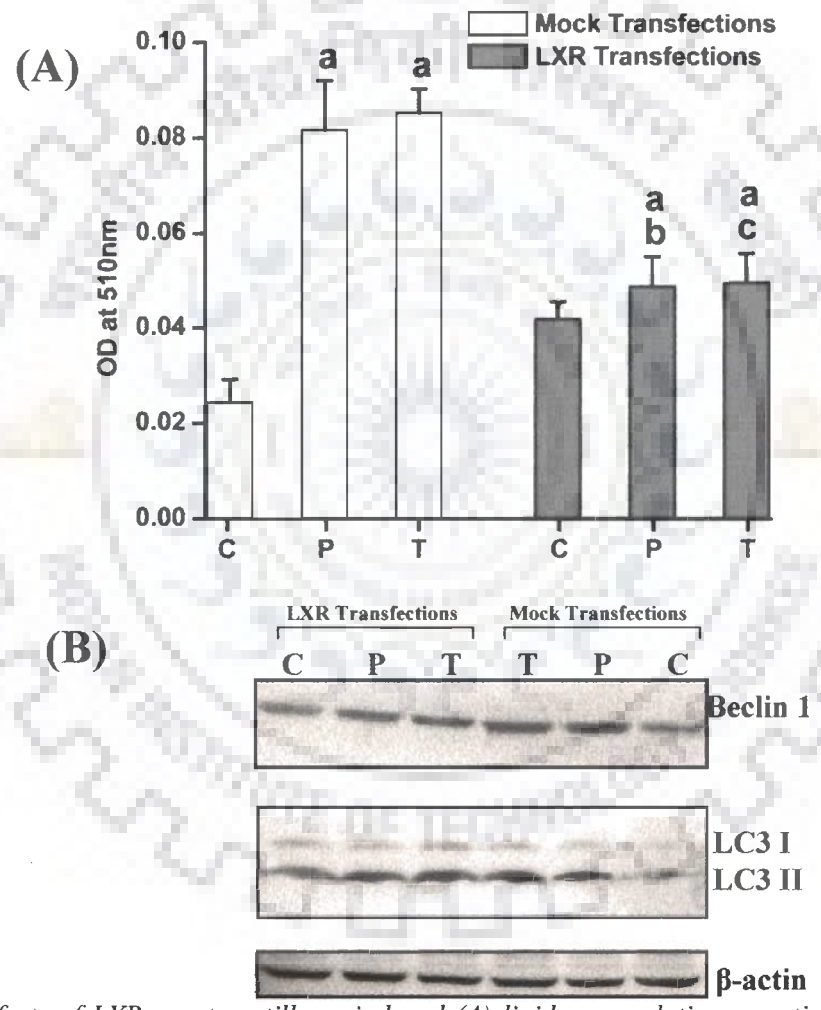


Fig. 7.14: *Effects of LXR on pterostilbene induced (A) lipid accumulation as estimated by intensity of ORO stain extracted from the cells and (B) expression of autophagic marker proteins. C, P, T, represents, vehicle, pterostilbene and tamoxifen treated cells respectively. The data represent the average from independent triplicate experiments (±S.E.M.). a, b and c represents statistically significant difference with respect to vehicle, pterostilbene and tamoxifen treated mock transfected MCF-7 cells respectively at $P<0.05$.*

7.3.9 Differential role of pterostilbene in tumor cells of breast and normal breast cells

Although pterostilbene caused accumulation of neutral lipids in MCF-7 cells along with subsequent differentiation, it was obvious to check the same effect on breast tumor cells and the normal mammary gland cells. Primary culture of tumor breast cells and normal mammary gland cells showed differential lipid accumulation after 72 h of pterostilbene exposure. As shown in figure 7.15, the lipid content of the tumor cells increased (as determined by the ORO staining of cells) with incubation of pterostilbene as compared to the vehicle treated control cells. On the contrary, lipid accumulation of the normal (non-tumorigenic) breast cells decreased possibly due to the hypoglycemic action of pterostilbene under normal conditions (Fig 7.15).

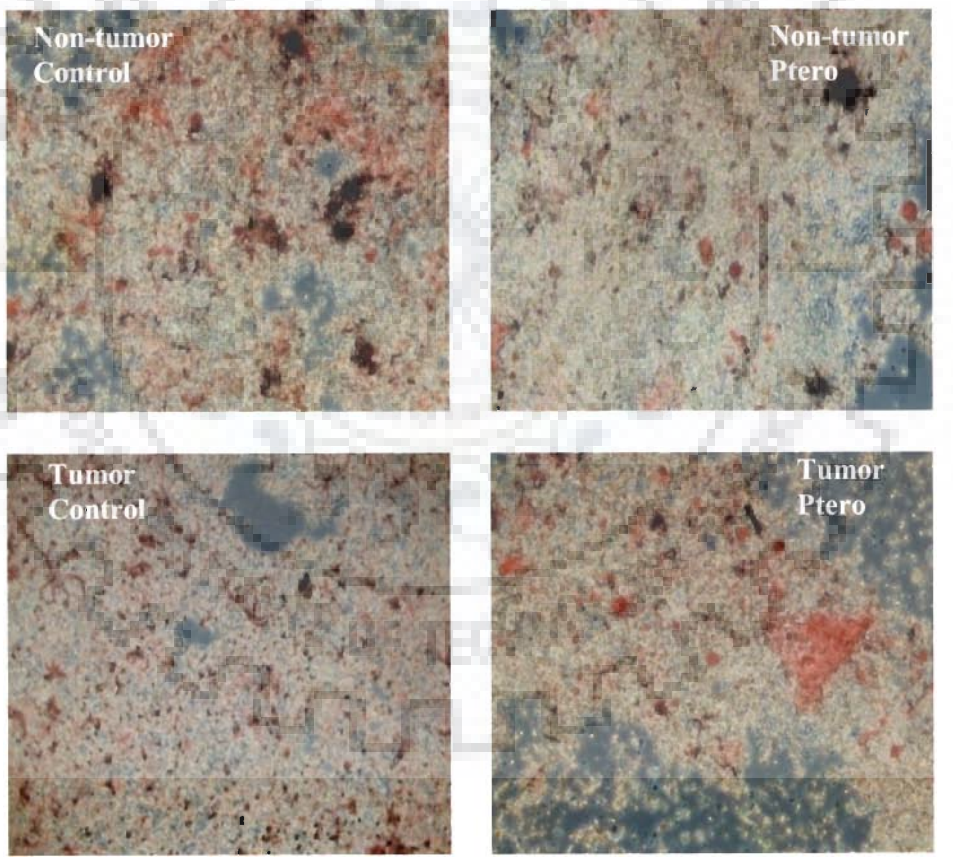


Fig. 7.15: *Effects of pterostilbene on the lipid accumulation in the mammary gland organ culture. ORO-stained cells as observed by light microscopy (10X objective magnification). The organs were collected from tumor and normal glands of MNU and vehicle treated rats respectively. Ptero represents pterostilbene.*

7.4 Discussion

It has been previously established that pterostilbene is a potent anticancer molecule having multiple targets of actions. In continuation to that, the present study was conducted to understand the probable mode of action of this phytochemical as cellular growth inhibitory molecule. Pterostilbene is reported to produce reactive oxygen species (Alosi et al., 2010; Chakraborty et al., 2010). Various studies reported that ROS itself act as the effecter signal and is involved in the control and regulation of cell death pathways (Wong et al., 2010). The fact that ROS can act as effecter molecules in cell death in breast cancer, has also been supported by a recent study by Ullah et al. (2011), where it is shown that ROS scavengers inhibit the genistein-induced cell death. The redox status of the cell is determined by the balance between the rates of production and breakdown of reactive oxygen and/or nitrogen species (ROS: RNS) (Orrenius, 2007), such as superoxide anion ($O2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO) and hypochlorus acid (HOCl) (Kamata et al., 2005). Our previous studies have shown that pterostilbene produces H_2O_2 and singlet oxygen as the major ROS in MCF-7 cells (Chakraborty et al., 2010). Recently, tamoxifen, a potent antiestrogenic molecule, and 7-ketocholesterol, a precursor of cholesterol, have been shown to stimulate the production of reactive oxygen species through the stimulation of the expression of NADPH oxidase in human hepatoma cell lines (Lee et al., 2000) and in mouse macro-phages (Rosenblat and Aviram, 2002). NADPH oxidase has been reported to be highly inducible in MCF-7 cells and is thus responsible for carrying out potent phosphorylation and death activation pathways in cancerous cells (Alexandre et al., 2007). The generation of ROS causes the accumulation of sterols which are the precursors of cholesterol and they in turn produces oxysterols within the cell (Payre et al., 2008). These are necessary, but not sufficient to trigger the differentiation and the growth control of breast cancer cells (Payre et al., 2008). Our data showed that pterostilbene can inhibit proliferation of MCF-7 cells simultaneously by inducing its differentiation and autophagy as a result of prolonged exposure for 72 h at a low dose.

The MCF-7 cells accumulated 2 fold more lipid molecules in their cytoplasm as compared to the control cells when exposed to sub-acute concentrations of pterostilbene. This effect was lost if catalase, actinomycin D or cycloheximide were co-administered with pterostilbene. Catalase is an enzymatic scavenger of hydrogen peroxide. Catalase thus scavenges ROS which is induced in MCF-7 cells as a result of pterostilbene thereby reducing the

intracellular lipid accumulation produced by ROS. The growth inhibitory effect of pterostilbene due to the above fact is also obliterated in the presence of actinomycin D (transcriptional inhibitor) and cycloheximide (translational inhibitor). Similar result has also been reported with tamoxifen. The lipid accumulation in MCF-7 cells is supported by the fact that tamoxifen and few known anti-cancer agents like ansamycins and suberoylanilide hydroxamic acid induce high amount of lipid production (as high as 5-folds in case of ansamycins) and accumulation of triglycerides resulting in the differentiation of MCF-7 cells into more of epithelial like cells (Münster et al., 2001a, 2001b; Payre et al., 2008). Pterostilbene at a low dose not only attenuates high mitotic acitvity but also the cells became more flattened and columnar in shape with less nuclear: cytoplasmic ratio. This morphological change is mainly observed to be initiated after 60 h of continuous exposure to pterostilbene. In fact the lipid droplets accumulation is further supported by our observation that the gene expression pattern of CCAAT- enhancer binding protein (c/EBP) expression increased in pterostilbene and tamoxifen treated cells after 72 h of exposure. The c/EBPβ is essential for differentiation of mammary secretory epithelial cells and is required for the activation of milk protein genes (Robinson et al., 1998). It also negatively regulates the cell proliferation and inhibits androgen receptor and the rate of DNA synthesis in prostate cancer cells (Chattopadhyay et al., 2006). The sterol accumulation in breast cancer cells is specific to the cancer morphology only as the normal breast cells are not found to accumulate sterols and differentiate as a result of pterostilbene. This provides a clue that the action of pterostilbene is specific to a type of cell which might be responsible to cause the growth arrest of these cancerous cells. The breast carcinoma cells or the malignant epithelial cells have been known to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of the expression of essential adipogenic transcription factors, i.e., PPARγ and C/EBPα, β, γ (Meng et al., 2001). It can therefore be assumed that pterostilbene induced 10-folds induction of c/EBPα mRNA is one of the prominent marker of the differentiation of the malignant MCF-7 cells into epithelial like lactating morphology. However, we did not find substantial increase in PPARγ in MCF-7 cells after pterostilbene treatments (data not shown).

Mammary gland, in specific, undergoes the process of involution which results in the clearance of lactating cells by the process of autophagy (programmed cell death type II (Lockshin et al., 2000; Motyl et al., 2007). Autophagy is a bulk proteolytic degradation of cellular organelles and cytoplasm by lysosomes forming autophagic vesicles (Jin and White,

2007). It has been shown that tamoxifen causes accumulation of sterols (Payre et al., 2008) which lead to the appearance of multilamellar bodies in the cytoplasm and there by the stimulation of the overexpression of autophagic protein Beclin 1 (Medina et al., 2009). Microtubule-associated protein 1 light chain 3 (LC3), a mammalian homologue of yeast Atg8, also plays an indispensable role in macroautophagy formation and is a suitable marker for this process (Karim et al., 2007). The accumulation of sterols leading to macroautophagy is already a phenotypic characteristic present in Nieman-Pick type C disease (which leads to autophagy) (Liao et al., 2007; Pacheco et al., 2007). The images of intracellular structures clearly showed the presence of unilamellar and multilamellar vesicles by pterostilbene and tamoxifen in MCF-7 cells under transmission electron microscopy. This result was further validated by the overexpression of Beclin 1 and LC3 proteins in response to pterostilbene. The production of these proteins within MCF-7 cells could be attributed to the continuous exposure of the cells to pterostilbene. The expression of Beclin 1 and LC3 ceased if the MCF-7 cells were pretreated with catalase which scavenges the hydrogen peroxide molecules. As expected, the expressions of these two autophagic proteins were also down-regulated by both transcriptional and translational inhibitors (actinomycin D and cycloheximide). Similar results have also been reported for silibinin, a natural product which is a mixture of flavono-lignans extracted from milk thistle (*Silybum marianum*) while causing autophagy in human fibroblast HT 1080 cell line (Duan et al., 2010). Antioxidant molecules like catalase and vitamin E induced inhibition of autophagy in response to silibinin (Duan et al., 2010) and tamoxifen (Medina et al., 2009) respectively. These results support the fact that the reactive oxygen species are mainly responsible for the oxidation of sterols into oxysterols which accumulates in the multilamellar bodies thereby causing fusion with lysosomes and autophagy. Our results showed that pterostilbene induces an early phase of autophagy which begins with the accumulation of Beclin after 12 h and continues till 60 h. However the expression of LC3 continues constantly till 72 h. This shows that although differentiation and autophagy are both induced by pterostilbene in MCF-7 cells, the cells fails to bear with the load of oxysterols and undergo massive autophagy after 72 h. Here we would like to specify that the mode of action of pterostilbene differs from its analogue resveratrol, which induces autophagy in breast cancer cells via the non-canonical pathway which is independent of Beclin 1 (Scarlatti et al., 2008). Beclin 1 is part of the PI3 kinase class III (PI3KC3) lipid kinase complex that plays a central role in the induction of autophagy (Levine et al., 2008). Wortmannin, a potent PI3K inhibitor of class I & III (Stack and

Emr, 1994; Walker et al., 2000; Wymann et al., 1996) inhibits the intracellular accumulation of macroautophagic vesicles thereby inhibiting Beclin 1 mediated autophagy caused by pterostilbene. We observed in this study that wortmannin on the other hand induced differentiation in MCF-7 cells with 1.4-folds increase of neutral lipid accumulation. Although at this point it is difficult to decipher the exact reason for the increase in lipid accumulation, but the data is in accordance with an earlier report by Munster et al. (2001,b) which showed that the PI3K inhibitors caused enhanced accumulation of neutral lipids intracellularly thereby causing growth arrest by MCF-7 cell differentiation.

The next obvious question was to identify the sterols involved in this process. The GC-MS data revealed that 7-dehydrocholesterol is the main sterol accumulated due to pterostilbene in MCF-7 cells. Apart from other sterols like zymosterol, lathosterol and cholesterol which are also detected in GC-MS, there was no significant difference in the pterostilbene treated lysates with that of the vehicle treated MCF-7 cells. Although zymosterol is a potent cholesterol precursor that induces cell growth arrest in MCF-7 cells due to tamoxifen treatments, our results showed that pterostilbene mainly resulted in the accumulation of 7-dehydrocholesterol unlike tamoxifen. Tamoxifen is known to cause intracellular accumulation of zymosterol and 7-dehydrocholesterol due to its inhibitory effect on two enzymes 3β-hydroxylsterol-Δ^8-Δ^7-isomerase (D8D7I) and 3β-hydroxylsterol-Δ^7-reductase (DHCR-7) which converts zymosterol to cholesta-7,24-diene-3β-ol and 7-dehydrocholesterol to desmosterol and cholesterol respectively (Payre et al., 2008). These enzymes are part of microsomal antiestrogenic binding sites which play a major role in postlanosterol cholesterol biosynthesis (Kedjouar et al., 2004). Further the transcriptional analysis showed that pterostilbene induced inhibitory effect on the DHCR-7 expression levels. This might probably explain the accumulation of the sterol intracellularly. Barnes (2010) reviewed that genistein is also inhibitory to the steroid biosynthetic enzymes. Although it is reported that steroidogenic enzymes are inhibited by ROS through the upregulation of c-jun in rat testis (Lee et al., 2009), the link between oxysterols production due to the upregulation of ROS is not considered in the study by Lee et al. (2009). Therefore, it can be assumed that the inhibitory effect of pterostilbene on DHCR-7 decreases the de novo cholesterol biosynthesis and the production of intracellular ROS on the other hand causes a synergistic effect in this process. Among the metabolites of tamoxifen, PBPE is known to induce effective growth inhibition in MCF-7 cells due to the induced accumulation of intracellular zymosterol and 7-dehydrocholesterol (Payre et al., 2008). Literature reports show that 7-

dehydrocholesterol is very susceptible to cleavage by even mild oxidation and it results in highly complex mixtures of corresponding 3-oxo compounds or the oxysterol moieties (Batta et al., 1997). Hydrogen peroxide and the singlet oxygen molecules induced by pterostilbene transform 7-dehydrocholesterol motifs into a complex mixture of oxysterols. On the other hand oxysterols are known modulators of cholesterol metabolism (Schroepfer, 2000) and are also known activators of LXR (Yang et al., 2006). LXR are already known to regulate genes involved in lipid metabolism (Kim et al., 2003; Schroepfer, 2000). Our hypothesis that induced oxysterols are produced inside the cell as a result of pterostilbene was further validated by the fact that not only the oxysterol binding protein was highly expressed (7.2-folds), the LXR expression also increased as compared to the normal cancerous MCF-7 cells. The MCF-7 cells in order to maintain homeostasis against this high levels of accumulated oxysterol probably over-expresses ATP-binding cassettes (ABC) transporters mainly G1. ABCs are generally responsible for efflux of lipid, sterols and oxysterols (Baldan et al., 2006; Rudolph et al., 2007; Schroepfer, 2000; Tall, 2008). Recently it has been reported by Trasino et al. (2009) that cholesterol precursors and small molecular oxysterols may result in as high as 10-folds increase in the mRNA transcript of ABC transporters. This study also refers to the fact that ABCG1 is mainly controlled by LXRβ. Therefore, it could be propably concluded from the present data that LXRβ may be up-regulated as a result of pterostilbene with the consequent increase in ABCG1.

Our hypothesis that pterostilbene causes accumulation of sterols and oxysterols in the MCF-7 cells which results in MCF-7 differentiation and growth control by autophagy was further validated by the fact that when the cells are transfected with LXR full length construct, the sterols induced by pterostilbene or tamoxifen reduced down to a basal level. This might probably be due to the efflux of sterols and oxysterols that become active as a result of the overexpression of LXR. On the other hand there was no significant change in autophagy of the cells. It has been reported that LXR suppresses messenger RNA and/or protein expression of Skp2, cyclin A2, cyclin D1 and estrogen receptor (ERα), whereas it increases the expression of p53 at the protein level and maintain the retinoblastoma protein in a hypophosphorylated active form in MCF-7 cells (Vedin et al., 2009). Further, LXR maintains the homoestasis of sterol moieties inside the cell. It activates the key lipogenic genes including sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase and stearoyl-coenzyme A desaturase 1, leading to increased triglyceride production in MCF7 cells (Vedin et al., 2009) and upregulates the ABC transporters to efflux the triglycerides and sterols formed inside the cell (Baldan et al.,

2006; Rudolph et al., 2007; Schroepfer, 2000; Tall, 2008). The ultimate oxidative stress thus produced inside the cell due to the ROS moieties and oxysterols also induces the expression of the death receptor CHOP after 3 days. CHOP is known as a death effecter induced in cells as a result of endoplasmic reticulum toxicity due to intracellular ROS (Verma et al., 2010; Zinszner et al., 1998). Further it has been also reported to be expressed in macrophages due to cholesterol overload induced toxicity inside the cell (Feng et al., 2003).

A study by Wong et al. (2010) revealed that chemotherapy induced ROS load on the tumor cells resulted in simultaneous induction of autophagy and apoptosis involving ERK and JNK activation. On the other hand, increase in CHOP is related to increased activation of JNK (Verma et al., 2010). However in the present study, the pterostilbene had no significant effect on the triglyceride induction in presence of inhibitor to MEK/ERK pathway, PD98059. Moreover, the MEK inhibitor marginally inhibited the autophagy caused by pterostilbene. The present data thus confirms further that pterostilbene may involve the activation of MEK/ERK pathway at some particular stage in regulating autophagy but this findings further warrants more detailed study to understand the mode of action of pterostilbene which is beyond the scope of this thesis.

In conclusion, to the best of our knowledge, this study shows for the first time that pterostilbene inhibits cholesterol biosynthesis by regulating the expression DHCR-7. It induces the overproduction of oxysterols which are the key effecter molecules involved in MCF-7 cell differentiation and cell growth control by autophagy. There might be a cross talk between sterol accumulation, LXR and autophagy which further needs to be investigated. Finally, this study also shows that pterostilbene induced a growth control with characteristics of autophagy and differentiation in breast cancer cells and stimulate the events involved in the chemoprevention of cancers that support its therapeutic and prophylactic use after further validation of this phytochemical using both in vitro and in vivo animal models.

CHAPTER 8

EFFECTS OF PTEROSTILBENE ON PROGESTERONE RECEPTOR

8 Effects of pterostilbene on progesterone receptor

8.1 Introduction

Breast is a specific target where the steroid hormones mainly estrogen and progesterone acts and causes the development of the both normal mammary gland and breast cancer (Lapidus et al., 1998). During development of cancer in breast tissues, few cells become modified and tend to form a solid mass due to uncontrolled growth, loss of contact inhibition and apoptosis. They finally evade the host immune surveillance processes and attain invasive properties. Breast cancer is most common in middle aged women (Rose et al., 2007). It is often linked with over-expression of estrogen and/or progesterone receptors (ER and/PR). Almost 75% of breast cancers are ER positive and out of that majority (about 65%) are also progesterone receptor-positive (Hoskins et al., 2009). Early menarche was more consistently associated with ER/PR-positive breast cancers (Althius et al., 2004). In the middle aged women the hormone replacement therapy (HRT) is one of the major choices for the cure and management of several diseases, which utilizes a combination of estrogen and progesterone as therapy. On the contrary, to the use of sex steroids as therapeutic agents, some studies have related the widespread use of progestins (also progesterone) in various clinical applications as the cause of breast, uterine and ovarian cancers (Kato et al., 2005; Lee et al., 2005; McGowan et al., 2007; Singh et al., 2007) due to the activation of progesterone receptors in these conditions.

Progesterone receptor (PR) is a major steroid hormone receptor which functions as a ligand mediated transcription factor. PR exists in two isoforms PR-A (~94kDa) and PR-B (~116kDa) which are slightly distinct in function, structure and intracellular distribution. The PR isoforms have differences in the transcriptional activities (Narayanan et al., 2005a; Pierson-Mullany and Lange, 2004; Shen et al., 2001; Takimoto et al., 1996). The expression of pure homodimers of PR-A and PR-B, act as repressors and activators of transcriptions, respectively (Mohammed et al., 1994). Apart from the transcriptional regulation, PR binds to a number of cytosolic proteins and regulates their actions. Progesterone-PRB complexes have been reported to activate PI3K/Akt/NFκB pathway to initiate cell cycle (Saitoh et al., 2005). PR-B interacts with several cell cycle regulators like cyclin D1 or src through its SH3 domain (McGowan et al., 2007). Moreover, recently discovered novel membrane progesterone receptors reportedly resemble and function as G-protein coupled receptors and are the upcoming potential therapeutic targets (Fernandes et al., 2008). Both these genomic and non-genomic actions of progesterone

have been shown even in breast and endometrial tissues (Ballare et al., 2006). In endometrial tissues the PR clearly regulates cell differentiation pathways through the wnt/β-catenin pathways (Cloke et al., 2008). Therefore, it is proposed that anti-progestagens in PR positive breast cancer cells may act effectively by blocking these activities (Chakraborty et al., 2010a). The dietary polyphenols have a wide range of role in inhibiting cell proliferation through the mediation of different pathways with minimal side effects (Lee et al., 2011). In hormone sensitive cell lines the specific interaction of polyphenols (catechin, epicatechin, quercetin, and resveratrol) to the steroid receptors is reported to be linked with cancer cell growth inhibition (Damianaki et al., 2000). One of such dietary polyphenol can be referred to the stilbene family members who have been studied for its cytotoxic and anti-inflammatory properties. Pterostilbene is a dimethyl ester of resveratrol. It has higher oral bioavailability (Mannal et al., 2010) and enhanced potency as compared to resveratrol (Chiou et al., 2011). Pterostilbene has been shown to inhibit Akt/NFkB pathway in breast cancer cells (Pan et al., 2011) and thus suppresses/delays and reverses the process of carcinogenesis. It also inhibits various proteases like matrix metalloproteinases which are essential for metastasis (Chakraborty et al., 2010b). Although the anti-metastatic, anti-tumor and anti-leukemic property of this stilbene molecule has been well established in different types of cancers, there is not much report on the action of pterostilbene on the cancer proliferation mediated by steroid hormones. Recently, it is reported by Wang et al. (2010) that pterostilbene arrests the proliferation of LNCaP cells due to the inhibition of the androgen stimulated production of prostate specific antigen (Wang et al., 2010).

The present study was aimed to check the effect of pterostilbene on a PR-positive breast cancer cells and to determine its cross-talk with PR. Our initial data showed that the growth of T47D cells was inhibited by pterostilbene. Here, we report that pterostilbene bears an anti-PR activity in both *in vitro* and *in vivo*.

8.2 Brief experimental protocols

8.2.1 Cell lines

T47D cell lines were used to study the interaction of pterostilbene and PR. For PR transactivity studies, the ER and PR negative cell line, MDA-MB-231 was used. MCF-7 cells were used for PR localization studies. The culture conditions of the cell lines are described in chapter 3.

8.2.2 Dose and duration of exposure

The T47D cells were exposed to a low dose of 30 μM for 24 h, unless otherwise mentioned. For in vivo studies, pterostilbene was dissolved in DMSO and injected (s.c.) to female rats at a dose of 10, 20 and 30 mg/kg body weight for 2 days. Progesterone (10 nM) and RU486 (10 nM) were used as standard positive and negative regulators of PR.

In order to check the effect of pterostilbene on PR, alkaline phosphatase assay, RT-PCR, immunoblot analysis, dot blot analysis, transfection studies and ChIP assay were performed according to the methodology described in chapter 3.

8.2.3 In vivo anti-progestagenic assay

Initially the ovarectomized female rat model was developed (section 3.5.3). The treatment groups were specified as follows:

Group I : Sham control
Group II : Vehicle control
Group III : Progesterone (0.3 mg/kg bw) gavaged
Group IV : Pterostilbene (10 mg/kg bw) s.c. injection in the nape of the neck (0.02 ml)
Group V : Pterostilbene (20 mg/kg bw) – do –
Group VI : Pterostilbene (30 mg/kg bw) – do –
Group VII : RU 486 (3 mg/kg bw) – do –
Group VIII : Estadiol (0.3 mg/kg bw) gavaged

The in vivo effect of pterostilbene was checked on ovarectomized female rats by analyzing the expression of uterine complement 3 (C3) mRNA by RT-PCR (section 3.26).

8.3 Results

8.3.1 Pterostilbene inhibits PR and PR- regulated breast cancer marker kallikrein 4

In order to check the effect of pterostilbene on the PR-positive breast cancer cells, it is always essential to see the PR expression status in these cells in response to pterostilbene. As shown in Fig. 8.1 A, the expression of PR was down regulated significantly by 30 μM pterostilbene (~2-folds) ($P < 0.01$) (Fig. 8.1 D) and was comparable to RU486 with respect to vehicle treated cells after 24 h of treatments. A similar trend of inhibition was observed in the transcriptional analysis by RT-PCR where pterostilbene repressed the transcription of PR (2-folds) in T47D cells (Fig. 8.1 B, E) ($P < 0.01$). Kallikrein 4 (KLK4) is a breast cancer marker, which is regulated by PR. The metastasis inducer protease KLK4 was down-regulated

significantly by 1.8-folds ($P<0.05$) in presence of pterostilbene after 24 h (Fig. 8.1 C, E). The anti-progestagen, RU-486, also caused the down-regulation of this gene transcript.

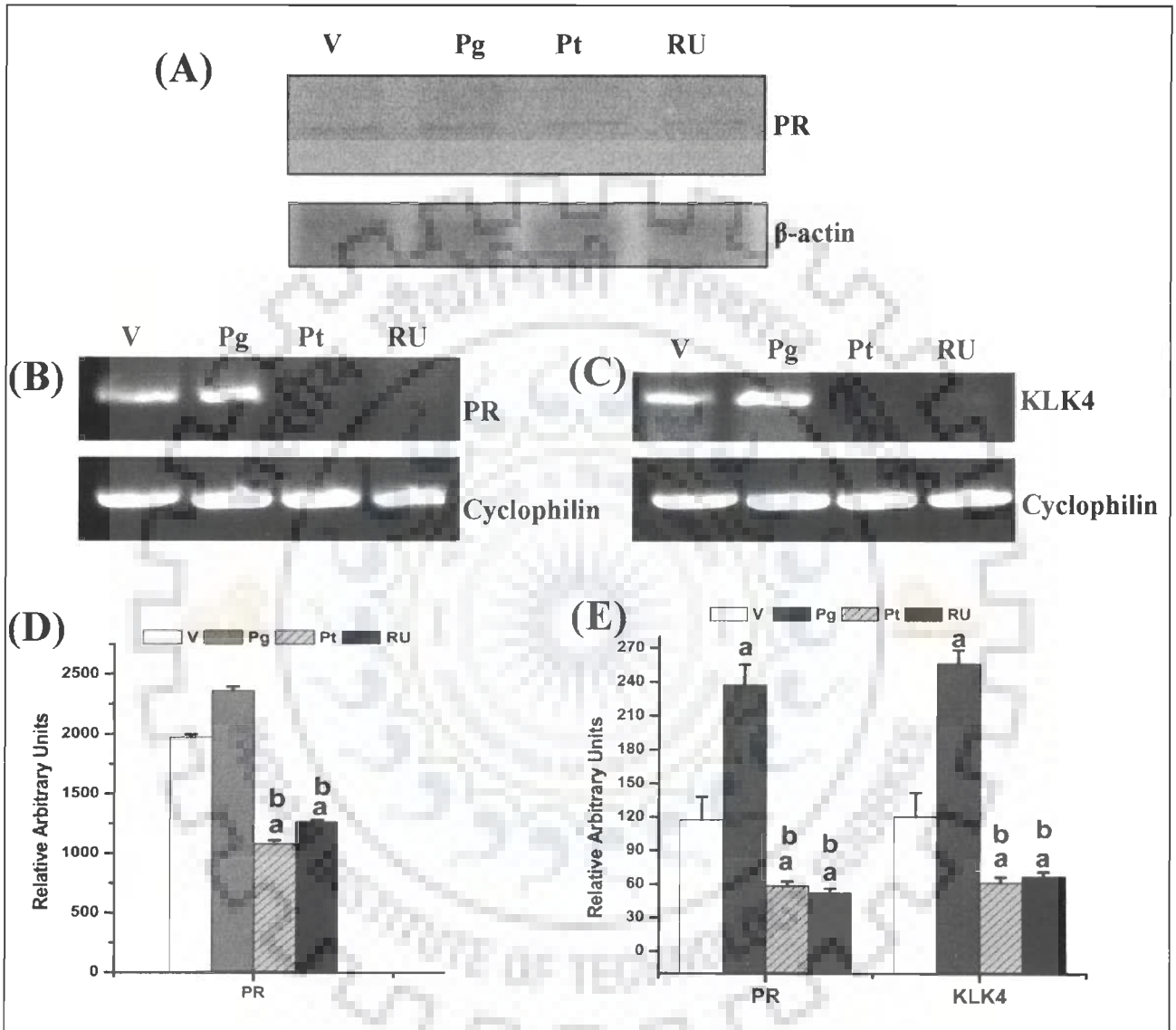


Fig. 8.1: *Effect of pterostilbene on the expression of progesterone receptor and progesterone regulated gene Kallikrein 4. Translational analysis of progesterone receptor (A) and transcriptional analysis of progesterone receptor (B) and Kallikrein 4 (C) after 24 h of treatment with pterostilbene. The histogram representing mean ± S.E.M. of arbitrary pixel intensities of respective treatments as analyzed by immunoblot (D) and RT-PCR (E). V, vehicle; Pg, progesterone, Pt, pterostilbene ; RU, RU486 .The data is a representative of 3 individual experiments. a and b, respectively represent statistically significant difference as compared to vehicle treated and progesterone treated T47D cells ($P < 0.01$).*

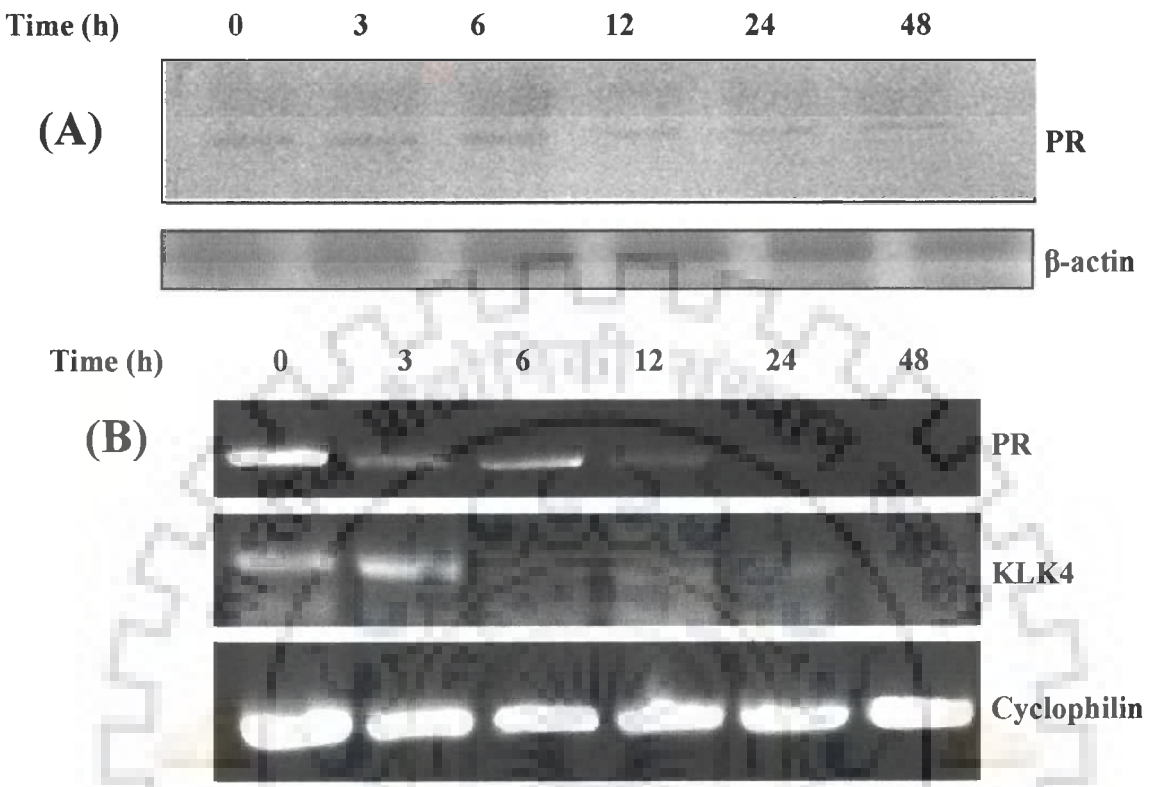


Fig. 8.2: *Time dependent translation (A) and transcription (B) of progesterone receptor Kallinkerin 4. T47D cells were exposed to pterostilbene (30 μM) followed by transcriptional and translational analysis. The data represent the average from independent triplicate experiments.*

The time dependent study of PR expression showed that its expression decreased after 12h of pterostilbene exposure, which continued further till 48h (Fig. 8.2 A). This fact was further supported by the transcriptional analysis (by RT-PCR) of PR, which also showed a similar trend (Fig. 8.2 B). It was also found that a corresponding decrease in the transcription of KLK4 mRNA occured at different time points (Fig. 8.2 B) thereby showing that both PR and PR regulated gene expressions were inhibited by pterostilbene (30 μM) with longer exposure (48 h).

8.3.2 Pterostilbene interferes with the PR mediated alkaline phosphatase activity

Alkaline phosphatase is a prominent marker, which shows the PR activation status in a cell. Due to the fact that pterostilbene repressed the PR expression both at transcription and translation levels, it was necessary to check the effect of pterostilbene on alkaline phosphatase. Pterostilbene inhibited progesterone induced alkaline phosphatase activity in a dose dependent

manner (Fig. 8.3 A). It was found that the production of para nitrophenol (pNP), the product formed by the alkaline phosphatase activity, decreased to 57, 43 and 38% respectively in presence of 10, 20 and 30 μM pterostilbene as compared to 10 nM of progesterone treatment ($P<0.01$). Although inhibition trend of pterostilbene on phospatase activity was not as efficient as RU486 (10 nM), which reduced enzymatic activity to almost 19%, the former was significantly different as compared to only progesterone treatment ($P<0.01$). It was thus observed that 30 μM pterostilbene reduced the progesterone stimulated alkaline phosphatase activity at the cellular level by less than 50% after 24 h. When checked for the time dependent action of pterostilbene on alkaline phosphatase activity in presence or absence of 10 nM progesterone, it was found that 30 μM of pterostilbene inhibited alkaline phosphatase activity to 45% initially, after 6 h in the absence of progesterone which was even lesser than that in the vehicle treated cells (Fig. 8.3 B).

Progesterone induced activity of alkaline phosphatase was significantly inhibited by ptersotilbene from 6 h and was comparable to potent PR antagonist (RU486) till 12 h. At 24 h although pterostilbene demonstrated a significant inhibition (about 40%) but it efficacy was lesser than RU486, which showed an inhibition of 27% (Fig 8.3 B). The present data therefore shows that the effect of pterostilbene on alkaline phosphatase activity of the normal T47D cells is only basal but when the cells are activated by progesterone, pterostilbene inhibits the alkaline phosphatase activity in these breast cancer cells.

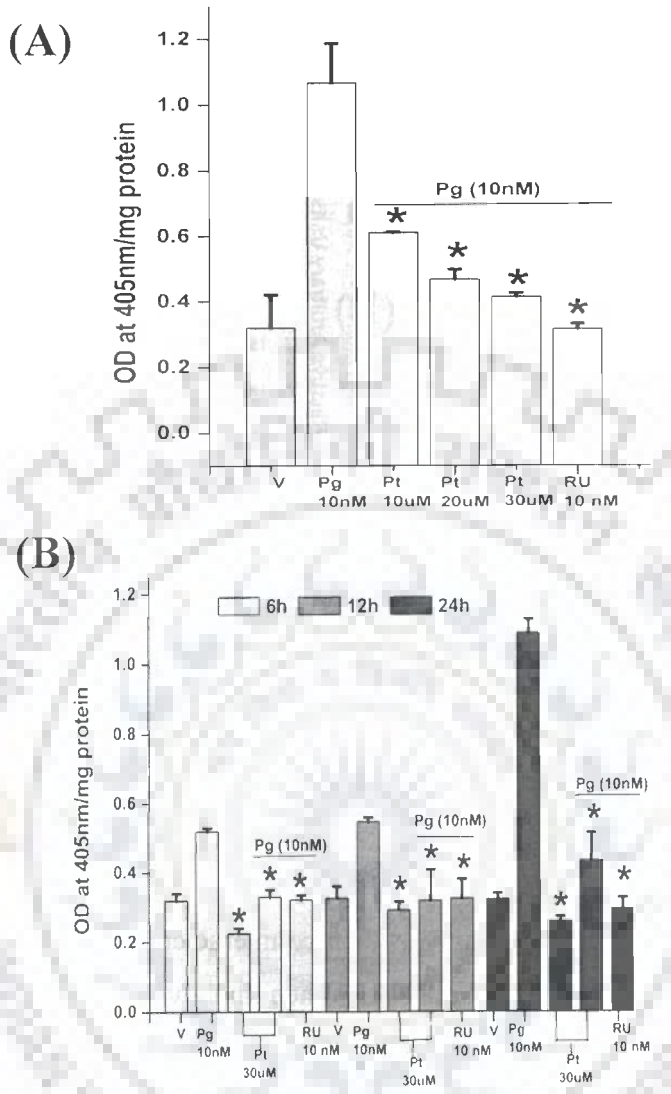


Fig. 8.3: *Effect of pterostilbene on PR-regulated alkaline phosphatase activity in T47D cells. (A) Dose-dependent and (B) Time-dependent alkaline phosphatase activity in presence of various test chemicals. Data are mean ± S.E.M. of three independent experiments. V, vehicle; Pg, progesterone; RU, RU-486 (10 nM) and Pt, pterostilbene. * indicates statistically significant (P<0.01) with respect to progesterone treatments.*

8.3.3 Rat complement C3 assay for anti-progestagenic activity

In order to check the anti-progestagenic activity, Lundeen et al. (2001) reported the development and characterization of the rat uterine complement component C3 assay for the evaluation of progestins activity in the uterus. According to this assay, estradiol (0.3 mg/ kg bw) induced expression of C3 was inhibited specifically by progesterone.

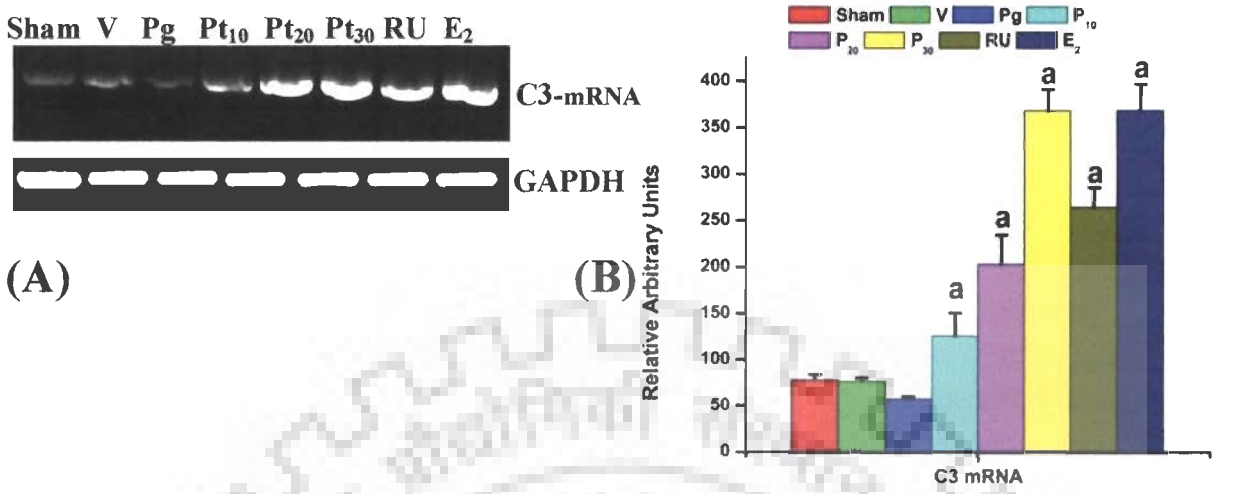


Fig. 8.4: *(A) Transcriptional analyses of rat complement C3 mRNA in ovarectomised rats.(B) Histogram representing mean ± S.E.M. of arbitrary pixel intensities of respective treatments as measured by ImageJ software . Sham, sham-operated; V, vehicle treated; Pg, progesterone treated (0.3 mg/kg BW); RU, RU-486 (3 mg/kg BW); E2, only estradiole treatment (0.3 mg/kg BW); Pt10, Pt20 and Pt30, pterostilbene treated groups at 10, 20 and 30 mg/kg BW, respectively. a represents statistically significant difference of C3 mRNA expression with respect to sham operated group at P <0.01.*

This inhibitory action of progestin was then counteracted by anti-progestins like RU486 thereby causing the expression of C3 mRNA. When the ovarectomised rats were injected subcutaneously with pterostilbene (10, 20 and 30 mg/kg bw) for 2 consecutive days a clear trend of increase by 1.7, 2.7 and 4.9- folds in the C3 transcript was observed with respective increasing doses (Fig. 8.4 A, B) ($P<0.01$). Potent anti-progestagen RU 486 (3 mg/ kg bw) also caused a 3.5- fold increase in the C3 mRNA. Therefore, it can be concluded that at highest dose (30 mg/kg bw) of pterostilbene induced high expression of C3, similar to the estradiol positive control without progesterone (Fig. 8.4 A, B). The sham operated and vehicle treated controls showed minimal expression of the C3 mRNA. These results were further confirmed when the total uterine weights were analyzed. It was found that the uterine weight increased by 2, 3.4 and 3.9- folds as compared to progesterone treatments (Table 8.1) with increase in the dosage of pterostilbene. This data thus confirmed that pterostilbene antagonizes the action of progesterone in rat uterus.

Table 8.1: The weights of intact uterus of ovarectomised groups of animals after respective treatments.

Groups	Weight of uterus (g)
Sham-operated	0.149 ± 0.039
Vehicle	0.177 ± 0.01
Progesterone	0.109 ± 0.003
Ptero (10 mg/kg)	0.237 ± 0.01*
Ptero (20 mg/kg)	0.380 ± 0.008*
Ptero (30 mg/kg)	0.431 ± 0.006*
RU-486	0.373 ± 0.005*
E_2	0.432 ± 0.01*

*Data are Mean ± S.E.M. of the respective group of animals. * represents significant difference with respect to progesterone treatments ($P<0.01$); n=6.*

8.3.4 Pterostilbene induced inhibition of PR mediated transcription of luciferase activity

In order to check the anti-progestagenic activity of pterostilbene, PR positive T47D cells were transiently transfected with pGL3 hPRE-Luc construct. Ligand-activated PR binds to PRE, and its functional activation was tested using a luciferase reporter gene linked to its promoter (PRE). The effect of progesterone (10 nM) on the luciferase activity was expressed as 100% transactivation in the assay. Consistent with the results described above, pterostilbene alone did not induce transcriptional activation at any of the concentrations tested (10-100 μM). However, as shown in Fig. 8.5 A, pterostilbene exhibited a dose-dependent anti-progestagenic activity by inhibiting the progesterone induced transactivation to almost 50% at the hightest dose tested (100 μM) as compared to only progesterone treatment. In order to confirm the anti-progestagenic activity of pterostilbene further, similar transactivation was performed in ER and PR negative MDA-MB 231 cells. The cells were co-transfected with full length PR cDNA construct (pSG5-hPR) and pGL3 hPRE-Luc constructs. As shown in Fig. 8.5 B, the progesterone mediated luciferase activity was reduced by 18, 22, 43 and maximum to 44% in presence of 10, 20, 30 and 50 μM of pterostilbene, respectively. The inhibition of progestagenic activity did not reach more than 44% even at the highest concentration of pterostilbene (100 μM). In this context, it is worth mentioning that RU 486 on the other hand reduces the luciferase activity to the basal level. This shows that although pterostilbene effectively exhibits anti-

progestagenic activity in both PR positive and negative cell lines, it cannot completely inhibit the steroid receptor action thus differing from the general trend of anti-progestins like RU486.

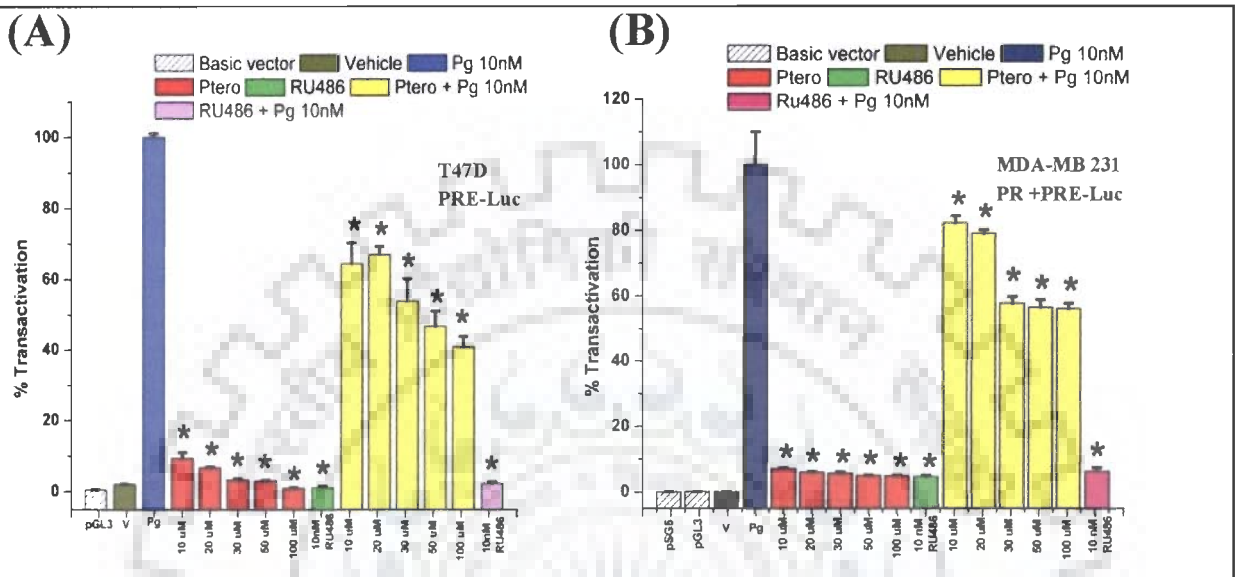


*Fig. 8.5: Effect of pterostilbene on the transactivation of progesterone receptor mediated expression of luciferase reporter gene in (A) PR positive T47D cells and (B) PR negative MDA-MB 231 cells. V, vehicle treated control cells; Pg, progesterone; Ptero, pterostilbene. * indicates statistically significant (P<0.05) with respect to progesterone treatments.*

8.3.5 Inhibition of steroid co-factors by pterostilbene

In order to determine if pterostilbene has any direct or indirect roles in affecting the expression of steroid receptor co-activators, the expression profiles of two prominent co-activators, steroid receptor co-factors (SRC1) and glucocorticoid receptor and progesterone receptor co-activator (GRIP-1) were tested in T47D cells. Both SRC1 and GRIP-1 were significantly down regulated by 2.7 and 1.6- folds respectively ($P<0.05$) as a result of 24 h incubation of the T47D cells with pterostilbene (Fig. 8.6 A, B). When tested for the time dependent expression parameters of these two co-factors, it was found that the expression of SRC1 started decreasing after 12 h, while for GRIP-1 it was after 48 h (Fig. 8.6 C). This data clearly showed that pterostilbene has a negative effect at least on the expression of two steroid receptor co-factors.

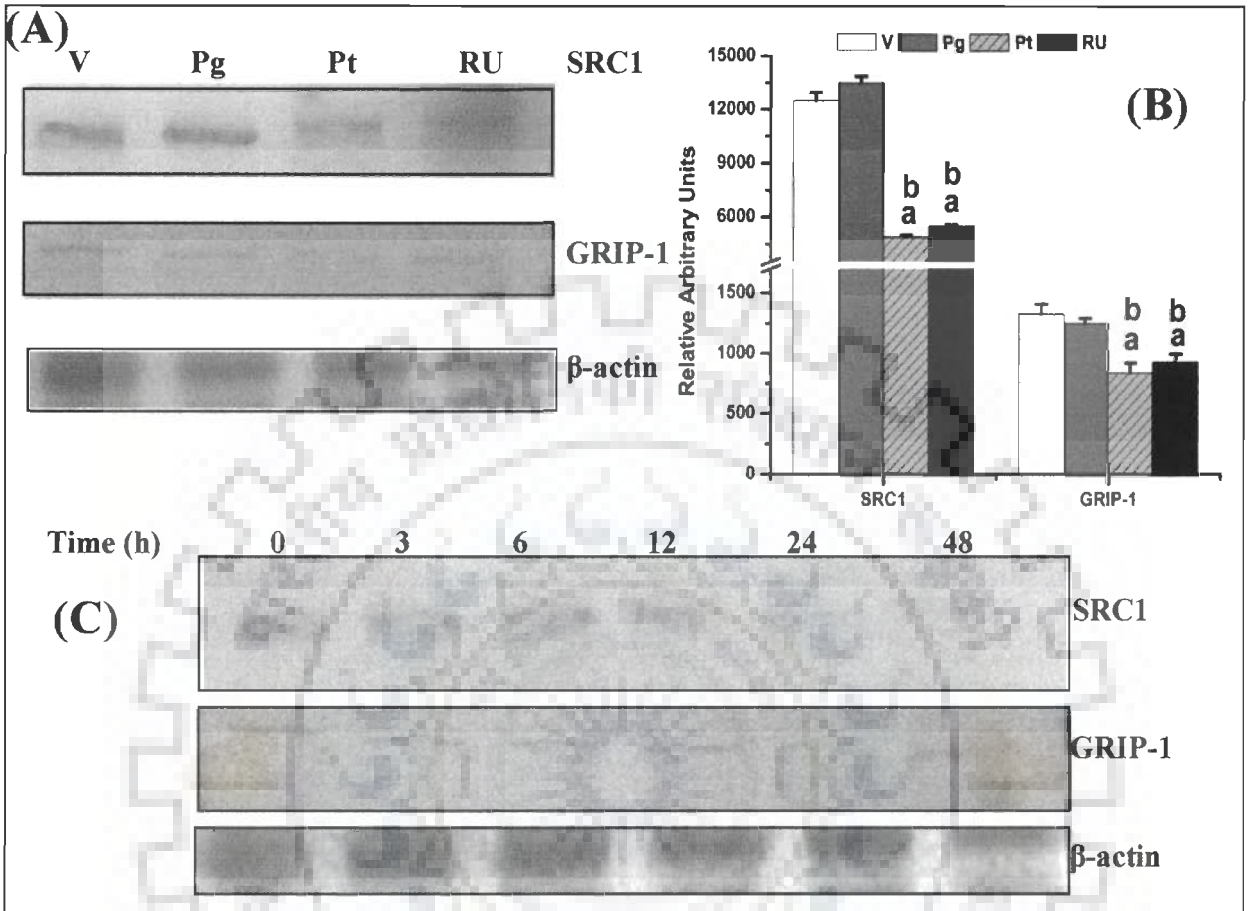


Fig. 8.6: *Immunoblot analysis for the expression of progesterone responsive co-activators in response to pterostilbene in T47D cells (A) after 24 h and (C) after various periods of pterostilbene treatments. (B) Histogram representing mean ± S.E.M. of arbitrary pixel intensities of immunoblot developed from 24 h drug treatments and measured by Image J software. V, vehicle; Pg, progesterone, Pt, pterostilbene; RU, RU486. a and b, respectively represent statistically significant difference as compared to vehicle treated and progesterone treated T47D cells ($P < 0.01$).*

8.3.6 Role of pterostilbene in the inhibition of nuclear localization of PR

We next checked the effect of pterostilbene on the nuclear localization of PR. To test this, 24 h after transient transfection of a full length cDNA construct of human PRA and PRB forms fused to GFP (pEGFP-hPRA and pEGFP-hPRB respectively) to MCF-7 cells, they were treated with 10 nM progesterone in the presence or absence of pterostilbene (30 μM). The PR ligand is expected to cause the nuclear localization of PR plasmid within a 5 min of exposure (Graham et al., 2009; Mockus et al., 1983). It was found that with co-treatment of pterostilbene and progesterone there was a diffused cytoplasmic localization of the GFP-PR A and B proteins even after 30 min, whereas only progesterone treatment showed prominent nuclear localization within 10 min which was completed by 30 min (Fig. 8.7). This data indicated that pterostilbene inhibited the migration of ligand bound PR to nucleus.

In order to exactly locate the PR proteins within the cells, a dot blot analysis was performed separately with the nuclear and cytoplasmic fractions. When equal amount of total protein from the cytoplasmic and nuclear extracts from the respective treatments at 0, 10 and 30 min were loaded on a PVDF membrane and blotted with PR antibodies, it was observed that in the presence of pterostilbene, the progesterone receptor could not be translocated to nucleus even after 30 min, whereas only progesterone stimulated the localization of PR to the nucleus (Fig. 8.8 A). In case of only progesterone treatments the cytoplasm contained about 0.6- folds of the remaining PR, whereas the nucleus had almost 2- folds more PR than initial contents in the cytoplasm and nucleus respectively. While in case of pterostilbene treatment it was observed that even after 30 min the PR content in both cytoplasm and nucleus increased only by 1.1-folds. Equal loading of proteins on the membrane is checked by the image of ponceau S staining (Fig. 8.8 B).

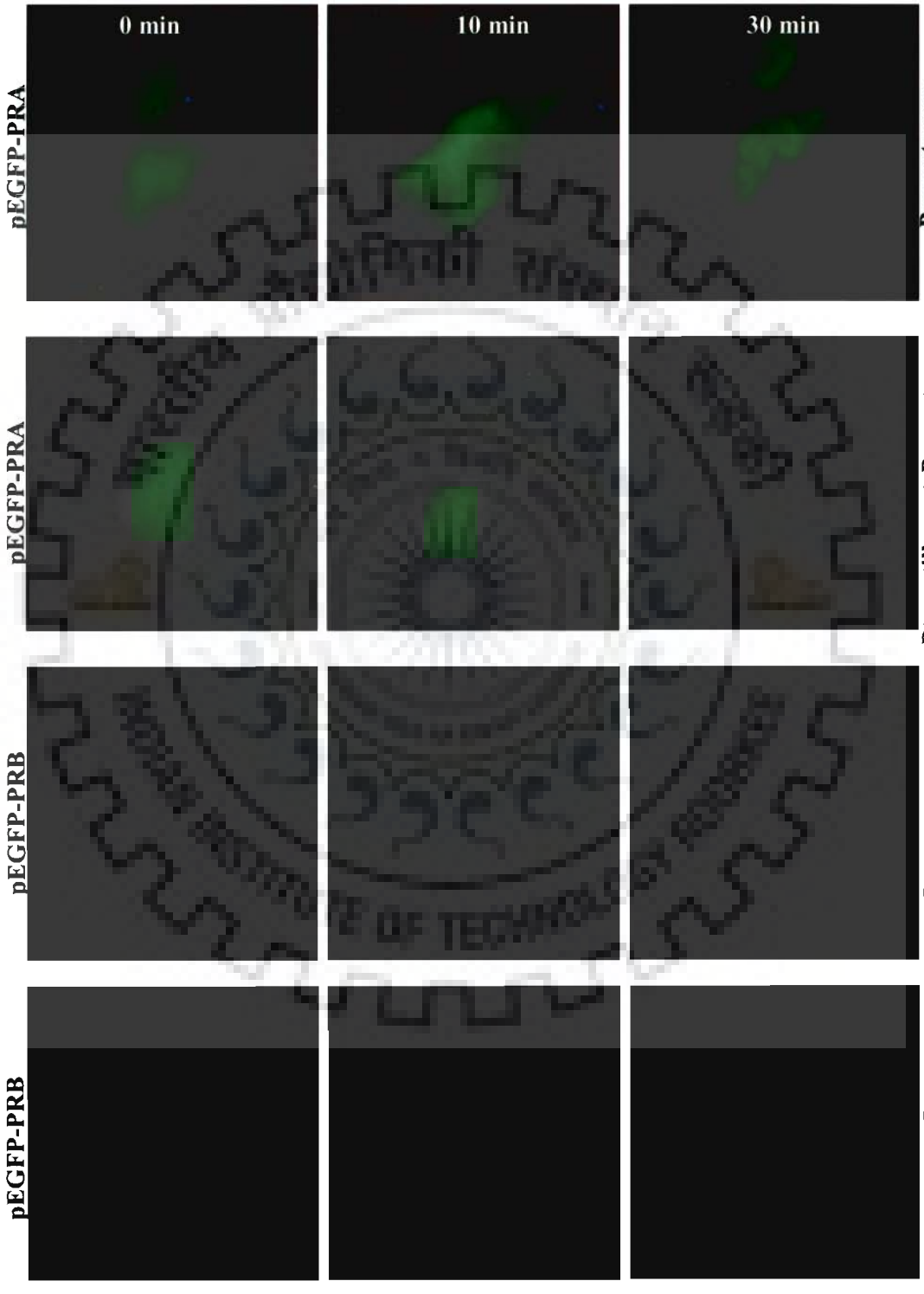


Fig. 8.7: *Localization of progesterone induced PR in presence or absence of pterostilbene. (40X objective magnification). The imaged is a representative of 3 individual experiments.*

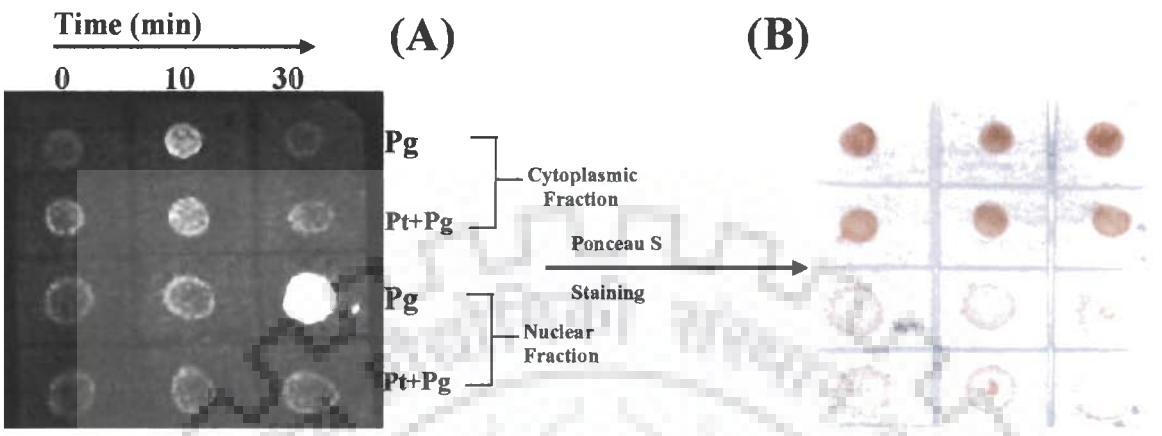


Fig. 8.8: *Dot blot analysis of nuclear and cytoplasmic fractions with PR specific antibody. After treatment with pterostilbene of T47D cells, nuclear and cytoplasmic fractions were separated at various time points for immunoblot analysis. The right-hand panel is the Ponceau S stained membrane for normalization purposes. Pt, pterostilbene; Pg, progesterone.*

8.3.7 Effects of pterostilbene in the binding of PR to its response element

The inhibition of the transactivational activity of PR by pterostilbene was further proved by the fact that in the presence of the stilbene molecule, PR-bound-PRE was much reduced in the nuclear fraction as revealed by the chromatin immunoprecipitation (ChIP) assay. The PCR amplification from imput chromatin of T47D cells although showed equal expression of PRE specific sequence but the PR antibody precipitated chromatin revealed that almost no binding is observed in the vehicle treated or pterostilbene treated cells. This was further validated by the fact that pterostilbene also inhibits the binding of PR to its response element in presence of progesterone (Fig. 8.9). From the present study, it was thus established that pterostilbene inhibits the progesterone mediated binding of PR to the PRE sequence of c-myc promoter, whose activity is regulated by progesterone receptor in breast cancer cells, causing their proliferation. The ChIP assay also revealed that both pterostilbene and RU486 (Fig 8.9) have inhibitory activity for the trans-activational regulation of PR.

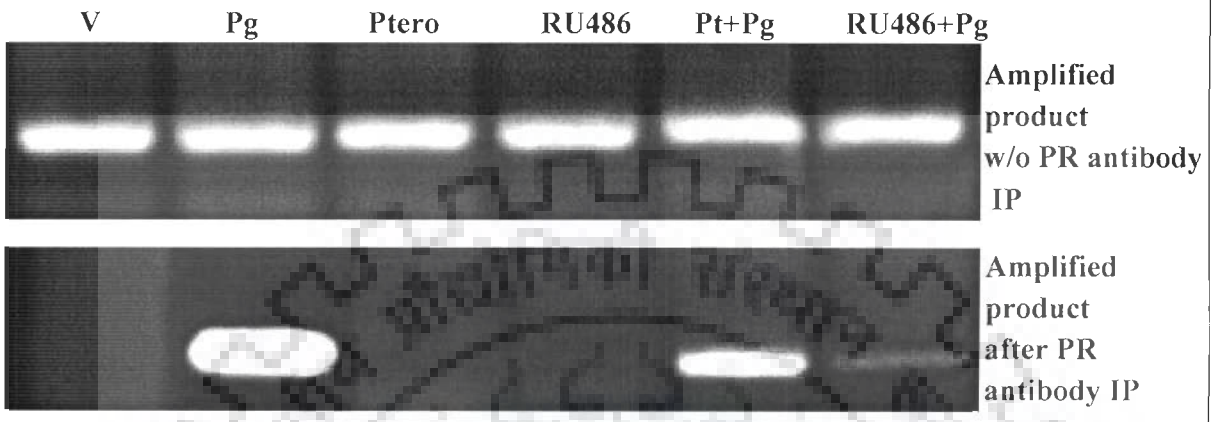


Fig. 8.9: *Effect of pterostilbene on the interaction of PR with its response element as determined by chromatin immunoprecipitation (ChIP) assay using PR antibody followed by a PCR amplification of its response element (PRE). V, vehicle control; Pt, pterostilbene; Pg, progesterone*

8.4 Discussion

The main objective of this part of study was to determine the effect of pterostilbene on the PR positive T47D breast cancer cell lines. PR has been known to negatively regulate apoptosis. It interacts with another transcription factor FOXO1 and essentially upregulates the cell survival genes like wnt, insulin receptor binding protein etc. (Labied et al., 2006; Takano et al., 2007). Previously (chapter 6), it was shown that the T47D cells treated with pterostilbene undergo a dose dependent cytotoxicity. This cytotoxicity was even higher than the endogenously ER expressing MCF-7 cells (Table 6.1). The induction of cytotoxicity at higher concentrations can be directly linked to an early inhibition of mitotic potential at sub-acute concentrations in these breast cancer cells. T47D cells are well-established PR positive breast cancer cell line (Sartorius et al., 1994). Solid tumors like that of breast cancers and endometrial cancers exhibit elevated mitogen activated protein kinase (MAPK) activity (Hoshino et al., 1999; Sivaraman et al., 1997) which is up-regulated by progestins (Lange et al., 1998). Cell proliferative factors like c-myc, c-fos and cyclin D1 and E are expressed in T47D cells and are also under the regulation of PR (Groshong et al., 1997; Musgrove et al., 1991; Skildum et al., 2005; Sutherland et al., 1998; Thuneke et al., 2000). Progesterone has been reported to activate PI3K/Akt/NFκB pathway to initiate cell cycle (Saitoh et al., 2005). Pterostilbene on the other hand is already

pathway to initiate cell cycle (Saitoh et al., 2005). Pterostilbene on the other hand is already known to inhibit the above pathways (Pan et al. 2009, 2011). The increases in the cell cycle regulator proteins can therefore be correlated with the high levels of PR mRNA which are expressed in breast and endometrial cancers (Mauland et al., 2011). In the context of efficient cytotoxicity and inhibition of mitotic potential in T47D cells, the effect of pterostilbene on the growth responsive steroid hormone action was investigated with special emphasis on PR.

As observed by western blot and RT-PCR analysis, pterostilbene inhibits or down regulates the PR expression in T47D cells under normal growth conditions. The transcriptional down-regulation of PR was the basis to check the effect of pterostilbene on the PR-responsive genes. Alkaline phosphatase is one of the progestin regulated gene in T47D breast cancer cell line (Lorenzo et al., 1991). It was observed that increased expression of alkaline phosphatase activity in presence of progesterone was significantly inhibited by pterostilbene. On the other hand, effect of pterostilbene alone was insignificant ($P<0.01$) on the above enzymatic activity. It can therefore be presumed that pterostilbene inhibits specifically the progesterone induced PR response. A similar study has also been reported by Wagner et al. (1999), where two novel synthetic steroidal compounds (RTI 3021–012 and RTI 3021–022) were screened for their competitive inhibition of agonist binding to PR.

Based on these data, where pterostilbene was found to cause the down-regulation of PR and its genes, it was intriguing to check the effect of pterostilbene on the PR regulated breast cancer marker oncoproteins. The promoter region of oncoproteins like c-myc and proteases like kallikrein family bear progesterone responsive element, which is induced by the ligand-receptor complex (Katiyar et al., 2009; Lai et al., 2009) and are highly expressed in breast cancers. It was found from the RT-PCR analysis of the breast cancer marker, KLK-4, that this gene was sharply down-regulated by about 1.8- folds after incubation of the breast cancer cells with pterostilbene. Similar results has also been reported for the anti-androgenic effect of resveratrol in prostate cancer cells, where the anti-proliferative effect of resveratrol was linked to the inhibition of androgen regulated kallikrein 11 (Seeni et al., 2008). Unlike resveratrol, no previous report is available for the effect of pterostilbene on the expression of kallikrein although pterostilbene is also known to be a potent anti-androgenic molecule (Wang et al., 2010). Kallikrein superfamily proteins being recent breast cancer markers (Lai et al., 2009; Papachristopoulou et al., 2011), the

molecular targeting of the protease with pterostilbene can add to the efficacy of the molecule towards breast cancer.

The effect of pterostilbene was further checked on the progesterone mediated activity *in vivo*. In uterine tissues, estrogen and progesterone function as antagonist to each other (Chetrite et al., 2004). Thus uterine expression of estrogen induced C3 mRNA expression is inhibited by progesterone. Thus the antagonistic role of these two hormones is used in the screening of antiprogestagenic compounds (Lundeen et al., 2001). As observed from figure 8.4, pterostilbene at a concentration of 30 mg/kg bw completely obliterated the effect of progesterone and the expression of C3 was almost equivalent to that of estradiol alone, supporting our hypothesis that pterostilbene acts as an anti-progestagenic molecule even *in vivo*. Whether pterostilbene bears an estrogenic effect on the rat uterine C3 mRNA cannot be obliterated from the present study. Although this aspect was not investigated in the present study, this phytochemical is found to inhibit PR at a much higher concentration (10-30 mg/kg bw) as compared to RU 486 (3 mg/kg bw). It can be added here that at this concentration the molecule has other functional properties also (as mentioned in earlier chapters) and is absolutely free of any physiological toxicity (Ferrer et al., 2005). Moreover, it is worth mentioning here that the plasma concentration and bio-availability of pterostilbene is more than that of resveratrol at this concentration (Ferrer et al., 2005). Further, it has also been seen that at 20 mg/kg bw pterostilbene is highly effective against the metastatic activity of B16 melanoma cells. *In vivo* effect of pterostilbene as anti-progestagen therefore adds up to its anti-tumor properties. Although, we did not check the effect of this stilbene on the PR status in mammary gland of the rat models, the uterine C3 mRNA conclusively proved that pterostilbene is antagonist to PR functions. The plant based molecules like, genistein, is also reported to regulate the steroid receptor functions *in vivo* and *in vitro* in female reproductive tissues (mammary gland, uterus, vagina and others) but their mode of action is different from the steroid hormone or their inhibitor functions. These molecules (genistein, resveratrol etc.) do not act through the pituitary axis *in vivo* (Bottner et al., 2006; Rimoldi et al., 2007), but their actions are more target tissue specific. Thus it further supports the fact that not only the steroid molecule, some non-steroidal phytochemicals can also regulate the steroid receptor functions.

To further assess the antagonist activity, (anti) progestagenic action of pterostilbene were tested using the reporter based transactivation assays. The transiently transfected T47D cells

with PRE-Luc construct showed that pterostilbene (without progesterone) had no effects on the PR mediated gene activation, but it down-regulated the progesterone induced luciferase activity in a dose dependent manner in these cells which expresses endogenous PR. The same response was observed in the PR negative cells (MDA-MB 231), which were transiently transfected with both receptor and reporter cassettes. This was in accordance with some earlier studies which reported that not only the steroid-like molecules, but also several non-steroidal aromatic compounds can behave as PR agonists/antagonists (Du et al., 2010; Kern et al., 2007; Zhang et al., 2008).

In order to elucidate further on the probable cross-talk of pterostilbene induced inhibition of progesterone receptor transcriptional regulation it was interesting to check the expression status of steroid receptor co-factors. PR associates with steroid receptor co-activators such as SRCs to activate transcription. Three members of the SRC/p160 family have been identified and characterized in PR-dependent transcription: SRC-1 (Halachmi et al., 1994), SRC-2 (GRIP-1) (Dong et al., 1997), and SRC-3 (Anzick et al., 1997). Ligand-bound PR interacts preferentially with SRCs (Amazit et al., 2003; Li et al., 2003). It is worth-mentioning that both SRC1 and GRIP-1 were down-regulated by 2.7 and 1.6- folds respectively by pterostilbene. These co-activators of nuclear receptor interacts separately with PR to form the nuclear receptor transcription factor complex (Boonyaratanakornkit et al., 2001; Castoria et al., 1999; Migliaccio et al., 1998; Skildum et al., 2005). Inhibition of SRC-1 and GRIP1 is also reported to be essential for estrogen receptor function and decrease in the expression of these two co-activators by a protein (Hakai) is linked to the negative role in the development and progression of breast cancers (Gong et al., 2010). These results suggest that pterostilbene not only shows anti-PR activity due to its receptor antagonistic properties but it might also inhibit some other interacting proteins, which in turn regulates the PR transcriptional activity. Previously, pterostilbene and other methyl ester derivatives of resveratrol have been shown to be more potent inhibitor of the androgen and estrogen mediated pathways (Wang et al., 2010). In the next part, it was checked if pterostilbene has any effect on the PR localization to the nucleus. It was found that there was an inhibition in the localization of PR to the nucleus even 30 min after the stimulation with steroid in presence of pterostilbene. A prominent cytoplasmic fluorescence was observed in pterostilbene and progesterone co-treatments with no nuclear specific intensity. A similar pattern of inhibition of progesterone receptor nuclear location has also been observed in two lines of non-tumorigenic human ovarian surface epithelial cells by estrogen (Mukherjee et al., 2005).

Anti-mineralocorticosteroids (ZK9 1587, SC9420, 18-vinylprogesterone) is also reported to maintain cytoplasmic distribution of the steroid hormone receptor and inhibit its aldosterone-dependent nuclear localization (Lombes et al., 1994). In case of androgen receptor (AR) the antagonist does not inhibit its nuclear localization but in absence of critical co-activators AR traverses the DNA binding in a transcriptionally silenced state (Kumar et al., 2008).

PR is responsible for differentiation and cell survival. In the previous chapters it was clearly shown that pterostilbene produces ROS and increases the expression of cEBP. Although the role of ROS is not studied in terms of PR in this chapter, but existing literature reports that ROS induces a higher levels of cEBPβ in endometrial cells, which in turn leads to the differentiation of these cells and also inhibited the actions of PR through the JNK pathway (Al-Sabbagh et al., 2011; Leitao et al., 2010). All these data could provide some indirect support to our findings.

It can thus be concluded that pterostilbene inhibits the PR regulated pathways in breast cancer cells and affects proliferation of PR positive T47D cells through various pathways. To the best of our knowledge this is the first study to show that pterostilbene acts as PR antagonist. Although the molecule is not as effective as RU 486 at least in lower concentrations, yet it showed promising response in this study even at a concentration which was non-toxic and was close to the dose used by some earlier authors (Ferrer et al., 2005). Moreover, the transcriptional inhibition of PR could also be linked to the failure of PR to localize into the nucleus in presence of this phytochemical. However, the present report warrants further investigations to understand the exact mode of PR inhibition by this phytochemical since some unanswered questions remains, like the levels of cross talks with PR, metabolism of progesterone etc. Hence a comprehensive study on this phytochemicals based on the present findings could support the therapeutic and prophylactic use of this molecule in PR positive breast cancers in future.

CHAPTER 9

SUMMARY

9 Summary

Cancer develops when a large number of genetic alterations accumulate to give rise to 'oncogene addiction' in the cells whereby the cancerous cells and their associated cellular environments are programmed either to proliferate or help the adjacent cells to proliferate. As a result of this a number of signature pathways are activated simultaneously in cancer cells (Santilli et al., 2011). This rationalizes the design and development of a number of molecules that has the capacity to target multiple pathways.

The work in the present thesis focused on the anticancer properties and the mode of action of two major class of chemicals: (i) a copper Schiff base compound (CuP1) and (ii) a phytochemical i.e., pterostilbene for prospective breast cancer therapy. CuP1 is a planar compound. It is reported by several groups that planar compounds have an affinity to enter into the stacks of nucleotides in a DNA double helix and if bound to the nitrogen bases, they hinder in the normal replication process of the growing cells (Humphreys et al., 2002; Koichiro et al., 2004; Chen et al., 2007). The data as presented in this thesis also showed that CuP1 has DNA binding activities in accordance with the previously established hypothesis. The in vitro studies are well supported by the cell based analysis which revealed loss of DNA integrity after being incubated with CuP1 for 24 h. The single cell gel electrophoresis showed that there was an induction of strand breakage of all individual MCF-7 breast cancer cells in CuP1 treatments which was even more than that of the cisplatin. Microscopic analyses of the drug treated nucleus also revealed prominent chromatin condensations. It is already established that hindrance in the DNA replication process is sensed by various DNA repair enzymes like GADD45, GADD153, ATM etc. which accumulates at the replication bubble and finally activate the cytoplasmic caspase cascade and CAD (caspase-activated deoxyribonuclease) to produce massive DNA fragments which is a characteristic feature of apoptosis (Loo, 2003; Matsuoka et al., 2007; Yu et al., 2003). The biochemical and transcriptional analysis of CuP1 treated mammary tumor cells showed a significant activation of caspase 3 and bax. Therefore, the DNA binding activity of CuP1 and the induction of intracellular DNA fragmentations can be rightly correlated to the growth inhibitory activity of the novel molecule CuP1.

There are already several reports that various copper complexes act as proapoptotic drug in pancreatic and prostate cancer cell lines (Adsule et al., 2006; Ahmed et al., 2007; Ambike et al., 2007). These compounds act through their DNA damage activity, they have been found to

inhibit COX-2, VEGF, Bcl-2, NFκB, survivin and other growth promoting proteins at inhibitory concentrations of < 10 μM (Adsule et al., 2006; Ahmed et al., 2007; Ambike et al., 2007). In this regard it is worth mentioning that CuP1 was found to be active at a further lower concentration of 4 μM. CuP1 inhibited the specific growth rate of tumor which decreased by 44% with a simultaneous 2- fold increase in the tumor doubling time. This was probably through the inhibition of phosphorylation of Akt and p38 and their target genes since it is been already reported that MNU treated tumor bearing animals shows an increased phosphorylated Akt and MAPK (Ghayad et al., 2010; Liao et al., 2005). These pathways play a central role in activation of growth promoting proteins like c-myc, cyclin D, STAT, TNFα, growth factor receptors, HIF and metabolic enzymes like glycogen synthase, pyruvate dehydrogenase kinase etc. (Kim and Dang 2006). Although not all synthetic molecules act on the various protein targets, CuP1 has been found to affect the transcriptional status of Akt, TNFα, Wnt, MMP9 and AMACR in MNU induced rat tumor tissue. Moreover, the Bax:Bcl-2 ratio increased significantly in the tumor tissues. This study therefore showed that CuP1 can induce apoptosis in breast cancer cells by targeting DNA as well as few growth promoting kinases.

The development of mammary gland tumors by MNU is an excellent and well established animal model to check the anti-cancer properties of any molecules in vivo (Davoodpour et al., 2007; Thomson and Adlakha, 1991). The highly reactive nitroso group of MNU causes DNA adducts formations which later give rise to genetically altered tumor cells at the site of injection (Baird and Mahadevan, 2004). Previously it was established by Naiki-Ito et al. (2007) that peroxide ions are generated due to MNU treatments. Peroxidases are less active in cancer cells which results in the general hypoxic cellular environment. CuP1 further reduced the glutathione peroxidase in mammary tumor cells thereby increasing the ROS load in the breast tumor. ROS at higher concentrations changes the mitochondrial membrane potential to release cytochrome c in the cytosol (Filomeni et al., 2005). Higher concentration of ROS is thus an effecter signal for apoptosis. Unlike many other efficient metal complexes which induce systemic toxicity in animals, CuP1 had minimal hepatotoxicity or nephrotoxicity. All the above findings has been summarized in Fig. 9.1 which hypothetically shows the probable pathways by which CuP1 may act in a cancerous cell.

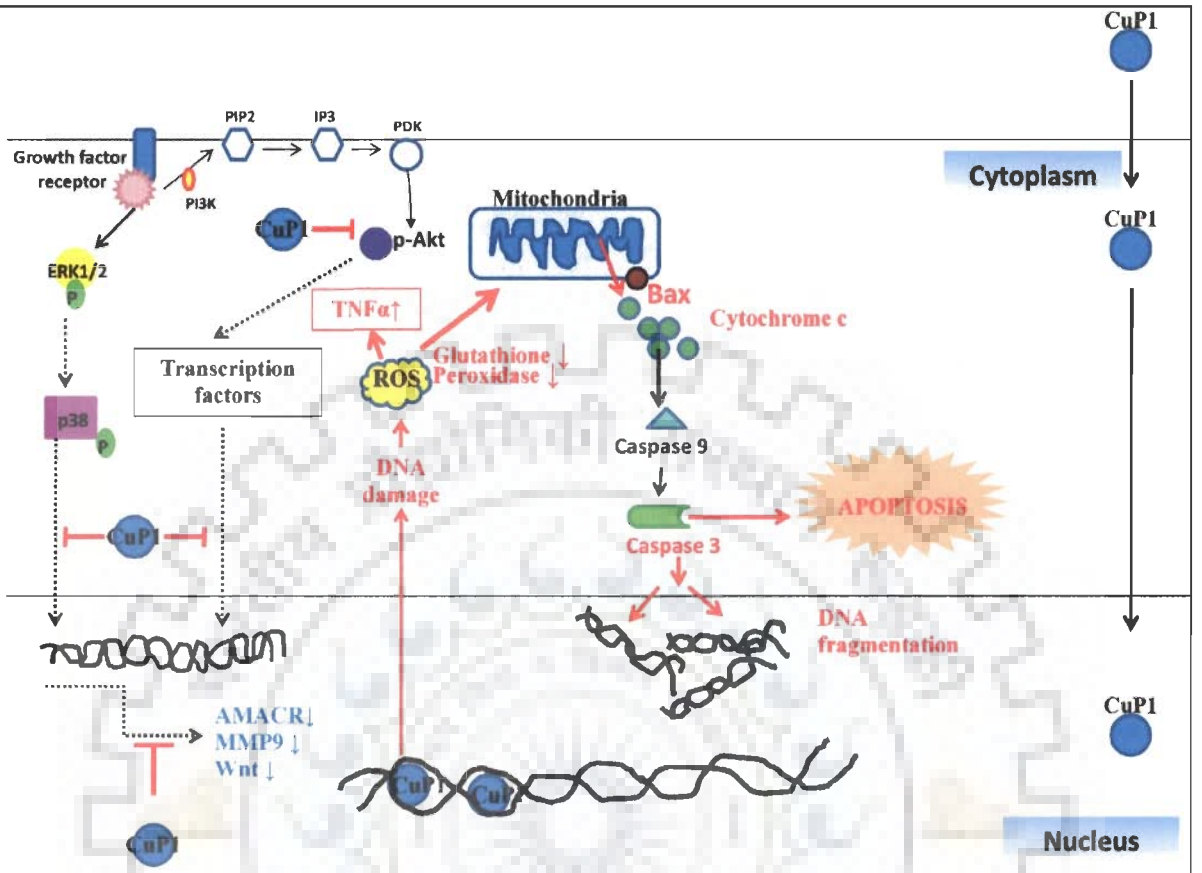


Fig. 9.1: *Schematic diagram representing the molecular pathways activated by CuP1 in rat breast tumor tissue. (All markings in red show the direct regulatory action of CuP1 (either activation – arrow heads or inhibition – blunt heads); black arrows represent the normal pathways and broken arrows represent the cancerous pathways that are indirectly inhibited by CuP1.)*

In the next part of the present thesis the anticancer properties and the molecular mechanism of a natural polyphenolic molecule, pterostilbene, was tested on breast cancer cell lines. Dietary polyphenols have multiple sites of action. As discussed previously, cancer therapy requires simultaneous targeting of various proteins as many cell survival pathways are activated in a genetically altered cell (Santilli et al., 2011). Due to this property polyphenols like curcumins, quercetin and resveratrol have been reported to be active against different types of cell lines (Sarkar et al., 2009). Of these the curcumin and its derivatives are already in clinical trials (Shehzad et al., 2010). In order to meet the requirements of the thesis, pterostilbene was purified from its natural source, *Pterocarpus marsupium*.

Similar to CuP1, the anticancer properties of pterostilbene were found to cause cellular DNA damage. It was found that pterostilbene caused DNA fragmentation and chromatin

condensation in MCF-7 cells. But when the DNA binding activity was checked in vitro, unlike CuP1, the stilbene molecule did not show any ethidium bromide displacements in solution. Therefore, it was presumed that the phytochemical has a different mode of action for causing cellular DNA fragmentation. The next obvious question answered was whether the molecule caused apoptosis. It was found that the pterostilbene molecule caused more efficient apoptosis which was linked to the up regulation of caspase activity and cell membrane blebbing even more than cisplatin. In gastric carcinoma cells pterostilbene is reported to induce both the extrinsic and intrinsic pathways of apoptosis due to the activation of more than one caspase types (caspase- 2, -3, -8, and 9) (Pan et al., 2007).

The developments of breast cancers are regulated by steroid hormones (mainly estrogen and progesterone) and hence majority of researches are focused on the development of anti-hormone therapies like tamoxifen. However, the biggest challenge with these drugs is that the breast cancer cells have a tendency to develop resistance towards these anti-hormone drugs (Dorssers et al., 2001). These limitations can be addressed with more generalized drugs like polyphenols which have multiple targets of action. The present data showed that pterostilbene arrested the growth of both ER and PR positive breast cancer cell lines and was more specifically antagonist to PR. Further it was able to inhibit a group of cancer related genes like Akt and Nkx3.1, AMACR, and MMP9 which are regulated directly or indirectly by Akt. Simultaneously it induced the expression of proapoptotic genes like p53, Bax and both caspase 3 and 9.

The most interesting feature of pterostilbene action in breast cancer cells tested was the up regulation of hydrogen peroxide and singlet oxygen species. Antioxidant action of pterostilbene was reported long way back by Charvet-Faury et al. (1998) which showed that it scavenges reactive oxygen species in vitro. On the contrary, the data presented in this thesis showed that apoptosis caused by pterostilbene in breast cancer cells is rather due to the generation of reactive oxygen species. It was further found that in addition to the increase in the intracellular ROS levels there was a release of mitochondrial cytochrome c into the cytoplasm. This finding was in accordance with a recent report which showed that pterostilbene induces the mitochondrial depolarization (Alosi et al., 2010). The anti-cancer properties of pterostilbene was abolished in the presence of a potent ROS scavenger i.e. catalase which further supported our hypothesis. Hence, from the present findings, it could be concluded that ROS is an effecter

molecule for the anticancer properties of pterostilbene. The ROS overload is sensed by the stress responsive genes like ASK1 which finally induces the mitochondrial membrane depolarization (Noguchi et al., 2005) and the MAPK mediated phosphorylation pathways which are exhaustively needed for apoptosis in case of oxidative stress (Filomeni et al., 2005). The generation of ROS by pterostilbene can be rightly linked to the phenoxyl radical generation of the polyphenol backbone which mediates the overproduction of hydroxyl ions (Loo, 2003). Although this theory is rarely considered in the anticancer properties of a polyphenolic molecule, the results of the present thesis was further is supported by the recent reports by Duo et al. (2011) and Ullah et al. (2011) which established that the actions of molecules like capsaicin and genistein are dependent on the intracellular ROS generation and that ROS scavengers inhibit their anticancer properties. The depleted glutathione and the low antioxidant enzymes in cancer cells which are insufficient to scavenge the amount of ROS overload produced in the cells cannot finally tolerate the oxidation insult (Loo, 2003). With decrease in glutathione levels, the ROS molecules generated by pterostilbene finally fails to nullify its adverse effects and undergoes apoptosis in breast cancer cells. The overall apoptotic pathways activated by pterostilbene in a breast cancer cell is schematically shown in Fig. 9.2.

ROS in addition to resulting in mitochondrial depolarization and apoptosis are also involved in changing the oxidation states of different cellular molecules like sterols. Since high dose of pterostilbene treatment showed promising anticancer proprieties it was intriguing to check the effect of this phytochemical at low dose but prolonged exposure. In order to further address the result under this condition the sterol content of the MCF-7 cells were analyzed. It was found that pterostilbene caused accumulation of neutral lipids and oxidized sterols and the breast cancer cells differentiated into more epithelial like morphology with simultaneous growth arrest. Further, the metastatic properties were also highly challenged by this sub-acute dose of pterostilbene. The accumulation of neutral lipids was more specific to the breast tumor cells and normal mammary gland cells were not found to accumulate these neutral lipids. The oxysterols have been reported to have two roles in breast cancer cell growth inhibition. Firstly, they induce breast cancer cell differentiation and secondly they cause autophagy (Payre et al., 2008; Medina et al., 2009) which is quite active in the development of normal mammary gland cells (Lockshin et al., 2000; Motyl et al., 2007). In the ER positive breast cancer cells pterostilbene was found to induce autophagy as evidenced from the expression of autophagic marker proteins (Beclin 1 and LC3II) as early as 12 h which was highest at 48 h and continues till 72 h. In addition to these

proteins there was remarkable appearance of autophagic vesicles and neutral lipids within the cells.

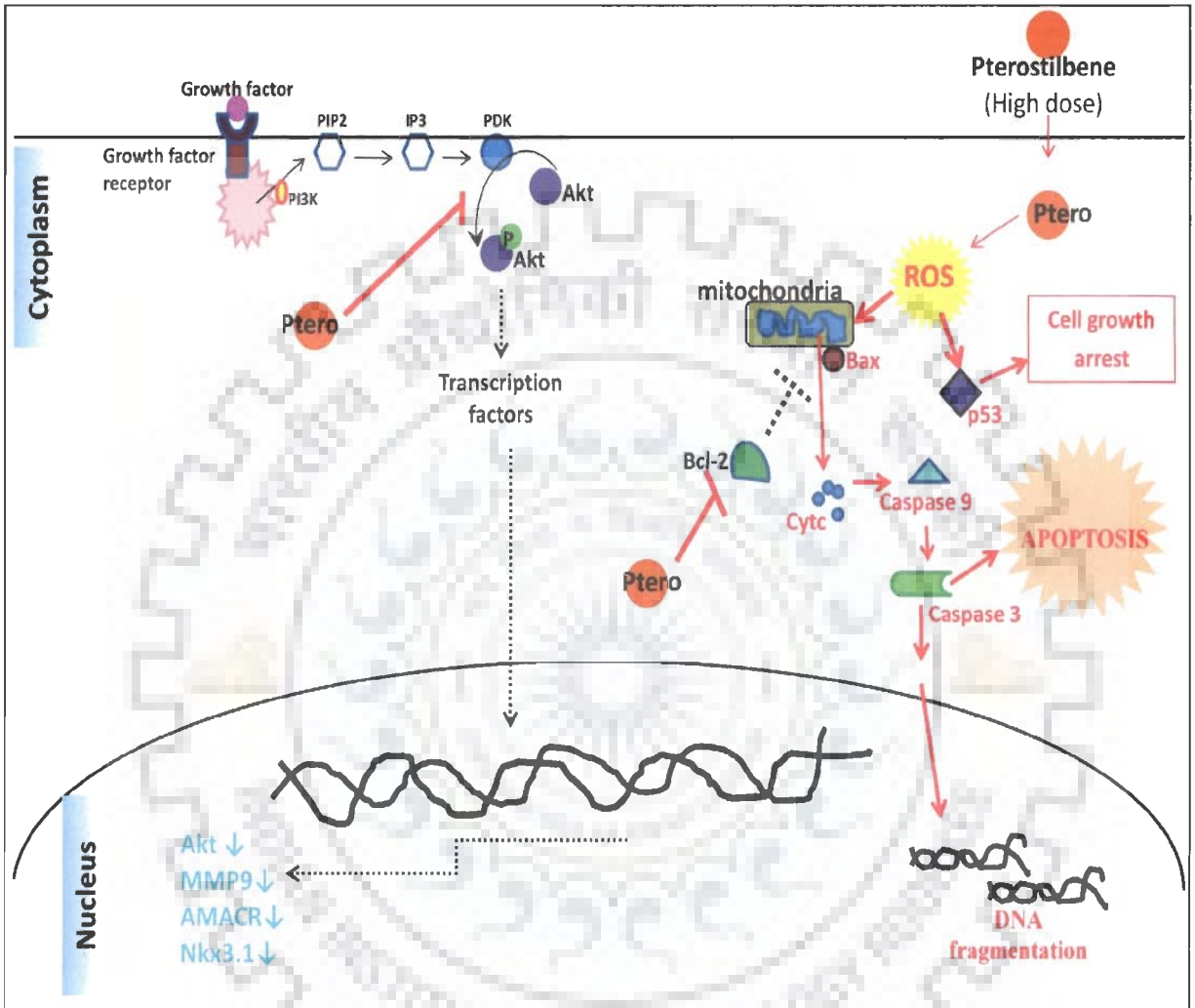


Fig. 9.2: *Schematic diagram representing the apoptotic pathways activated by pterostilbene. (All markings in red show the direct regulatory action of pterostilbene (ptero), either activation- arrow heads or inhibition- blunt heads ; black arrows represent the normal pathways and broken arrows represent the pathways that are inhibited indirecty by pterostilbene.)*

These findings are well supported by similar evidences as obtained in case of tamoxifen. Further, according to a recent report pterostilbene has been shown to induce autophagy in bladder cancer cell line even without causing any apoptosis (Chen et al., 2010). At the sub-acute dose of pterostilbene that was used here in the present study also did not show any significant apoptosis. Hence it could be presumed that when apoptosis is not activated by the low dose of pterostilbene it tends to inhibit the breast cancer cell proliferation by differentiating them and

inducing the phenomenon of autophagy. The involvement of Beclin 1 in pterostilbene mediated autophagy distinguishes this molecule from its analogue resveratrol in terms of the induction of autophagy (Scarlatti et al., 2008).

When ROS is one of the causes, the sterol production and its homeostasis should also be considered. It was found that oxysterol production requires active transcription and translation of proteins in pterostilbene treatments. The oxysterol metabolic regulator (LXR) is also highly expressed with simultaneous over-expression of the oxysterol transporters by pterostilbene to maintain the efflux of these oxysterols (Baldan et al., 2006; Rudolph et al., 2007; Schroepfer, 2000; Tall, 2008; Yang et al., 2006). However, due to the impairment of cholesterol producing enzymes (DHCR7) there was a prominent increase in the oxidation susceptible sterol molecule (7-dehydrocholesterol). This is regarded as the causative agent for the sterol overload inside the pterostilbene treated cells. The role of this sterol in growth inhibitory mechanisms is well supported by the studies of Payre et al. (2008). Finally a significant co-relation was found among the increase in neutral lipids, triglycerides, 7-dehydrocholesterol, the change in the cell morphology and the transcription of c/EBP, the differentiation marker and autophagy (Fig. 9.3). This study also showed that LXR over expression can protect the ER positive breast cancer cells from differentiating and accumulation of excess lipids but its role in the regulation of autophagy could not be clearly understood from the present findings.

As discussed before, effect of pterostilbene is not only restricted to the ER positive breast cancer cell line but the PR positive T47D cells have also been found to be effectively inhibited by pterostilbene at low concentrations. Progesterone is reported to cause growth in cancer cells while pterostilbene is already proved to be growth inhibitory in this study. Hence the effect of pterostilbene on PR regulated growth was worth investigating.

It is found from the present study that pterostilbene is anti-progestagenic in nature. It inhibited the expression and transactivation of PR. Inhibition of PR transactivation is usually used to screen the anti-progestagenic action of different compounds (Du et al., 2010; Kern et al., 2007; Zhang et al., 2008). Anti-progestagenic compounds are now focused as a therapy of breast, uterus and ovarine cancers in females (Chakraborty et al., 2010a).

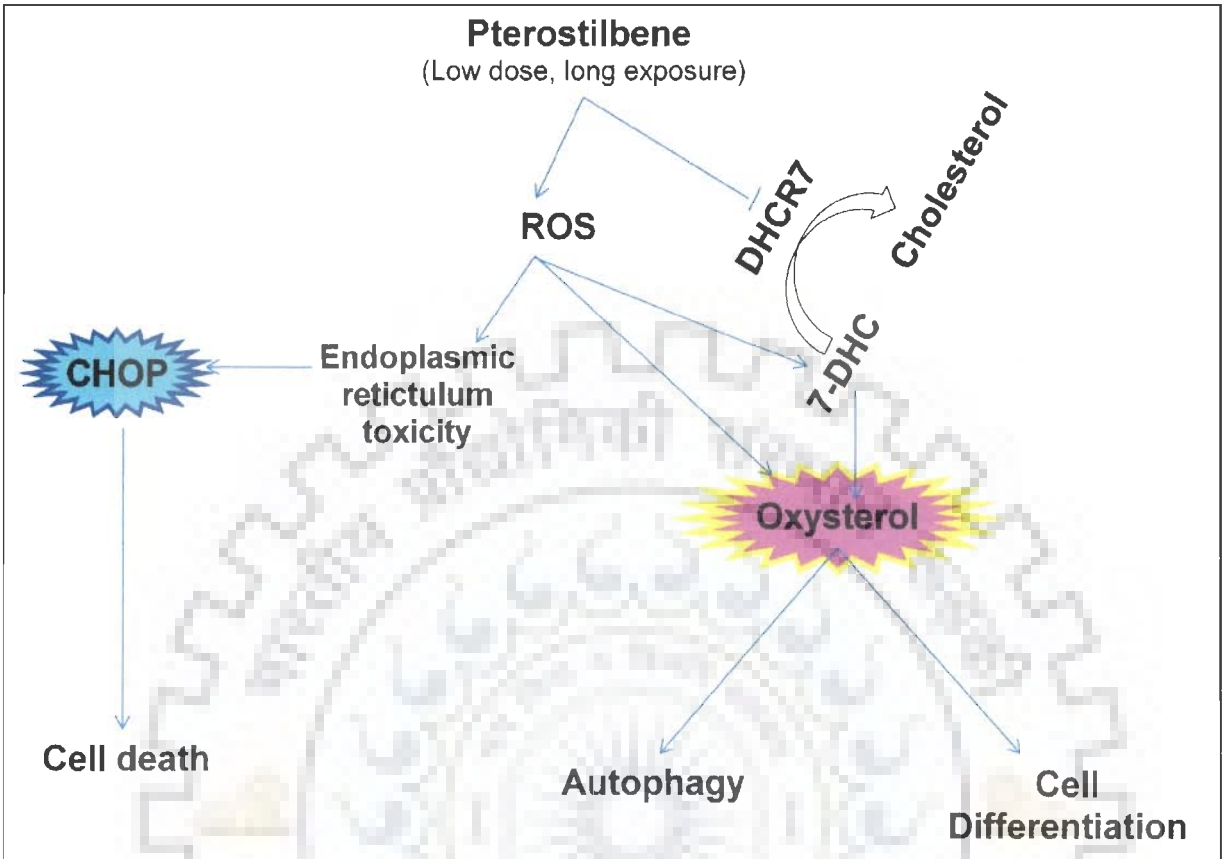


Fig. 9.3: *Schematic diagram representing ROS mediated induction of autophagy and differentiation in MCF-7 cells by pterostilbene.*

Although some natural phytochemicals bearing anti-progestagenic and anti-androgenic action like N-butylbenzene-sulfonamide (extracts of *Pygeum africanum*) (Papaioannou et al., 2010) are less studied so far, the new discoveries of such type of molecules will be of good help in various therapies due to less side effects. Moreover, these molecules act differentially on the cancer and the normal cells in a way that the normal physiology of the individual is usually not affected with even high doses of these molecules unlike the synthetic chemotherapeutic drugs. Here it is worth mentioning that the effective concentration of pterostilbene was much higher than that of mifepristone (PR antagonist) and that the former modulated the action of PR but could not bring the activity to basal level. However, this does not limit this phytochemical to be considered as potent anti-progestagen since there are enormous scope for its structural improvements

Earlier it was reported that pterostilbene inhibits AR activities in prostate cancer cell lines (Wang et al., 2010). Although these polyphenols have not been reported till date to bind

directly to the hormone receptors to inhibit their action but these molecules have been reported to modulate the transcriptional regulation of the hormone receptors. It is evident from the present study that the transcription of metastatic protease KLK4 and protein expressions of the PR co-factors were are down regulated by pterostilbene in a time dependent manner. Also the progesterone mediated enzymatic activity of alkaline phosphatase was inhibited in a dose and time dependent manner. The antagonistic activity of pterostilbene for PR was further linked to the inhibition ligand induced nuclear localizations of PR. PRA is more nuclear and PRB is localized in both nucleus and cytoplasm (Boonyaratanakornkit et al., 2007). Our data showed that the effect of pterostilbene on PRB was therefore more pronounced than that of PRA where it significantly inhibited the migration of the receptor to the nucleus. All these data suggested that pterstilbene has a potent anti-progestin like activity. This is the preliminary evidence in this direction which needs further validation which is beyond the scope of this thesis. However, if this concept of anti-progestin activity of pterostilbene is explored further for the development of anticancer molecule it may provide a drug with high efficacy but with least side effect since is active at very low dose.

In conclusion, in the present thesis various classes of chemicals were screened for their anticancer properties with special emphasis on breast cancer and an attempt was made to understand their modes of actions using both in vitro and in vivo approach. The present thesis especially reinforces the concept that various phytochemicals if tested properly could lead to the development of some potent anticancer drugs. However, given the complexities in targeting various signal transduction pathways involved in the development of cancer together with several other unknown factors, further investigations with in vivo and in vitro experimental models are required to get a final mode of action of these potent chemicals. The information as reported in this thesis could for sure provide a platform for taking up those two chemicals (Cupl and pterostilbene) for further detailed preclinical and clinical studies to test their efficacy in human systems.

CHAPTER 10

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10 Bibliography

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