EVALUATION OF BREAST CANCER CELL LINES (MCF-7 & T47D): INFLUENCE OF INSULIN AND METFORMIN ON GROWTH PARAMETERS

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BY

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CERTIFICATE

I declare that the dissertation entitled **"EVALUATION OF BREAST CANCER CELL LINES (MCF-7 & T47D): INFLUENCE OF INSULIN AND METFORMIN ON GROWTH PARAMETERS"** has been prepared by me under the guidance of Prof. P. Ramarao, Dean, School of Basic and Applied Science, Central University of Punjab, Bathinda and Dr. Sanjeev Thakur, Assistant Professor, Centre for Biosciences, Central University of Punjab, Bathinda. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Evaluation of Breast Cancer Cell Lines (MCF-7 & T47D): Influence of Insulin and Metformin on Growth Parameters

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Breast cancer is the complex and heterogenous malignancy caused as a result of interaction of hormonal, environmental and genetic factors. Major advances made in the field of breast cancer research have emanated from studies involving human breast cancer cell lines MCF-7 and T47D.

Insulin act as a potent mitogen in normal mammary tissue and breast cancer cells in culture. Insulin receptor is overexpressed in several human breast cancers and this overexpression results in transformed phenotype in human mammary epithelial cells. Experimental study has shown that Insulin induces significant mitogenic effect in MCF-7 and T47D cells in dose-dependent manner. 100nM concentration of Insulin enhances the growth of MCF-7 and T47D cells by ≈ 7.3 and ≈ 5 folds respectively in comparison to control.

The antidiabetic drug metformin lowers risk of breast cancer via direct action (Insulin independent) and indirect action (Insulin dependent). The treatment of the hyperinsulinemia with the metformin will lower the circulating levels of insulin and improve insulin sensitivity, thus results into potential decrease in the incidence of breast cancer. The results show that MCF-7 and T47D cells growth inhibited over control at high doses (10 mM concentration) of metformin. MCF-7 and T47D cells also show decrease in insulin induced cell proliferation at higher doses of metformin.

(Prateek Sharma) (Dr. Sanjeev Thakur) (Prof. P. Ramarao)

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Prateek Sharma

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Adenosine diphosphate	ADP
2	Adenosine monophosphate	AMP
3	AMP activated protein kinase	AMPK
4	Androgen receptor	AR
5	American type culture collection	ATCC
6	Adenosine triphosphate	ATP
7	Dulbecco's modified eagles medium	DMEM
8	Dimethyl sulfoxide	DMSO
9	Ethylene diamine tetraacetic acid	EDTA
10	Estrogen receptor	ER
11	Estrogen receptor positive	ER+ve
12	Fetal bovine serum	FBS
13	Human epidermal growth factor 2	HER-2
14	Insulin receptor	IR
15	Insulin receptor substrate	IRS
16	Mitogen activated protein kinase	MAPK
17	Minimum essential medium	MEM
18	Mammalian target of rapamycin	Mtor
19	Millimolar	mM
20	Nanomolar	nM
21	Phosphate buffered saline	PBS
22	Phosphoinositide dependent kinase	PDK
23	Phosphatidylinositol-3-kinase/Phosphoinositide-3-kinase	PI3K
24	Phoshatidylinositol-3-phosphate	PIP3
25	Protein kinase B	PKB
26	Protein kinase C	PKC
27	Progesterone receptor	PR

28	Src homology-2	SH2
29	World health organization	WHO
30	Microlitre	µL
31	Micromolar	µM
32	Degree Celsius	°C
33	Molar	M
34	Millilitre	mL
35	Millimolar	mM
36	Type 2 diabetes	T2D

CHAPTER 1

INTRODUCTION

1. Introduction

Cancer is a leading cause of deaths globally. According to World Health Organization (WHO) cancer factsheet, in 2008 about 7.6 million deaths (around 13% of all deaths worldwide) occur because of cancer malignancy (World Health Organization Fact Sheets 2011). The term "Cancer" is used for group of many diseases resulting due to the failure/breakdown of fundamental regulatory mechanism that usually control the normal growth and proliferation of cells. The loss of growth control exhibited by cancer cells is the net result of accumulated abnormalities in multiple cell regulatory systems. It is caused by multiple factors, like as genetic predisposition, chronic persistent inflammation, environmental factors, life style, ageing etc. There are more than 100 different types of cancer. Most of the cancers are named after the cell type or organ in which they start.

1.1 Breast Cancer

Breast cancer is a cancer that forms in the tissues of the breast usually the ducts (tubes that carry milk to the nipple) and lobules (milk forming glands). It occurs in both men and women, although the cases of male breast cancer are very rare. It is a complex and heterogenous malignancy caused as a result of interaction of hormonal, environmental, genetic and lifestyle factors. Today breast cancer has become the second most common cause of female deaths in western world.

1.1.1 Anatomy of the breast

The female breasts are modified sweat glands composed of lobules/lobes (glandular tissue) and ducts (small tubes) interspersed with adipose tissue (fatty tissue) and connective tissue.

Lobules are the functional secretory units (having milk glands) where milk is made. Ducts drain out from each lobule and converge to form a lactiferous duct. The lactiferous ducts merge together just beneath the tip of the breast i.e. nipple to form a

lactiferous sinus. Ducts carry the milk from lobules to nipple. Each duct has an epithelium lining surrounded by a thin layer of myoepithelial cell.

Under the skin, a part of breast tissue extends into the armpit (axilla). The armpits have a collection of lymph nodes (lymph glands), which are part of the lymphatic system. Lymph nodes are also present just beside the breast bone and behind the collar bones.

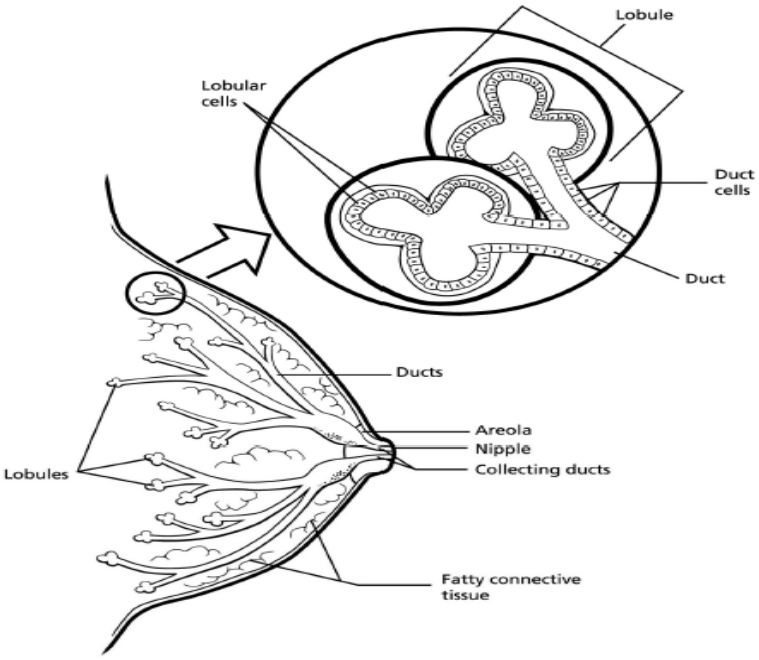

Figure 1.1 Anatomy of the breast (Source: Adapted from Breast Cancer Facts & Figures 2009-2010, American Cancer Society, 2011)

1.1.2 Breast cancer statistics

Breast cancer is the most common cancer in women in developed countries, while its incidence is continuously rising in developing countries at an alarming rate. According to the International Agency for Research on Cancer (IARC) report in 2008, 1384155 women were diagnosed with breast cancer all over the world and approximately 458503 died from this neoplasm (Global Cancer Statatics Fact Sheets-International Agency for Research on Cancer, 2011). According to the report of American Cancer Society, 207090 new cases of breast cancer in women were

diagnosed in the America in 2010 and nearly 40,000 women died because of this malignancy (Cancer Facts & Figures- 2010- American Cancer Society, 2011). Recent studies and statistics have suggested that breast cancer has become reason for the third most number of cancer-related deaths in Europe (Ferlay *et al*. 2010).

In India also breast cancer is the second most common neoplasm in women after lung cancer and is spreading at faster rate especially in urban societies. Epidemiological studies have shown that the incidence of breast cancer vary greatly in different geographic regions. In general, women in developed/industrialized countries are more prone to the risk of breast cancer in comparison to the women of developing/third world countries. In Indian scenario since more and more women are beginning to work outside their homes, allowing various risk factors of breast cancer to come into play, resulting into higher incidence of breast cancer cases.

1.1.3 Molecular classification of breast cancer

Array techniques and gene expression profiling have led to the development of molecular classification of breast cancer. Various subtypes of breast cancer were determined using cDNA microarrays, which are differentiated in their pattern of genetic expression and in prognosis. Breast cancer is divided into two divisions based on the basis of the presence/ absence of genetic expression of Estrogen receptor (ER), which has been identified as the important discriminating factor of molecular subtype. The genetic expression profiling has proposed three ER +ve subtypes: luminal A, B and C, although the stability of luminal C is still unclear. ER −ve subtypes are: HER-2, basal type and normal type. The normal type subgroup may represent only an extension of the expression profile between HER-2 and basal type (Andre and Pusztai 2006).

1.1.4 Risk factors for breast cancer

Although a number of epidemiological risk factors have been identified, the exact cause of breast cancer is still unknown. However, there are numerous primary risk

factors that have been responsible for incidence of breast cancer. These involve: age (Kelpin *et al*. 2009, Key *et al*. 2001) gender (Key *et al*. 2001), race (Key *et al*. 2001, Benjamin *et al*. 2003), ethnicity, family history of the disease(Family History, Inheritance, and Breast Cancer Risk Fact Sheet, 2011), genetical factors (inherited mutations in the BRCA1 and BRCA2 genes) (Oesterreich and Fuqua 1999), long estrogen exposure (Yager and Davidson 2006, Clemons and Goss 2001), obesity, excessive alcohol intake, menstrual history, environmental factors involving carcinogens like tobacco or radiation, life style factors, contraceptive factors and many more (Breast Cancer Facts & Figures 2009-2010, American Cancer Society, 2011).

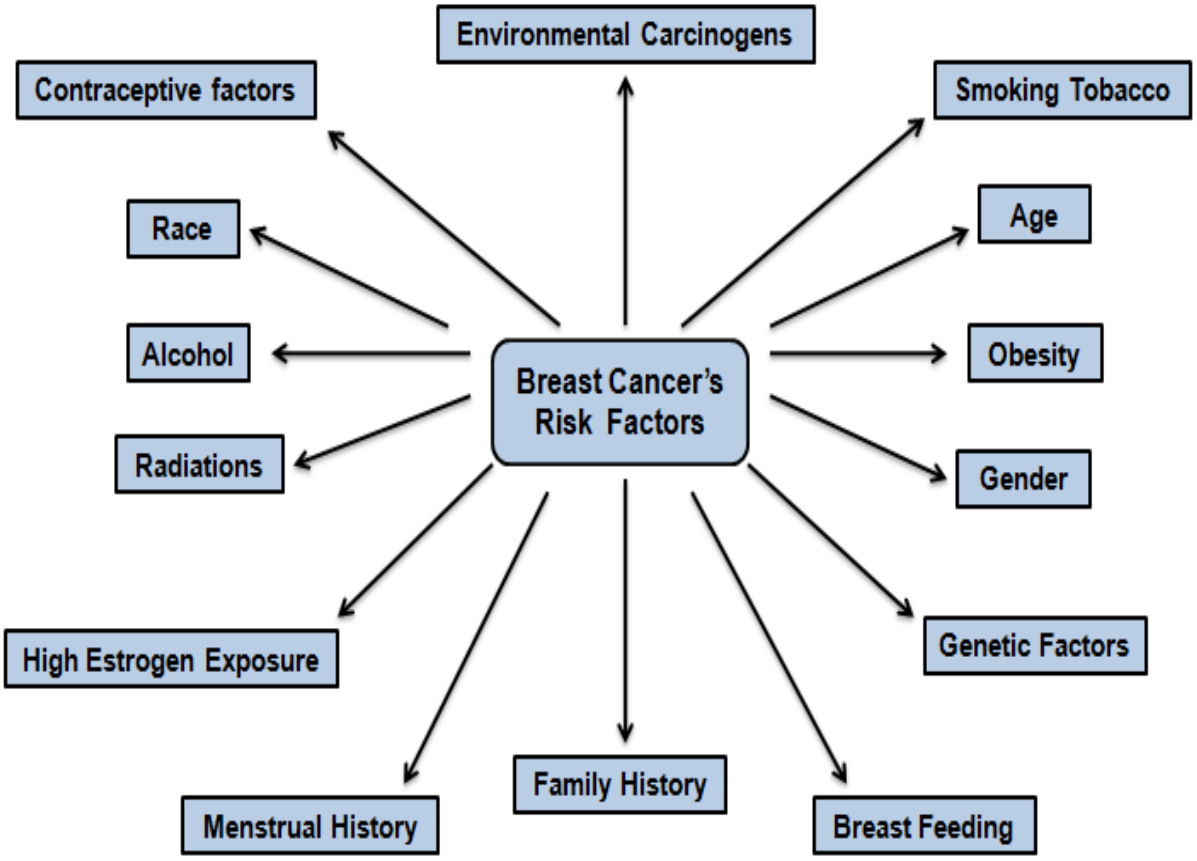

Figure 1.2 Risk factors for development of breast cancer

1.2 Breast Cancer: Diabetes

Insulin is also a growth-promoting hormone with potent mitogenic effects in both normal and malignant breast tissue (Gliozzo *et al.* 1998, Bentel *et al.* 1995, Yang *et al.* 1996, Pollak *et al.* 1990). Insulin suppresses IGF binding protein-1 and thereby increases bioavailable Insulin like Growth Factor-1 (IGF-1). The effect of estradiol on hormone-dependent breast cancer cell proliferation may depend on the presence of insulin or IGF (Conover *et al.* 1992).

Insulin resistance coupled with an insulin secretory defect causes the metabolic disorder type 2 diabetes (T2D). Hyperinsulinemia with insulin resistance also has been associated with increased risk of breast cancer (Papa and Belfiore 1996, Milazzo *et al.* 1992). Obesity is also associated with type 2 diabetes and leads to a rise in endogenous estrogen levels.

With the worldwide increase in breast cancer and type 2 diabetes, an association between type 2 diabetes and breast cancer might have public health implications.

1.2 Cell line

Major advances have been made in the field of breast cancer biology, a significant proportion of which have emanated from studies involving human breast cancer cell lines. These cell lines are widely used to study different aspects in laboratory/ clinical research and particularly as experimental models in breast cancer research (Burdall *et al.* 2003). Cell lines provide a number of advantages; for example, they are easy to handle and represent an unlimited self-replicating source that can be grown in almost infinite quantities. In addition, they exhibit a relatively high degree of homogeneity and are easily revived from frozen stocks if lost through contamination (Osborne *et al.* 1987).

However, there are also some disadvantages associated with the cell lines. Cell lines exhibit genotypic and phenotypic changes during their continual culture. These

changes are particularly recorded in frequently used cell lines, especially those that have been deposited in cell banks for several years. Subpopulations may arise and cause phenotypic changes over time by the selection of specific, more rapidly growing clones within a population (Bahia *et al*. 2002).

Also variations in cell growth rate, karyotype and clonogenicity are shown, despite the cells appearing morphologically identical. This creates a serious problem, especially if the cell lines are to be regarded as valid models for evaluating the pathobiology of cancer and/or the likely response to novel drug therapies. Therefore in order to eliminate all these irregularities from the cell lines, it is vital to confirm the identity of the cell lines and exclude the possibility of contamination .i.e. their Validation is necessary. Validation of cell line involves authentication, characterization, and the demonstration of the lack of contamination in a cell line. Validation of cell line is carried out by measuring different inherent properties/ characteristics of the cell line such as cell morphology, proliferation/growth rate, yield, viability, plating efficiency, quantitation, ease of harvesting, stability etc (Freshney 2005).

The present study is also carried out in the cell lines with a purpose to further investigate the link between the breast cancer and diabetes.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of Literature

2.1 Breast Cancer and Cell lines as a Model System

Cell lines are widely used in research as in vitro model system for mechanistic studies in the cancer research. Initially the propagation and establishment of human breast cancer cells in long term culture conditions was proved to be very difficult, even when significant advances were arising from other types of cancers.

The first stable, well documented human breast cancer cell line, BT-20, was established in 1958 (Lasfargues and Ozzello 1958). This achievement was followed by the establishment of the CaMa cell line by Dobrynin in 1963 (Dobrynin 1963). During the early 1970s several new cell lines such as HBT-3 and HBT-39 were isolated but they were having contamination of HeLa cells (Nelson-Rees and Flandermeyer 1977, Engel and Young 1978). The last of these early breast cancer cell lines to be developed was the SKBr-3 cell line (Trempe and Fogh 1973, Fogh and Trempe 1975), which became a popular experimental model during the decade of 1990s due to amplification and overexpression of the HER-2/erbB2 receptor tyrosine kinase, an important oncogene in human breast cancer.

An important development in this field occurred when Cailleau et al. in 1974 discovered that breast cancer cells derived from pleural effusions could more readily adapt to in vitro culture conditions in comparison to the cells derived from explants of solid tumors. This discovery led first to the propagation & establishment of the MDA MB-134, MDA MB-175 and MDA MB-231 cell lines and subsequently to many others (Cailleau et al. 1974). The success cell lines derived from pleural effusions highlighted the limitations of establishing continuous cultures from solid tumor explants, i.e. the low yield of viable epithelial cells and overgrowth by fibroblasts. This development subsequently led to the discovery and establishment of several more breast cancer cell lines.

However, despite the significant development carried out through these carcinoma cell lines to the field of breast cancer biology, none of them was responsive towards estrogen, a critical factor in the of breast cancer (Yager and Davidson 2006,

Clemons and Goss 2001, Henderson et al. 1988). Hence, it was the discovery of the ER positive cell line, MCF-7 at the Michigan Cancer Foundation (from which it derives its name) in 1973 (Soule et al. 1973) and its response and sensitivity towards various hormones and growth factors that has started the exploitation of the breast cancer cell line as the predominant tool in the study of human breast cancer biology (Levenson and Jordan 1997). Equally importantly, the subsequent studies showing that breast cancer cells express a wide range of receptors for steroid and peptide hormones, growth factors and cytokines, and often retain responsiveness to their cognate ligands, has extended the use of these cell lines far beyond breast cancer. The cell lines are used to study the receptors themselves, their signalling pathways, their gene expression regulation and cellular function.

Today apart from MCF-7, the most commonly used breast cancer cell line worldwide, a number of other cell lines are routinely used as breast cancer in vitro models, such as BT20 (Ozzello et al. 1974), MDA MB-231 (Cailleau et al. 1974), MDA MB-435, MDA MB-468 (Cailleau et al. 1978), SKBr-3 (Trempe and Fogh 1973, Fogh and Trempe 1975) and T47D (Keydar et al. 1979). Moreover, most of the in vitro studies using breast cancer cells are based on a few well characterized cell lines, such as MCF-7, T47D and MDA MB-231, which have been established in culture for over 30 years. Thus breast cancer cell lines have become major experimental models, not only for breast cancer research but for dissecting basic molecular mechanisms controlling diverse aspects of cancer biology. MCF-7, T47D and MDA MB-231 are the long established breast cancer cell lines and are the most commonly used breast cancer cell lines all over the world, having the most number of citations in scientific literature over a defined period of time (Lacroix and Leclercq 2004).

In the last few years major advances have been made in breast cancer research, a significant proportion of which have arise from studies involving these cell lines as experimental models.

2.1.1 MCF-7 cell line

MCF-7 breast cancer cell line was isolated from a 69-year-old Caucasian female patient Frances Mallon after her death in 1970. The cell line was first of all established at Michigan Cancer Foundation institute, Detroit by Herbert Soule et al in 1973, hence the acronym “MCF-7” was assigned to the cell line (Soule et al. 1973, Levenson and Jordan 1997, Glodek 1990).

MCF-7 cell line has several properties of differentiated mammary epithelium, including the presence of estrogen receptor, progesterone receptor and the capability to form characteristic morphology of dome/cluster (ATCC Advanced Catalog Search 2010, Karimi-Busheri et al. 2010) (Fig. 2.1).

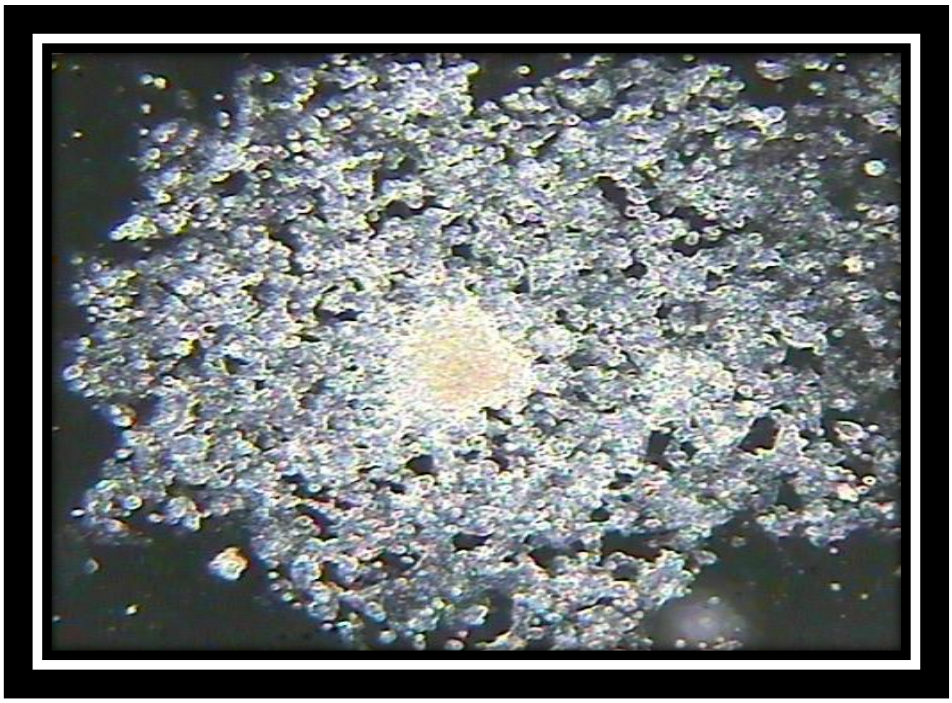

Figure 2.1 MCF-7 cells

2.1.2 T47D cell line

The breast cancer cell line T47D was isolated from a pleural effusion obtained from a 54 year old female patient in 1979. The cell line was first of all established by Keyder and coworkers in 1979 (Keydar et al. 1979). This differentiated epithelial cell

line express estrogen receptor, progesterone receptor, androgen receptor along with calcitonin receptor, glucocorticoid receptor and prolactin receptor. These cells shows epithelial morphology and form monolayers in culture (Fig 2.2). The cell line displays a relatively high colony forming efficiency (ATCC Advanced Catalog Search 2010).

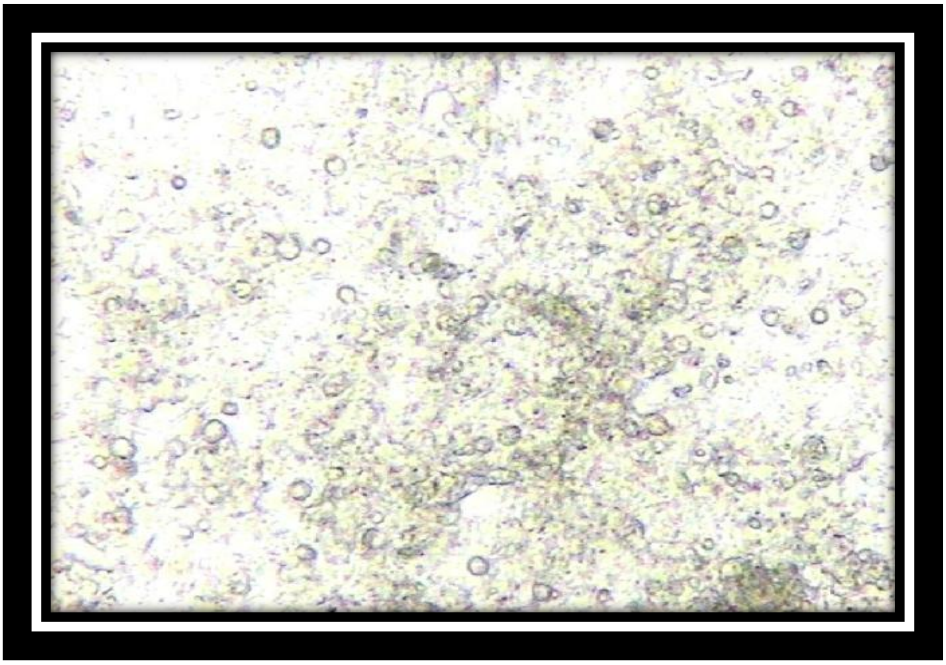

Figure 2.2 T47D cells

Some of the inherent characteristics of both these cell lines are as follows-

Table 2.1

Cell line	MCF-7	T47D
Organism	Homo sapiens	Homo sapiens
Gender	Female	Female
Ethnicity	Caucasian	-
Patient age	69 years	54 Years
Tumor stage	IV	IV
Primary site	Breast	Breast
Specimen site	Pleural effusion	Pleural effusion
Disease	Adenocarcinoma	Ductalcarcinoma

Cell type	Epithelial	Epithelial
Growth properties	Adherent	Adherent
Morphology	Dome/Cluster shaped	Monolayer
Receptors	Estrogen receptor, Progesterone receptor Expressed (ER+ve), (PR +ve)	Androgen receptor, Progesterone receptor, Estrogen receptor expressed (AR +ve), (PR +ve), (ER +ve), (Also express Calcitonin receptor, Glucocorticoid receptor, Prolactin receptor)
Biosafety level	1	1
Antigen expression	Blood Type O; Rh+	-
Culture medium	MEM/DMEM medium	RPMI 1640 medium
Authentication	Isozyme analysis	Isozyme analysis
Doubling time	29 hours	32 hours
Serum starvation period	24 hours	24 hours
Storage temperature	Liquid nitrogen vapour phase	Liquid nitrogen vapour phase
Availability	ATCC	ATCC
Primary reference	Soule et al., 1973	Keyder et al., 1979

Source: Information adapted from ATCC- Advanced Catalog Search

2.2 Insulin

2.2.1 Introduction

Insulin is a peptide hormone intricately linked to cellular metabolism and plays a critical role in the growth and development of cells, tissues and regulates overall

growth. Insulin was discovered by Fredrick Banting, Charles Best, J.J.R. Macleod and James Collip (Rosenfeld 2002). It is composed of 51 amino acids and has a molecular weight of 5808 dalton. It was the first protein to have its amino acid sequence sequenced in 1955 by Frederick Sanger for which he was awarded by Nobel Prize in 1958 (Stretton 2002). Insulin is secreted by beta cells of islets of langerhans of pancreas in response to raised levels of nutrients in the blood. Insulin triggers the uptake & storage of glucose, fatty acids and amino acids into muscle, liver and adipose tissue and promotes synthesis of glycogen, lipids, and protein.

2.2.2 Structure of insulin

The primary structure of insulin was determined by Frederick Sanger. Insulin is a small globular protein consisting of two peptide chains i.e. A-chain and B-chain. A-chain consists of 21 amino acids and has glycine at its N-terminus whereas B- chain involves 30 amino acid residues and has phenylalanine at its N-terminus. Within A-chain, there is an internal disulfide bond between cysteine 6 & cysteine 11. B-chain does not have any internal disulfide bridges; indeed it is linked to A-chain by two disulfide bonds between amino acid residue B7-A7 and B19-A20.

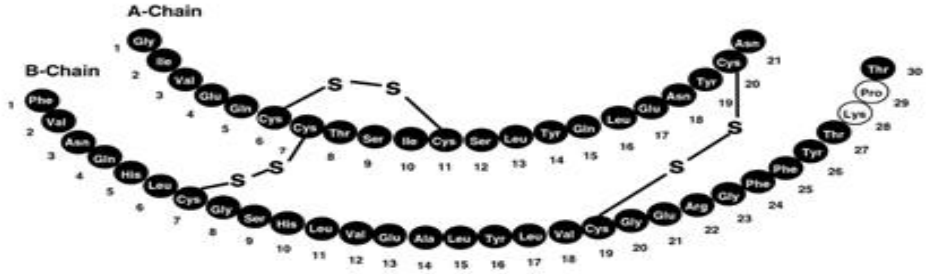

Figure 2.3 Structure of insulin

(Source: Adapted from Dailymed.nlm.nih.gov archive)

2.2.3 Overview of insulin signalling pathway

As described above insulin signaling pathway is activated when nutrients are available. The action of Insulin is mediated by a complex network signal transduction

via the insulin receptor (IR) located on the surface of insulin sensitive cells (Lizcano, and Alessi 2002). In humans, IRS is widely expressed in normal and cancerous tissues. Normal tissues express B isoform whereas cancer cells preferentially express the A isoform of IRs which shows difference with B isoform in its affinity for different ligands. Also cancer cells fail to downregulate the insulin receptors in condition of hyperinsulinemia, potentially explaining the influence of insulin levels on cancer prognosis.

Binding of Insulin to Insulin Receptor:

Once secreted from beta cells of pancreas, insulin binds to IR resulting into the initiation of a cascade of intracellular signalling events which regulates multiple biological processes like glucose and lipid metabolism, gene expression, protein synthesis and cell growth/differentiation/survival. The IR is a heterotetrameric transmembrane glycoprotein which belongs to the Receptor tyrosine kinase (RTK) superfamily. It consists of two extracellular alpha subunits and two transmembrane β-subunits linked together by disulfide bonds. The binding of insulin to IR results into activation of IR (Dipl-Pharm and Zierath 2005).

Activation of Insulin Receptor:

Binding of insulin to alpha subunits leads to a conformational change resulting into activation of tyrosine kinase residues in β-domain. This results in the binding of adenosine triphosphate (ATP) to the receptor and finally the autophosphorylation of tyrosine residues on the β subunits (White 2003).

Insulin Receptor Substrates (IRS):

The activation of IR is followed by phosphorylation of key tyrosine residues on a series of downstream elements including Insulin Receptor Substrate (IRS) family, Shc, c-Cbl protooncogene, $p60^{dok}$, Src homology 2 (SH2) domains APS, Grb2-associated binder-1 (Gab1) etc. IRS are cellular proteins similar in functional properties but different in sequence pattern. IRS family includes four substrates i.e. IRS-1, IRS-2, IRS-3 and IRS-4 (Dipl-Pharm and Zierath 2005). In humans, IRS-1 and

IRS-2 are chief mediators of insulin-signal transduction to metabolic and gene regulatory processes. Human expresses nonfunctional/ degenerated IRS-3 gene thus lacks the required genetic information for coding the phosphotyrosine binding domain. The role of IRS-4 in propagation of insulin action in humans is largely unknown (Dipl-Pharm and Zierath 2005).

IRS contain an NH2- terminal pleckstrin homology (PH) domain and a phosphotyrosine-binding domain, COOH-terminal tyrosine residues which creates SH2 protein-binding sites, proline-rich regions that engage Src homology 3 (SH3) domains or WW domains (protein modules that bind proline-rich ligands) and serine-threonine-rich regions that bind with other proteins.

The signal generated via the insulin receptor is propagated through three chief pathways involving:

- Phosphatidylinositol 3 kinase (PI3K) pathway.
- Cbl-associated protein (CAP)/ Cbl pathway.
- Mitogen-activated protein kinase (MAPK) pathway.

PI3K is considered to be the key player of the metabolic and mitogenic actions of insulin whereas the CAP/Cbl pathway mainly play role in insulin-mediated glucose transport. The MAPK pathway is principally involved in gene regulatory responses (cell growth and differentiation) in insulin-sensitive cells (Long et al. 2004).

PI3K signalling pathway:

PI3K signalling pathway play significant role in insulin–mediated glucose uptake, glycogen synthesis, protein synthesis, inhibition of gluconeogenesis, cell proliferation and apoptosis (Siddle 2011).

Phosphatidylinositol 3 Kinase (PI3K) is a heterodimeric lipid kinase consisting of two subunits i.e. catalytic subunit p110 (3 isoforms) and regulatory subunit p85 (2 isoforms). The regulatory subunit contains 2 SH2 domains that bind to phosphotyrosine motifs on phosphorylated receptor tyrosine kinases of IR or IRS proteins. IRS-1 and IRS-2 dock with the p85 regulatory subunit, which results into

activation of catalytic subunit p110. The p110 subunit then phosphorylates the PI3K substrate, phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3'-OH position of the inositol ring to produce phosphatidylinositol-3,4,5- triphosphate (PIP3) (Foukas and Shepherd 2004).

The elevated concentration of phosphatidylinositol-3,4,5- triphosphate (PIP3) induce the recruitment of PH domain-containing proteins such as the serine-threonine kinases like Phosphoinositide-dependent kinase-1 (PDK1), PDK2 (mTOR-RICTOR protein complex) and Akt/Protein kinase B (PKB). Once recruited, PDK-1 and PDK-2 activates Akt by phosphorylating it on Thr^{308} and Ser^{473} respectively (Dipl-Pharm and Zierath 2005). Once become fully active, Akt is known to play a role in insulin signalling to glucose transport/glycogen synthesis/protein synthesis. It mediates the insulin induced phosphorylation of glycogen synthase kinase 3 (GSK-3) leading to inactivation of GSK-3 which in turn prevents phosphorylation and inactivation of glycogen synthase (GS), the major substrate of GSK-3, resulting in increased glycogen synthesis.

Akt also promotes protein synthesis in the cell by interacting with mTOR signalling pathway. Another key action of activated Akt is stimulation of glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, from intracellular storage to plasma membrane.

Cbl-associated protein (CAP)/ Cbl pathway:

This signalling pathway is mainly involved in glucose uptake by recruitment of GLUT4 to the plasma membrane (Saltiel and Kahn 2001). It can work in both PI3K pathway dependent and independent manner. The Cbl protooncogene is direct intracellular substrate for the insulin receptor. Its phosphorylation requires the presence of another protein which recruits Cbl to the IR, which is likely to be the adaptor protein APS. APS with its SH2 domain interacts with phosphorylated IR and subsequently undergoes tyrosine phosphorylation. Upon phosphorylation, APS recruits Cbl through SH2 domain of Cbl that leads to the phosphorylation of Cbl, which in turn forms a complex with Cbl associated protein (CAP). Following

phosphorylation, the complex translocates to lipid rafts in the plasma membrane and activates members of the GTP-binding protein family, TC10. TC10 promotes translocation of GLUT4 to the plasma membrane, hence enhances glucose uptake by the cell (Chiang et al. 2001).

Mitogen-activated protein kinase (MAPK) pathway:

MAPK pathway is another important pathway activated by insulin mainly involved in gene regulatory responses in cellular system (Gehart et al. 2010). Activation of Ras protein is an important event in activation of the MAPK signalling pathway. Ras proteins are 21 kDa GTPases that are attached to the cell membrane by prenylation. Ras become active when it gets switched from GDP-bound state to GTP-bound state (Lowy and Willumsen 1993). Mutations in the Ras proteins resulting in constitutive activation of the Ras GTPase as found in 20% to 30% of human tumours. Activation of Ras involves activation of the Grb2-Sos complex. Grb2 is an adapter protein and Sos is a guanine nucleotide exchange factor which interacts with Grb2 by its SH3 domain. Studies have shown that the Grb2-Sos complex can be activated by at least two pathways. The phosphorylated IRS-1 protein serves as a docking site for Grb2 which then recruits Sos to the plasma membrane for activation of Ras. Alternatively, Grb2 can bind to Shc protein and this leads to the activation of Grb2-Sos complex. Once recruited to the plasma membrane, Sos activates Ras protein by exchanging GDP with GTP. To this activated Ras binds c-Raf, a serine-threonine protein kinase also called Raf-1. c-Raf phosphorylates and activates mitogen activated protein kinase (MEK) (Kyriakis et al.1992). Activated MEK catalyses phosphorylation of mitogen activated protein kinase (MAPK) at threonine and tyrosine residues. MAPK (also known as extracellular signal-regulated kinase, Erk) is a serine/threonine kinase and has two closely related isoforms, Erk1 and Erk2 (Ahn et al.1992).The phosphorylated Erk1 and Erk2 enter the nucleus and has been demonstrated to activate transcription factors like FOS, MYC and JUN which are well known for their role in cell proliferation. Erk1 & Erk2 also increases expression of genes like Cyclin D1 that are required for cell cycle entry. Additionally, Erk1 & Erk2 are involved in a

negative-feedback loop of insulin action by phosphorylating IRS-1 on serine residues. In summary, MAPK pathway is primarily involved in mediating mitogenic actions of insulin (Gehart et al. 2010).

2.2.4 Insulin-breast cancer connection

First of all Nobel Prize winner Otto Heinrich Warburg proposed the idea indicating a strong connection between cellular metabolism and cancer (Warburg 1956). Signalling pathways controlling metabolism and neoplasm have shown that both are intricately linked with each other, suggesting a close relationship between metabolic disorders and tumor malignancies. Insulin is the main anabolic peptide hormone regulating cellular metabolism. Due to absolute and relative deficiency of insulin metabolic disturbance namely diabetes develops. The more prevalent diabetes, type 2 diabetes (T2D) and cancer are diseases with several overlapping factors (Lorenzo et al. 2003). Studies have strongly suggested that pathophysiology of T2D enhances the risk of cancer (Stern et al. 2004, Godsland 2010, Tran et al. 1996, Bowker et al. 2006, Hjalgrim et al. 1997). Epidemiological studies have reported that besides other malignancies, T2D potentiates the risk of breast cancer resulting into adverse outcome and poor prognosis (Lipscombe et al. 2006, Yancik et al. 2001, Satariano and Ragland 1994, Wolf et al. 2005). Several case control studies have concluded that T2D increases the relative risk (RR) of breast cancer by 10% to 20% (Lipscombe et al. 2006, Key 2001). This T2D-Breast cancer connection is more prevalent in post menopausal females, where hyperinsulinemia is used as a marker of breast cancer risk (Gross et al. 1984, Heuson et al. 1967, Osborne et al. 1976, Stoll 1996). Insulin mediates its metabolic responses in both normal and transformed cells (Gliozzo et al. 1998, Bentel et al. 1995, Yang et al. 1996, Pollak et al. 1990). Insulin and hyperinsulinemia can promote the onset of cancer through insulin receptor in epithelial tissues. Since breast epithelial cells distinctly express the insulin receptors, they are more prone to enhanced growth in response to insulin, ultimately results into breast cancer (Vigneri and Goldfine 1992, Papa and Belfiore 1996, Milazzo et al. 1992).

2.3 Metformin

2.3.1 Introduction

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral antidiabetic drug belonging to biguanide class of compounds. It was historically derived from the herb Galega officinalis (Witters 2001, Hadden 2005) and today it is widely used as the front line therapy for treatment & management of hyperglycemia and type 2 diabetes (T2D) (Ben Sahra et al. 2010). Metformin lowers levels of circulating glucose, increases insulin sensitivity and reduces insulin resistance associated hyperinsulinemia, hence also referred as ‘‘Insulin Sensitizer’’ (Holland et al. 2004). Other than diabetes, the use of metformin is also found to be effective in cancer malignancies, obesity and polycystic ovarian syndrome (Ben Sahra et al. 2010, Evans et al. 2005, Jiralerspong et al. 2009, Diamanti-Kandarakis et al. 2010). Several studies have reported that metformin showed strong anticancer properties by inhibiting growth of cancer cells, including breast cancer in vitro and of tumors in vivo (Alimova et al. 2009, Zakikhani et al. 2006, Dowling et al. 2007, Evans et al. 2005, Ben Sahra et al. 2008).

2.3.2 History

The story of metformin began for the first time in ancient Egypt and then further in medieval Europe, where Goat's rue flower Galega officinalis (also known as French lilac/Italian finch) was prescribed by physicians for the treatment of frequent & excessive urination (polyurea) & halitosis (a sweet odour on breathing). Nothing was known about the active ingredients of the herb at that time. The light of modern period leads to the identification of the active chemical composition of the plant. After several experiments and trials, the biguanide (two linked guanidine rings) metformin along with its chemical cousins phenoformin and bufoformin were developed (Witters 2001, Hadden 2005, Ben Sahra et al. 2010, Holland et al. 2004). Up to 1950’s all the three were available and used as diabetes therapeutics. However, later on phenoformin and bufoformin were withdrawn due to emergence of frequent lactic acidosis leading to toxicity (Cusi et al. 1996). Metformin shows excellent therapeutic

index for type 2 diabetes, hence was introduced in Britain in 1958, Europe in 1970's, Canada in 1972 and in 1995 approved by U.S. Food and Drug Administration (FDA) for use in America. Today it is one of the most commonly prescribed drug for type 2 diabetes on the planet with nearly 120 million prescriptions filled yearly worldwide (Vazquez-Martin et al. 2010).

2.3.3 Chemistry of metformin

Metformin HCl is white crystalline compound with the molecular weight of 165.62. The molecular formula of metformin HCl is $C_4H_{11}N_5$.HCl and has the chemical structure as shown in the figure. The pKa of metformin is 12.4. It is freely soluble in water and slightly soluble in alcohol (95% in ethanol) and practically insoluble in acetone, chloroform, ether etc. The pH of 1% aqueous solution of metformin HCl is 6.68.

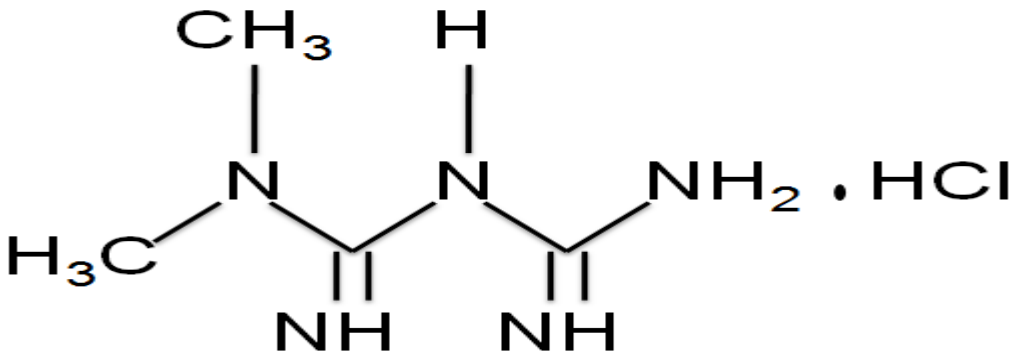

Figure 2.4 Chemical structure of metformin

2.3.4 Mechanism of action of metformin

The antineoplastic effects of metformin in breast cancer comes into play through two distinct mechanisms: Direct mechanism (insulin independent) and Indirect mechanism (insulin dependent) (Figure 2.5) (Goodwin 2008, Goodwin et al. 2009). The Indirect (insulin dependent) action of metformin is achieved through the

activation of “fuel gauge”/ ”cellular energy sensor” Adenosine monophosphate-activated protein kinase (AMPK) by disrupting complex 1 of mitochondrial respiratory chain resulting into decreased Adenosine triphosphate (ATP) synthesis and increase in AMP level (Pollak 2008, El-Mir et al. 2000, Towler and Hardie 2007). Activation of AMPK leads to the suppression of the fundamental biological processes heavily dependent on ample cellular ATP supply, such as hepatic gluconeogenesis, biosynthesis of cellular biomolecules including proteins, fatty acids, cholesterol etc (Zhou et al. 2001, Fisher et al. 2002). This suppression inhibits the transcription of gluconeogenesis genes in liver and stimulates the uptake of glucose in muscles, thereby systematically lowering the levels of circulating glucose & serum insulin, enhances insulin sensitivity and reduces hyperinsulinemia, ultimately resulting in diminishing of negative effect of insulin on cancer development & growth.

The direct action (insulin independent) exhibiting the antitumorogenic effect of metformin is mediated through the inhibition of mammalian target of rapamycin (mTOR) signalling pathway (Dowling et al. 2007). mTOR pathway is central to the cell growth & proliferation by integrating the growth factor and nutrient signalling. mTOR uses two opposing upstream pathways i.e. the Phosphatidylinositol-3-kinase/Protein kinase B/Akt (PI3K/PKB/Akt) signalling pathway, which signals availability of nutrients and the AMPK pathway, which signals energy deficiency. AMPK effects mTOR pathway via phosphorylation and stabilization of the tumor suppressor Tuberous sclerosis complex 2 (TSC2 or tuberin), which integrates regulatory inputs and transfer them to mTOR. These regulatory inputs involve oxygen dependent signals and growth factor dependent signaling pathways like as PI3K/Akt pathway (Inoki et al. 2003). Activation of Akt causes phosphorylation of TSC2 which negatively regulates the mTOR pathway (Kalender et al. 2010).

Metformin impairs the mitochondrial process of ATP production resulting into Liver kinase B1 (LKB1) mediated activation of AMPK signalling pathway (El-Mir et al. 2000, Fisher et al. 2002). AMPK activation leads to an inhibition of mTOR signalling pathway, a reduction in phosphorylation of its major downstream effectors, the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks), and an inhibition of protein synthesis & proliferation of tumor cells.

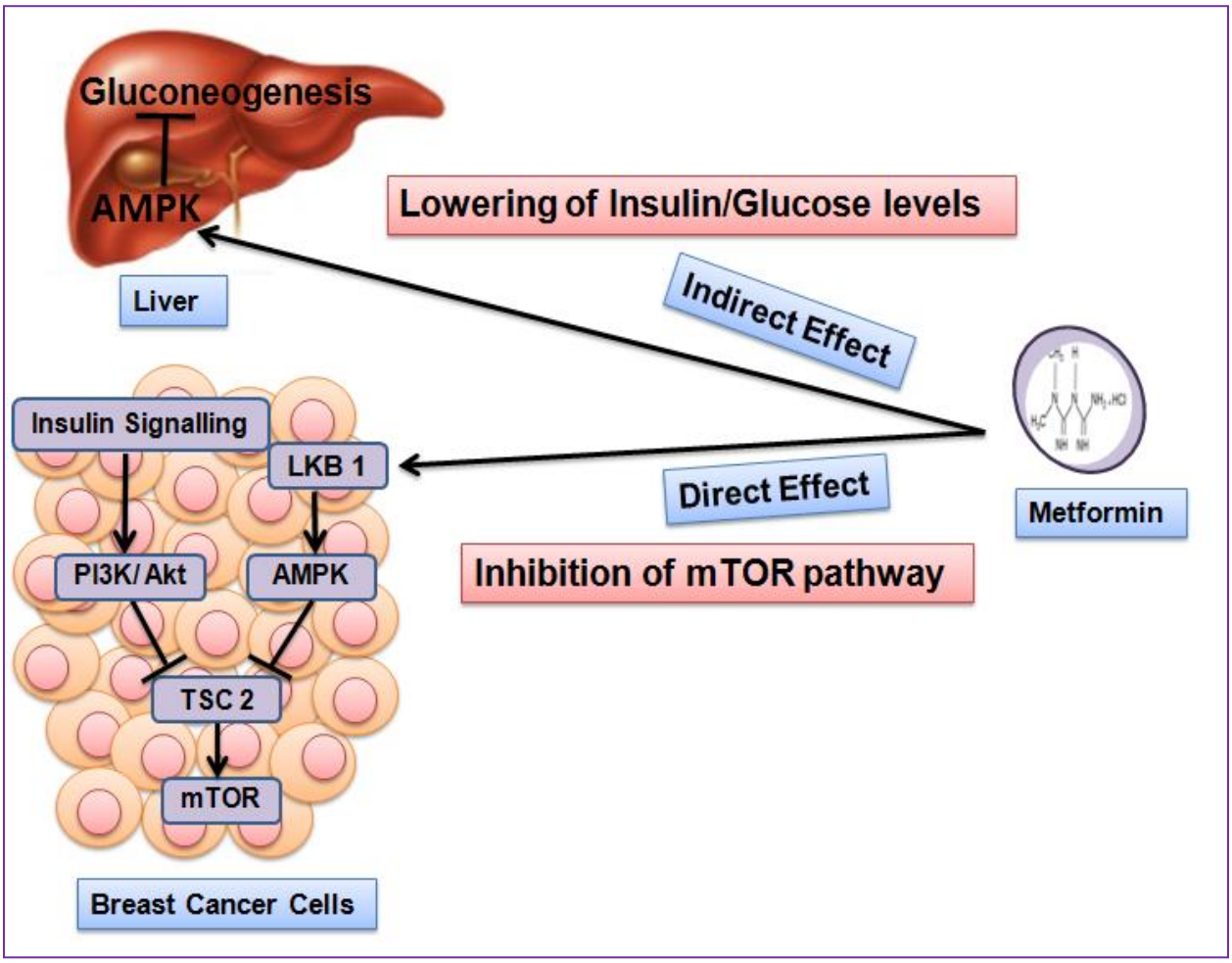

Figure 2.5 Mechanism of action of metformin

2.3.4 Metformin and breast cancer

Clinical and epidemiological studies have shown a strong connection between hyperinsulinemia, insulin resistance and type 2 diabetes to worsen breast cancer outcomes. Several experimental studies have demonstrated that metformin can inhibit the growth of breast cancer cells, in vitro and in vivo (Alimova et al. 2009, Zakikhani et al. 2006, Dowling et al. 2007, Ben Sahra et al. 2008). Metformin may also have role in regulation of breast cancer initiating ontogeny as the drug transcriptionally represses the process of epithelial to mesenchymal transition. Metformin reduces the growth of breast tumor xenografts in mice & also suppresses

the progression of breast cancer in transgenic mice (Anisimov et al. 2005, Tomimoto et al. 2008).

2.4 OBJECTIVES OF THE STUDY

On the basis of above review of literature, the main focus of the study is to determine the effect of hyperinsulinemia on breast cancer development and also to examine the effect of antidiabetic drug metformin on normal breast cancer cell proliferation and insulin induced breast cancer cell proliferation. Therefore, the objectives of the study are:

- To determine the in vitro effects of insulin on cell proliferation.
- To determine the in vitro effects of metformin on cell proliferation.
- To determine the in vitro effects of metformin on insulin stimulated cell proliferation.

2.5 PROPOSED HYPOTHESIS

On the basis of epidemiological, in vivo and in vitro studies there is strong evidence to support the notion that high levels of circulating insulin has strong correlation with increased incidence and poor prognosis of breast cancer. Although normal breast epithelial cells and the majority (80%) of malignant mammary epithelial cells express insulin receptors, it remains unknown whether in women with insulin resistance and hyperinsulinemia, these cells remain sensitive to insulin. In this condition a general hypothesis can be proposed that breast cancer epithelial cells can be stimulated/ proliferated in response to high levels of insulin. The objective of the study was to examine the effect of hyperinsulinemia on breast cancer development and progression using cell line models (MCF-7 and T47D) of breast cancer. Also it was hypothesized that antidiabetic drug metformin lowers risk of breast cancer via direct action (Insulin independent) and indirect action (Insulin independent). The treatment of the hyperinsulinemia with the metformin will lower the circulating levels of insulin and improve insulin sensitivity, thus results into potential decrease in the incidence of breast cancer.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

The materials and methods used in the present study entitled **"Evaluation of Breast Cancer Cell Lines (MCF-7 & T47D): Influence of Insulin and Metformin on Growth Parameters"** are outlined in this chapter. This section involves the experimental model (cell lines), devices, reagents, chemicals, culture media, plastic wares and protocols followed.

3.2 Materials

All the materials used during experimentation were of highest quality available and the chemicals and reagents used were of analytical grade & cell culture grade.

3.2.1 Cell culture medium

Table 3.1

Name of Cell culture medium	Name of the Company
Dulbecco's Modified Eagles Medium	Gibco, Invitrogen, India
RPMI 1640 Medium	Gibco, Invitrogen, India

3.2.2 Cell lines

Table 3.2

Name of Cell lines	Name of the Scientist/Institute
MCF-7 Cell line (Passage No.- 22)	Dr. K. B. Tikoo Department of Pharmacology & Toxicology National Institute for Pharmaceutical and Educational Research (NIPER) Mohali Chandigarh, Punjab, India
T47D Cell line Passage No.- 71)	National Centre for Cell Sciences (NCCS) Pune, Maharastra, India

3.3 Methods

3.3.1 Preparation of culture medium

3.3.1.1 Dulbecco's modified Eagle's medium (DMEM)

Complete DMEM medium was prepared as follows:

- Taking 45 mL of liquid DMEM and dispense in 50 ml falcon tube.
- Mixing 5 mL filtered fetal bovine serum (FBS) (10%) (Gibco, Invitrogen, India) to the DMEM medium.
- Adding 50 µL of penicillin/streptomycin (Gibco, Invitrogen, India) antibiotic to the medium.
- Filter sterilize media through a 0.2 µM filter disc (Pall Corporation, USA) and store at 4°C

3.3.1.2 RPMI 1640 medium

RPMI-1640 medium was developed at Roswell Park Memorial Institute, hence the acronym RPMI. Complete RPMI 1640 medium was prepared as follows:

- Taking 45 ml of liquid RPMI 1640 and dispense in 50 mL Falcon tube.
- Mixing 5 mL filtered FBS (10%) to the DMEM medium.
- Adding 50 µL of penicillin/streptomycin antibiotic to the medium.
- Filter sterilize media through a 0.2 µM filter and store at 4°C

(Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.2 Preparation of Metformin stock

Metformin (free gift from Ranbaxy Laboratories Ltd., India) was diluted in pure milliQ water (Millipore, India) to prepare a stock solution 0f 10^{-2} molar in 10 ml. The stock solution was further serially diluted to prepare a range of concentrations at 1µM, 10 µM, 100 µM, 1mM and 10 mM.

3.3.3 Preparation of Insulin stock

A 10 mg/ml stock solution of human insulin recombinant (Himedia Laboratories Pvt., India) was prepared by diluting it in phosphate buffered saline-acetic acid (Loba chemie Pvt. Ltd., Mumbai, India) solution. It was used across a range of concentrations at 1nM, 10 nM, 100 nM, 1000nM diluted in media.

3.3.4 Propagation of Human breast cancer cell lines

3.3.4.1 MCF-7 cell line

MCF-7 cells were grown in plastic T-25 and T-75 flasks (Orange Scientific, Borosil, India) containing Dulbecco's modified Eagles medium with 1% L-glutamine, 1% penicillin/streptomycin, supplemented with 10% heat inactivated FBS. Culture was maintained at 37°C with humidified 5% CO_2 environment in CO_2 incubator **(**Macro Scientific Works Pvt. Ltd**.,** India).

3.3.4.2 T47D cell line

The T47D cells were maintained at 37°C in a humidified 5% CO_2 environment in RPMI 1640 medium with 1% L-glutamine, 1% penicillin/streptomycin, supplemented with 10% heat inactivated FBS.

3.3.5 Revival of Cell lines

Frozen cells kept in cryovials were taken out of the liquid nitrogen and thawed immediately in order to maintain viability and quick recovery of cells. Clean the vial with 70% alcohol (Jebsen & Jessen GmbH & Co., Germany) and loose the cap of vial a quarter to release any trapped residual liquid nitrogen and internal pressure. Again retighten the cap. Quickly transfer the ¾ part of cryovial in water bath at 37°C and thaw the cells by gentle agitation until the silver lining starts to melt and only one or two small ice crystals remains (1-2 minutes). Pipette the whole content of the cryovial into a sterile centrifuge tube. Prewarmed media was added to centrifuge tube containing in dropwise manner. Centrifugation (Model 5430R, Eppendorf, Hamburg,

Germany) of cell suspension was done at 3000 rpm for 3 min and supernatant was discarded without disturbing the pellet. Gently resuspended the cells in growth media and then transfer to T-25 and T-75 culture flasks. Flasks were incubated at 37°C in humidified 5% CO_2 for 24 hours before the medium was refreshed. Change the media every other day on regular basis, until 60-90% confluence is not attained. (Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.6 Subculturing / Passaging of Cells

Cells were subcultured once they get 80-90% confluent using trypsin-EDTA (1X) (Himedia Laboratories Pvt., India). The cells were transferred to other flasks in order to propagate and proliferate the cell population. Following protocol was followed for subculturing of cells-

- Removal of old media from the flask by using a sterile pipette.
- Washed the cells twice with 5-10 ml of 1x phosphate buffer saline (PBS) to remove Ca and Mg impurities & traces of serum that will inactivate trypsin & then remove the Dulbecco's Phosphate Buffered Saline (PBS) (Himedia Laboratories Pvt., India) from the flask.
- Added 1-2 ml of trypsin-EDTA solution and left the flask for 5-10 minutes for detachment of cells from surface. Swirled the solution across the flask to ensure that trypsin reaches to all the cells.
- After cells detachment, fresh culture medium containing FBS was added to flask for inactivating the trypsin action. Pipet in/out the cell suspension in order to break up the cell clusters into single cells.
- Transferred the cell suspension to centrifuge tubes and centrifuged at 3000 rpm for 3 minutes. Decanted the supernatant and resuspended the obtained pellet in new medium.
- Transferred the 10^{6-8} cells in new flasks and incubate them at 37°C in 5% CO_2 environment.

  (Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.7 Cell Counting

For seeding, appropriate concentration of cells in cell suspension has to be determined hence cell counts were made using a haemocytometer (Improved Neubauer Slide). To count cells take a haemocytometer slide (ROHAM, India), wash & clean it with 70% ethanol and fix the coverslip over the counting chambers. About 10 µL of the cell suspension was loaded in each chamber with the help of micropipette (Tarsons Products Pvt. Ltd., India). Using the 10X objective of the microscope cells were focused on all sets of 16 corner squares of the haemocytometer. Calculation of cell number is based on the counting of total number of cells within the defined area underneath the cover slip. Total number of cells was calculated as follows:

Cells per mL = Average of total number of cells counted in four corner squares x 10^4

$$\text{Total number of cell} = \frac{\text{Cells}}{\text{mL}} \times \text{Total volume of cell suspension}$$

(Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.8 Viability testing

In order to determine the effect of test compounds (Insulin and Metformin) on MCF-7 & T47D cell's viability, cell viability assay was performed using the dye trypan blue (0.5%) (Himedia Laboratories Pvt, India). Trypan blue does not interact with the cell unless the cell membrane is damaged. Healthy undamaged cells exclude the dye, but it is readily absorbed by damaged cells and stained them in blue colour.

To determine the number of viable cells, the cell suspension was diluted in the ratio of 1:1 with Trypan blue dye and incubated for 5 minutes at room temperature. The non-viable cells were appeared as blue, while the viable cells remain unstained. The number of viable & nonviable cells was counted using the normal haemocytometer protocol.

$$\text{Percentage of viable cells} = \frac{\text{Number of unstained cells} \times 100}{\text{Total number of cells}}$$

3.3.9 Seeding of cells

Cells in media containing 10% FBS were seeded in 96-well plates (Tarsons Products Pvt. Ltd., India) at a density of $1x10^5$ cells per well and incubated for 24 hours at 37°C in order to allow the cells to adhere. After 24 hours, the complete media of cells was replaced with serum free medium i.e. serum starvation treatment was given to cells according to their respective serum starvation time period. Following serum starvation, cells were treated with media containing the different doses of test compounds insulin and metformin and incubated for 24, 48, 72, 96 hours at 37^oC.

3.3.10 Cryopreservation of cell lines

Cryogenic preservation (storage below -100°C) was used to keep stocks of cells at low passage numbers and to store/ maintain the reserves of cells.

Cells were pelleted down by centrifugation (Eppendorf) at 3000 rpm for 3 minutes and the supernatant was discarded. $2x10^{8-10}$ cells were resuspended in sterile cold freezing medium (Himedia Laboratories Pvt., India) containing 95% FBS and 5% DMSO. Cell suspensions were then aliquoted and stored in cryovials. These vials were insulated with propanol freezing canister and then transferred to -70°C deep freezer for overnight and then placed in liquid nitrogen for long-term storage.
(Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.11 Cell Proliferation Assay

The effect of insulin and metformin on MCF-7 and T47D cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Himedia Laboratories Pvt., India) assay. MTT assay is a rapid and convenient method for determining cell proliferation. The cells were seeded at a density of $1×10^5$ per well in 96-well plates and incubated for 24 hours at 37°C. After 24 hours, cells were given

the serum starvation treatment. Starved cells were treated with of insulin and metformin doses and incubated for 24, 48, 72, 96 hours at 37^{o}C. After requisite time period, 200 µL of filter sterilized MTT (5 mg/mL in PBS) was added to the wells and plates were incubated for 4 hours at 37^{o}C. Active mitochondrial reductase enzymes in viable cells reduce the tetrazolium salt of the MTT solution into blue-violet formazan crystals. Following incubation MTT was removed and 200 µL of fresh sterile DMSO (Himedia Laboratories Pvt., India) was added per well to dissolve the crystals. Absorbance was measured with an enzyme linked immunosorbent assay (ELISA) plate reader (Model 642, Systronics India Ltd., India) at 570 nm (using DMSO as blank). During the study only the inner rows of the 96-well plate were used in order to minimize the cell growth variations because of different medium evaporation rates at the periphery. The results for a single experiment were the average of triplicate determinations and the experiments were performed at least two times. The growth curves were plotted for absorbance vs. time and absorbance vs. concentrations.

(Protocol followed: Nicholas Piramal Experimental protocols sheet)

3.3.12 Statistical Analysis

All the results are expressed as mean ± SEM (Standard error mean) where applicable. These statistical calculations were performed by using the statistical software Sigma Stat 2.3.

CHAPTER 4

RESULTS AND DISCUSSION

4. Results and Discussion

4.1. Effect of insulin on the MCF-7 and T47D cells

In the initial experiments, we investigated whether high levels of insulin accelerate the growth of tumor cells or not. The direct effect of insulin on the proliferation of MCF-7 and T47D breast cancer cells was evaluated in a dose- dependent manner.

4.1.1 Dose-dependent effects of insulin

Insulin increases the MCF-7 cell proliferation by ≈ 4.75 folds over control when supplemented with 1 nM insulin in serum free media. Insulin stimulates a dose dependent effect on cell growth as 100 nM insulin enhanced the growth by ≈ 7.3 folds above control. Insulin above the 100 nM concentration does not elicit further effect on MCF-7 cell proliferation as growth reached the plateau at 1000nM (Fig. 4.1).

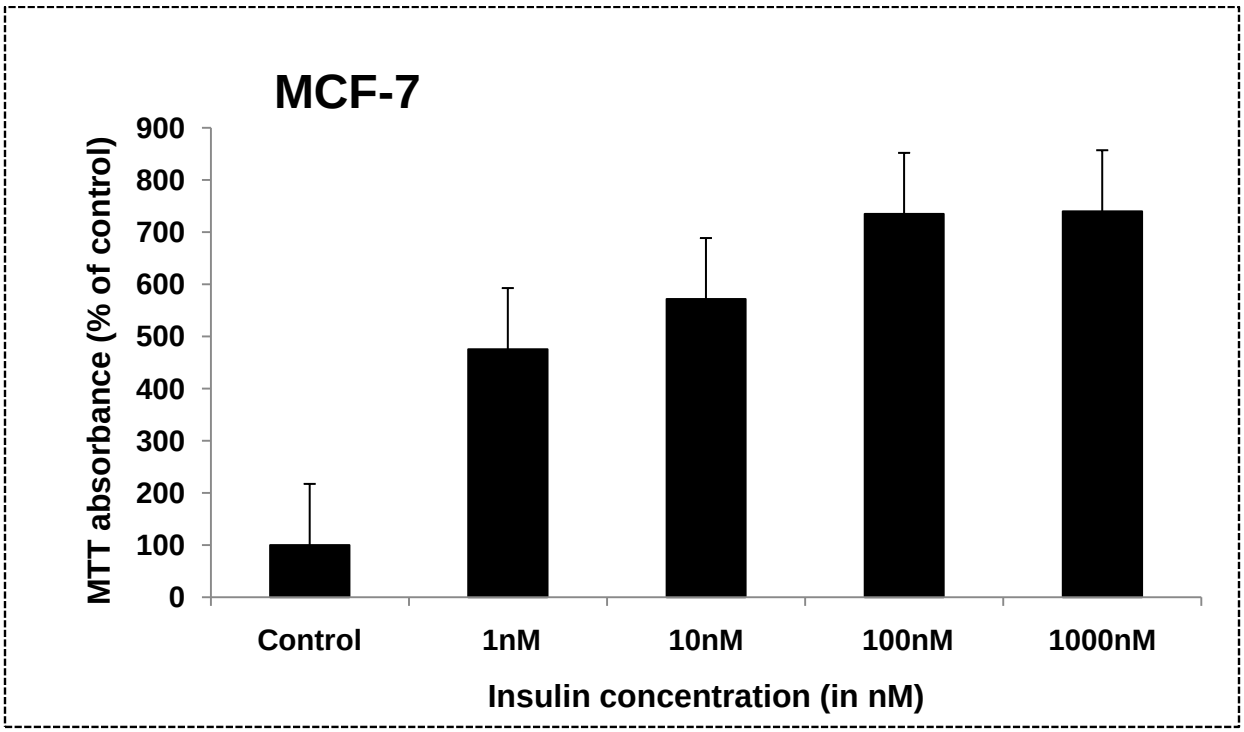

Figure 4.1 Effect of insulin (dose range from 1nM to 1000nM) on MCF-7 cells at 48 hrs. The data presented here are expressed as mean ± SEM (Standard error mean).

T47D cells show a dose-dependent proliferation in response to Insulin with ≈ 3.25 fold increase over control at 10 nM insulin concentration. The maximal effect was exhibited at 100 nM insulin concentration with a ≈ 5 fold increases over the control (Fig. 4.2).

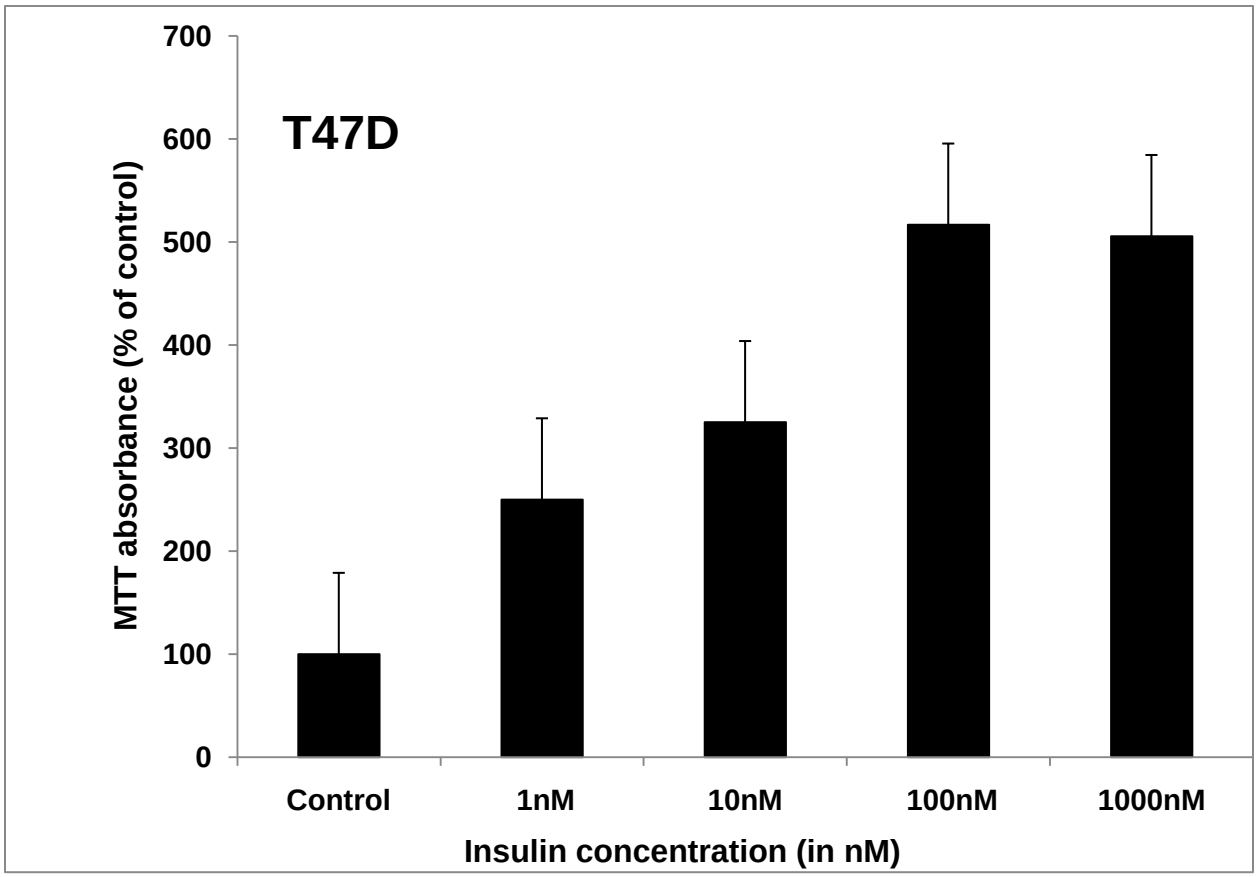

Figure 4.2 Effect of insulin (dose range from 1nM to 1000nM) on T47D cells at 48 hrs. The data presented here are expressed as mean ± SEM (Standard error mean).

The human breast cancer cell lines present an in vitro model system for studying the response of hormones and growth factors on breast cancer tissue. Insulin stimulates several cellular functions including cell proliferation in breast cancer cell lines (Osborne et al. 1976). The present work concludes that high levels of insulin (100 nM) stimulate the proliferation of cultured breast cancer cells. The high doses insulin has significantly enhanced the proliferation of MCF-7 and T47D cells in dose-dependent manner (Fig. 4.1 & 4.2). The results are in accordance with the previous studies showing the involvement of high circulating levels of insulin on breast cancer

development and progression (Heuson et al. 1967, Stoll 1996, Bowker et al. 2006). The results of the present study reconfirm the growth promoting effects of insulin on the breast cancer cells.

4.2. Effect of metformin on the MCF-7 and T47D cells

To examine the growth inhibitory effect of metformin on MCF-7 and T47D cells, cells were treated with different doses of metformin and change in the growth characteristics was examined using MTT assay. Level of control was calculated for each dosage of treatment. For estimating the inhibitory potency of metformin, the proliferation response obtained with metformin was compared with the proliferation response obtained with control. In order to examine the growth inhibitory effect of different doses of metformin in vitro, MCF-7 and T47D cells were treated with metformin concentrations in the range from 10µM-10mM. No significant inhibition was observed on the growth profile of MCF-7 cells at low metformin concentration. However cells show a ≈ 35% growth inhibition over control at 10 mM concentration of metformin after incubation of 72 hours (Fig. 4.3). T47D cells do not show some distinct changes in proliferation in response to lower concentrations of metformin. At 1mM and 10 mM concentrations, growth profile of T47D cells was inhibited up to ≈25 % and ≈30 % respectively (Fig.4.4).

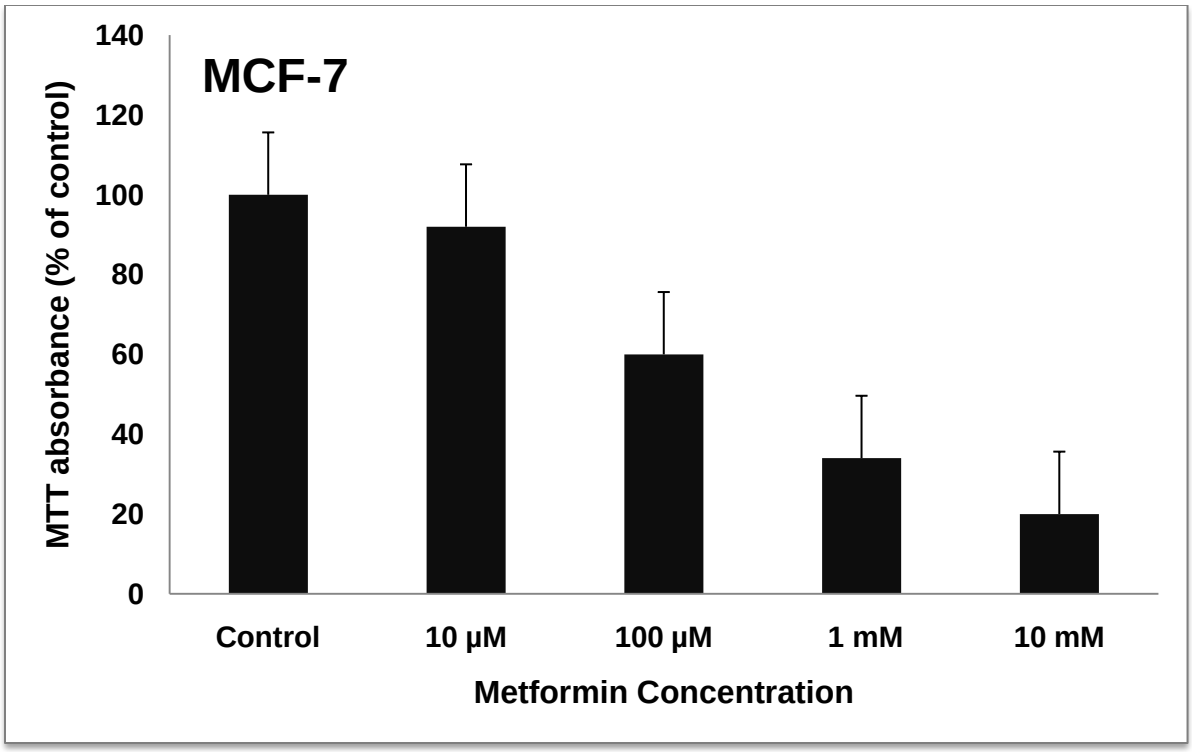

Figure 4.3 Effect of metformin (dose range from 10µM to 10mM) on MCF-7 cells at 72 hrs. The data presented here are expressed as mean ± SEM (Standard error mean).

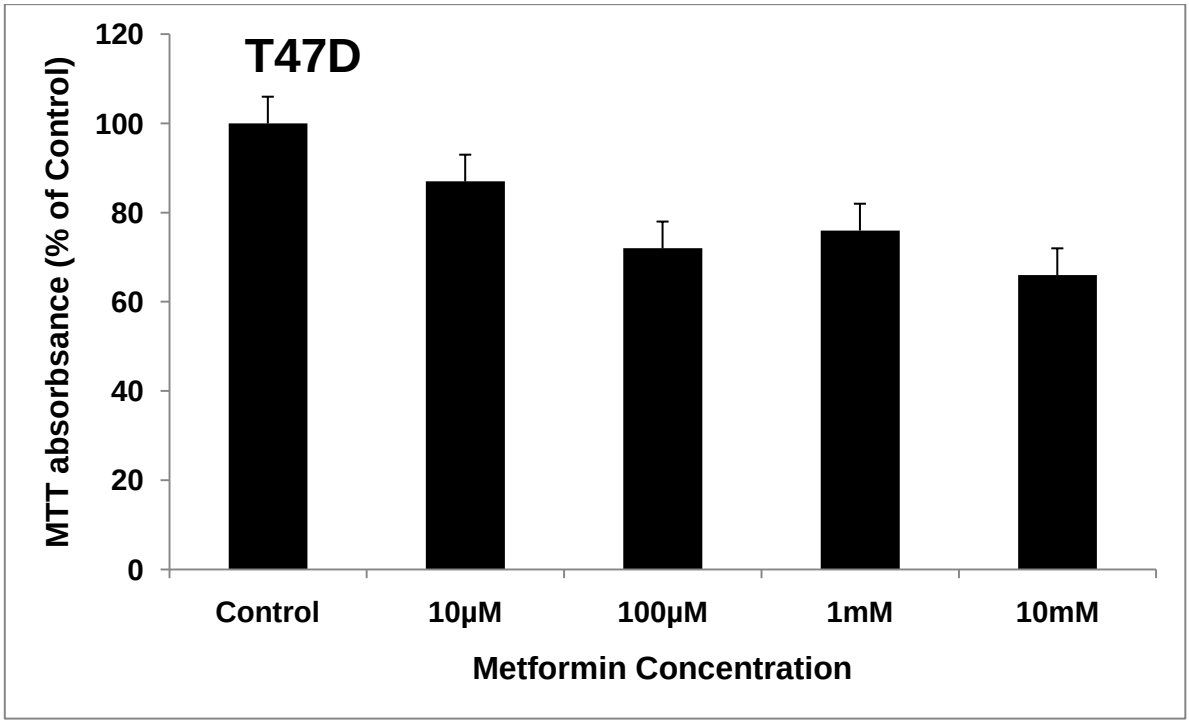

Figure 4.4 Effect of metformin (dose range from 10µM to 10mM) on T47D cells at 72 hrs. The data presented here are expressed as mean ± SEM (Standard error mean)

In order to test the hypothesis that metformin will lower the circulating levels of insulin and improve insulin sensitivity, MCF-7 and T47D cells were first treated with 100 nM insulin and incubated for 1 hour. Following incubation cells were given the dose of metformin concentrations in the range from 10µM-10mM (10 µM, 100 µM, 1mM, 10mM).

4.3 Effect of metformin on insulin stimulated proliferation of MCF-7 and T47D cells

Metformin was found to inhibit the insulin stimulated growth at higher doses i.e. 10mM. At 10mM concentration, metformin decreases insulin induced MCF-7 cell proliferation up to ≈75% in comparison to insulin treated control. No significant change was seen when cells were treated with other doses of metformin (Fig. 4.5).

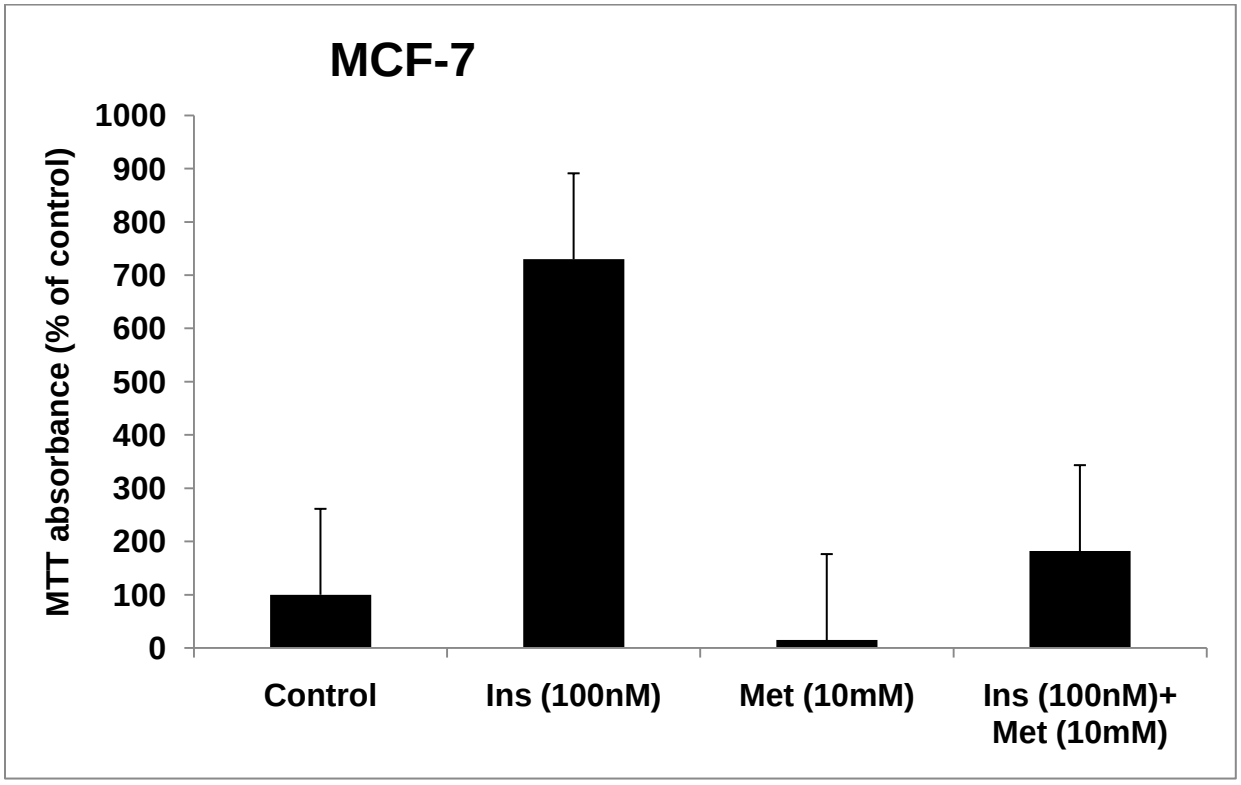

Figure 4.5 Effect of metformin on insulin stimulated proliferation in MCF-7 cells. The data presented here are expressed as mean ± SEM (Standard error mean).

In case of T47D cells, metformin inhibits the insulin stimulated growth at high concentrated dose. At 10mM concentration, metformin decreases insulin induced T47D cells proliferation up to ≈ 60% in comparison to insulin treated control (Fig. 4.6).

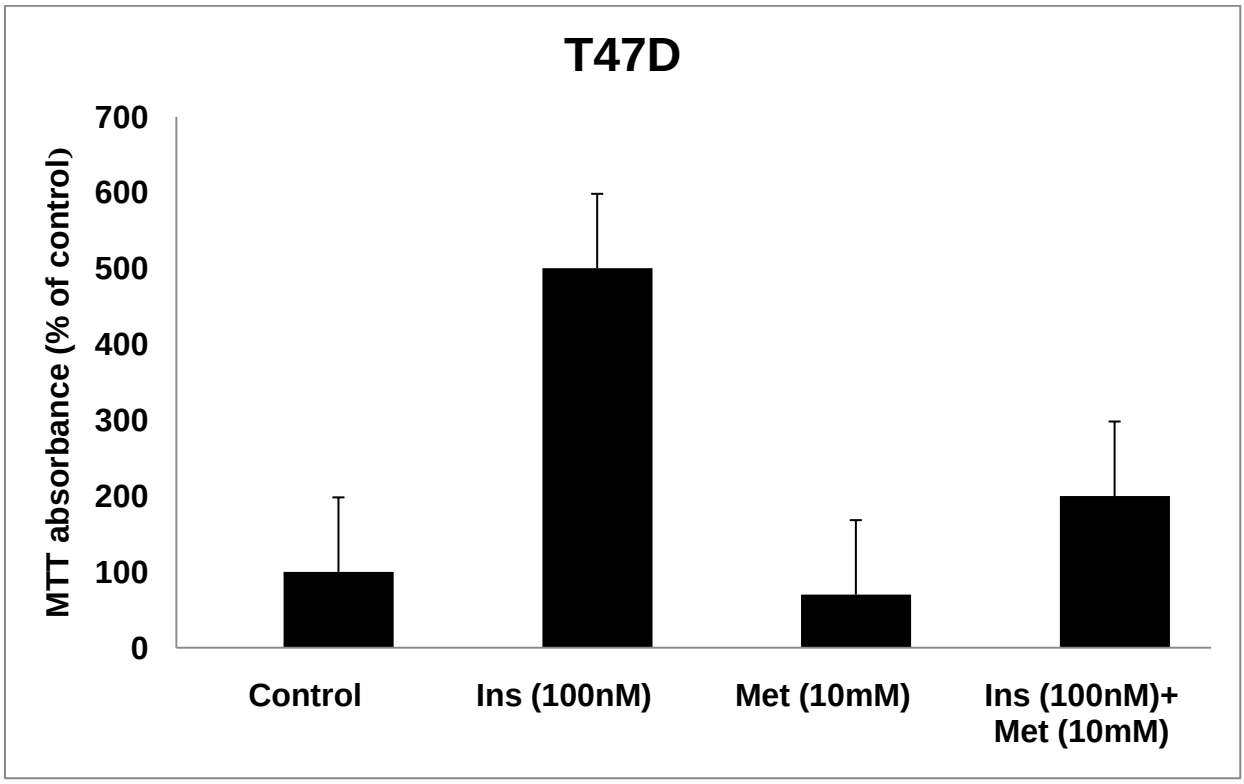

Figure 4.6 Effect of metformin on insulin stimulated proliferation in T47D cells. The data presented here are expressed as mean ± SEM (Standard error mean).

The high prevalence of the metabolic syndrome type 2 diabetes leading to adverse outcome of breast cancer has prompted us to investigate the effects of metformin (a widely prescribed anti-diabetic drug on breast cancer cells in the presence or absence of insulin. The evidences suggesting the potential role of metformin in breast cancer biology came from population, in vivo and in vitro studies (Alimova et al. 2009, Dowling et al. 2007, Ben Sahra et al. 2008). Experimental studies suggest that biguanide metformin shows antitumorogenic activity via insulin dependent as well as insulin independent mechanisms. It increases the glucose uptake, improves hyperglycemia and hyperinsulinemia by activating cellular energy sensor AMPK via its upstream element LKB1 (Alessi et al. 2006). As an insulin sensitizer, it lowers the

insulin levels, reduces insulin resistance which supports the view that it might be helpful in the reduction of the mitogenic effects of insulin on different cell types in insulin-resistant individuals. Metformin acts via activation of AMPK via its upstream kinase LKB1 (Zakikhani et al. 2006, Alessi et al. 2006). A number of studies have reported that activation of AMPK leads to a suppression of mTOR signalling pathway which ultimately leads to suppression of the proliferation of cells. Therefore, metformin as a potential target for breast cancer has prompted us to investigate its effect on the normal growth and insulin stimulated growth of MCF-7 and T47D breast cancer cells. Hirsch et al. reported that tumor forming, self-renewing cancer stem cells, which are resistant to well-defined chemotherapy, are exquisitely sensitive to metformin (Hirsch et al. 2009). Present results clearly demonstrate that metformin inhibits the growth of breast cancer cells per se as well as in the presence insulin.

CHAPTER 5

SUMMARY

5. Summary and Future Perspective

5.1 Summary

- Insulin increases the growth of breast cancer cells (MCF-7 and T47D) in a dose-dependent manner.
- Metformin inhibits the growth of breast cancer cells (MCF-7 and T47D) in a dose-dependent manner.
- Metformin inhibits the growth of breast cancer cells (MCF-7 and T47D) even in the presence of insulin.

5.2 Future Perspective

Studies are needed to examine the underlying molecular mechanism for the growth promoting effects of insulin using different pharmacological interventions such as insulin-receptor antagonist and receptor-isoform specific analogs. Further studies are warranted to better investigate the dose- and time depended effects of metformin on the breast cancer cells either in the presence or absence of insulin and insulin analogs.

CHAPTER 6

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