Modeling and Analysis of PI3K/Akt Pathway in Diabetes and Breast cancer

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A thesis submitted as a MS project in partial fulfillment of the requirement for the degree of Masters of Science in Industrial Biotechnology.

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List of abbreviations

GFAT	Glutamine fructose-6-phosphate amidotransferase
GFs	Growth Factors
GLUT	Glucose transporter
HBP	Hexoseamine Biosynthetic Pathway
IRS	Insulin receptor substrate
OGA	O-GlcNAcase
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase
PDK1	Phosphoinositide dependent kinase 1
PI3K	Phosphatidylinositol-3-OH kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate

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ABSTRACT

Epidemiological studies and clinical evidences have proved a positive association between diabetes and breast cancer. However, the possible biological links between the two heterogeneous diseases have not been fully determined yet. Recently, O-GlcNAcylation, a post translational modification of proteins has been proposed to be associated with diabetes and breast cancer progression. O-GlcNAc transferase (OGT) enzyme carries out O-GlcNAcylation by adding O-GlcNAc group to proteins. The OGT plays a crucial role in regulating PI3K pathway through activating/deactivating the Akt protein. In adipocytes, the overexpression of OGT attenuates Akt signaling as a result the efficiency of insulin signaling is reduced by impairing insulin responsive genes. However, increased expression of OGT results in Akt activation in breast cancer cells, leading to enhanced cell proliferation and inhibition of the apoptosis. Petri net modelling is an established technique for modeling of such complex biological systems, which is employed here to investigate the relationship and behaviors in diabetes and breast cancer systems, the role of PI3K pathway and OGT in progression and crosstalk between the two systems. We explored the overall behavior and dynamics of diabetes and breast cancer systems during insulin resistance and hyperglycemia. We also analyzed the effect of insulin resistance and hyperglycemia on OGT and cellular processes in breast cancer. Moreover, we evaluated the anti-cancer effects by OGT inhibition (shRNA and BZX) and anti-diabetic drug (Metformin), in breast cancer. Our model predicted that the best alternative therapeutics to combat breast cancer progression in diabetic patients could be a combination of OGT inhibitor (BZX) and Metformin. This therapy not only moderates breast cancer

cell proliferation, but also reduces the OGT expression which is a crucial target for treating diabetes and breast cancer.

INTRODUCTION

CHAPTER I: INTRODUCTION

1. INTRODUCTION

Recent epidemiological studies suggest that approximately 400 million people have Type II diabetes worldwide [1]. Moreover, breast cancer is the leading cause of cancer death among women belonging to developed countries. In Pakistan, 1 in 9 women, develops breast cancer at some stage of their life. Statistics show that Pakistani women have the highest risk of developing breast cancer in Asian population, coming second to Non-Arab Israeli women [2]. Risk factors of breast cancer include age, hormonal factors, obesity, benign breast disease, family history and genetics [3].

Recently, diabetes has been related to increased risk of cancer, among them breast cancer is the most common. According to Wolf et al., diabetic patients have 25% increased risk of breast cancer in cohort studies[3] Both the diseases are positively associated , heterogeneous in nature and multi factorial in origin [4]. Along with other risk factors such as obesity and hyperglycemia, insulin resistance in Type II diabetic patients enhances the possibility of cancer and cancer related mortalities.

One of the common associations between Type II diabetes and breast cancer is the abnormality in insulin signaling. Studies show that in diabetes hyperinsulineamia (a condition in which insulin rises above normal levels) is caused due to insulin resistance. It further causes hyperglycemia. Hyperglycemia is a strong inducer of cancer proliferation and progression as cancer cells alter their energetics via Warburg's effect[5]. Warburg Effect is

the phenomena in which malignant cells carry out increased glucose uptake due to enhanced glycolysis even in the absence of oxygen [6].

1.1 COMPUTATIONAL BIOLOGY TO STUDY DYNAMIC SYSTEMS

Biological systems are very dynamic in nature. Over the past decade, computational tools have been vigorously used to analyse biological systems in real time. The core of systems biology consists of computational modelling of systems to build an integrative and coherent picture. It not only helps in investigating the relationships and behavior of elements involved in a biological system but also explains how the system functions as a whole. Moreover, diagrammatic models summarizing biological systems improve mechanistic understanding of the observations.

An established technique for modeling of biological systems is Petri nets. Petri nets consider concurrency of a system which is vital to model biological systems. A Petri net contains two sets of vertices called places and transitions. Resources of the system are depicted by places while the events that change the resource's state are represented by transitions. In a Petri net, edges connect places to transitions and vice versa. A place hold tokens that might define for example the number of molecules involved in the system. Edges move the tokens causing a change in the system through a transition. Recently, Petri nets have widely been used to study and model metabolic pathways[7]. They have a number of benefits as they are more visual, offer variety in designing the system and also help us analyze systems through a range of tools. The following figure shows a simplified Petri net.

We have formulated a hybrid petri net model that describes insulin mediated phosphoinositide 3-kinase (PI3K) pathway in relation to insulin resistance and increased breast cancer progression in adipocytes as compared to breast cancer cells respectively.

For the sake of simplification the pathway has been divided into three states; Normal intact PI3K pathway; altered pathway in adipocytes and pathway over-activation in breast cancer. In breast cancer, the pathway has been studied under hyperglycemic condition. Moreover, the effect of OGT mRNA inhibition, OGT protein inhibition and Metformin was studied on various cell processes. When subjected to specific perturbations, the results reflect the alterations in protein expression and behavior.

The problem statement of the current study is

- Abberations in PI3K/Akt signaling due to abnormal O-GlcNAcylation imply that this pathway is a significant biological link between Type II diabetes and breast cancer. However, the role of OGT in regulation of insulin signaling is not well understood.

We have formulated a hybrid petri net model that describes insulin mediated phosphoinositide 3- kinase (PI3K) pathway in relation to insulin resistance and breast cancer progression in adipocytes as compared to breast cancer cells respectively.

1.2. RATIONALE

Increased rate of O-GlcNAcylation is observed in diabetes Type II and breast cancer. A major causative factor behind this abnormality is hyperglycemia. Inhibition of OGT and control on glucose levels in diabetic breast cancer patients can reduce disease burden.

1.3. RESEARCH OBJECTIVES

- Modeling and analysis of PI3K/Akt pathway and the significance of OGT enzyme in diabetes and breast cancer.
- To evaluate the anti-cancer effects of OGT inhibition and anti-diabetic drug in breast cancer.
- To study the overall behavior and dynamics of the system (diabetes and breast cancer) holistically and independently.

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF DIABETES

Diabetes mellitus is a complex, chronic illness requiring continuous medical care with multifactorial risk reduction strategies beyond glycemic control. Ongoing patient self-management education and support are critical to preventing acute complications and reducing the risk of long-term complications. Significant evidence exists that supports a range of interventions to improve diabetes outcomes.

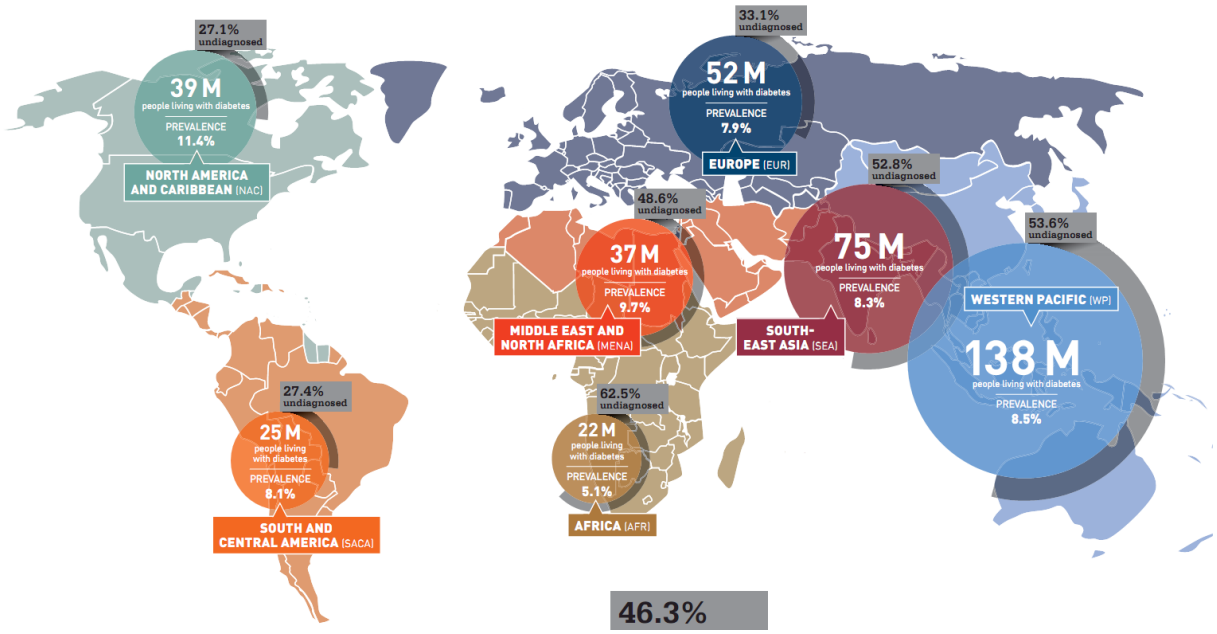


Figure 1 Global prevalence of Diabetes

The rate of diabetes is increasing all over the world. Type II diabetes mainly results due to influences like genetic predisposition, behavioral and environmental risk factors.

Many epidemiologic studies have revealed that having diabetes increases the risk of cancer development. Diabetes and cancer share common risk factors like obesity, age and hyperglycemia. Additionally the contribution of diabetes independently excluding high body

mass has not been studies in detail. Extensive research is required in this regard as obesity is ever increasing across the globe.

Tumor growth certainly increases due to increased insulin level in blood. In recent studies, insulin-like growth factor 1 (IGF-1) has been related to augmented cancer risk as IGF-1 may potentiate oncogenic cell growth. The strong associations between diabetes and breast cancer may be caused due to similar mechanisms.

2.2 EPIDEMIOLOGY OF BREAST CANCER

Cancer that commonly originates from ducts (ductal carcinoma) or the lobules of the breast (lobular carcinoma) is called breast cancer. There are two forms of Mammary ductal carcinoma, the first being Ductal carcinoma *in situ* and the second, invasive ductal carcinoma. Invasive ductal carcinoma is more dangerous as it can metastasize to other part of the body. Moreover, tubular carcinoma, medullary carcinoma and mucinous carcinoma are other rare forms of breast cancer.

Although breast cancer is most prevalent in females worldwide, it may also occur in males. According to International Agency for Research on Cancer, 1200 women in the age group 15-49 die every year because of breast cancer.

Figure 2 Global prevalence of Breast cancer

2.3 CROSSTALK BETWEEN DIABETES AND BREAST CANCER

About 60 years ago, it was observed for the first time that cancer, including breast cancer, is more commonly found in people with diabetes [8]. Recently, numerous studies have supported association between cancer and diabetes and have specifically identified a link between Type II diabetes and breast cancer risk [9]. Postmenopausal women of 50 years or older who have Type II diabetes, have around a 20-27 percent increased risk of breast cancer [10]. Diabetes causes certain changes in the body that

might increase breast cancer risk, for example, hyperglycemia, hyperinsulinemia and increased inflammation.

Moreover, the common risk factors of Type II diabetes and breast cancer include ageing, obesity, physical inactivity and unhealthy diet.

2.4 INSULIN SIGNALING PATHWAY

Insulin is a hormone that plays a crucial role in tissue development and growth and maintenance of glucose homeostasis [11]. Insulin is produced by pancreatic β cells in an inactive form called preproinsulin. The signal sequence present on preproinsulin directs its movement to secretory vesicles. Preproinsulin is converted to proinsulin as this signal sequence is proteolytically cleaved. Proinsulin is then converted to active insulin in response to increased blood glucose or amino acid concentration. Consisting of α and β chains, the active insulin molecule is held together by disulfide bonds. The active insulin molecule is a small protein that consists of α and β chains held together by two disulfide bonds [12]

Insulin stimulates transport of glucose into adipose and muscle cells while reducing production of hepatic glucose through gluconeogenesis and glycogenolysis. Insulin is not only required for amino acid uptake and protein synthesis but also lipid metabolism as it increases lipid synthesis in liver and fat cells.

Insulin signaling is centrally mediated by phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) (Figure 1).When insulin binds to the insulin receptor, a subtype of protein tyrosine kinase (RPTK), it leads to tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. IRS

activation is followed by recruitment of phosphatidylinositol-3-OH kinase (PI3K) to the cell membrane. Recruitment of PI3K further produces its lipid product, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) via phosphorylation of Phosphatidylinositol (4,5)-bisphosphate (PIP2). Production of PIP3 is followed by recruitment of signaling protein like phosphoinositide dependent kinase 1 (PDK1) and protein kinase B (Akt) to the plasma membrane. PDK1 activates Akt by threonine phosphorylation. Activated Akt performs a number of functions, among them cell survival is the most significant.

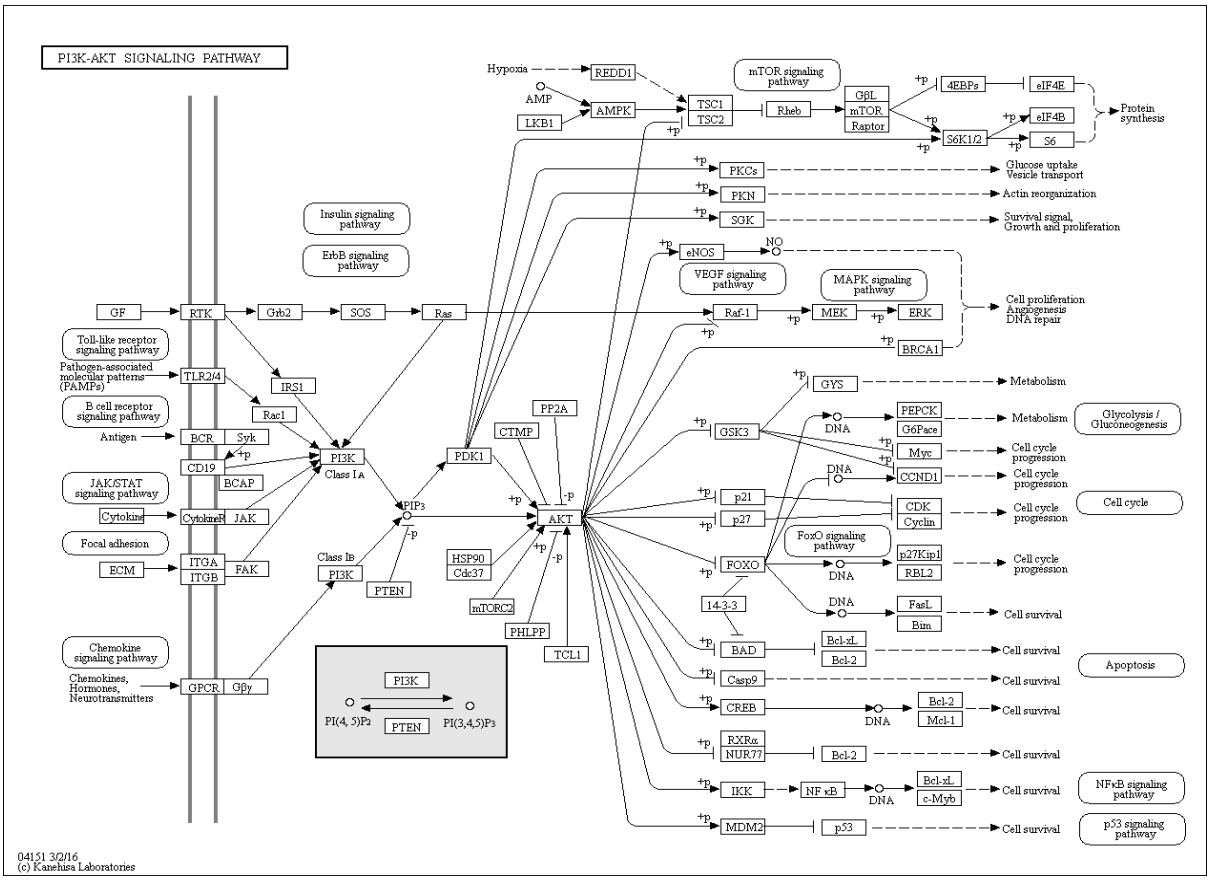


Figure 3: PI3K-Akt signaling pathway

Binding of insulin molecule to insulin receptor leads to auto-phosphorylation of tyrosine residues present on insulin receptor. Phosphatidylinositol-3 kinase (PI3K) is translocated to the cell membrane and activated. As a result phosphatidylinositol-3,4,5-triphosphate (PIP3) is produced,

which recruits Protein Kinase B (also known as Akt). The interaction of PIP3 with the PH domain of Akt induces conformational changes in Akt, thereby exposing the two main phosphorylation sites at T308 and S473. Phosphorylation at T308 and S473 by protein serine/threonine kinase 3'-phosphoinositide-dependent kinase 1 (PDK1) and mtor Complex 2 (mTORC2) respectively, is required for maximal Akt activation. Activated Akt translocates to the nucleus and carries out the activation and inhibition of various targets resulting in cellular survival and cell growth and proliferation.

2.5 ROLE OF PI3K PATHWAY IN INSULIN RESISTANCE

Insulin signaling results in important cellular functions such as cell differentiation and proliferation. Abnormalities in insulin function due to aberrant insulin signaling can cause insulin resistance.

Insulin resistance is a pathological state in which the efficiency of insulin signaling to regulate blood sugar decreases. Two types of insulin receptor isoforms are involved in insulin signal transduction: isoform A (IR-A) and isoform B (IR-B). IR-A responds generally to insulin and insulin like growth factors (IGFs), whereas IR-B is specific to insulin only and plays a significant role in glucose homeostasis [13]. Aberrant expression of IR-A has been observed in cancer cells, leading to increased responsiveness towards insulin and IGF-II resulting in enhanced cancer promoting effect due to hyperinsulinemia, a major hallmark of Type II diabetes.

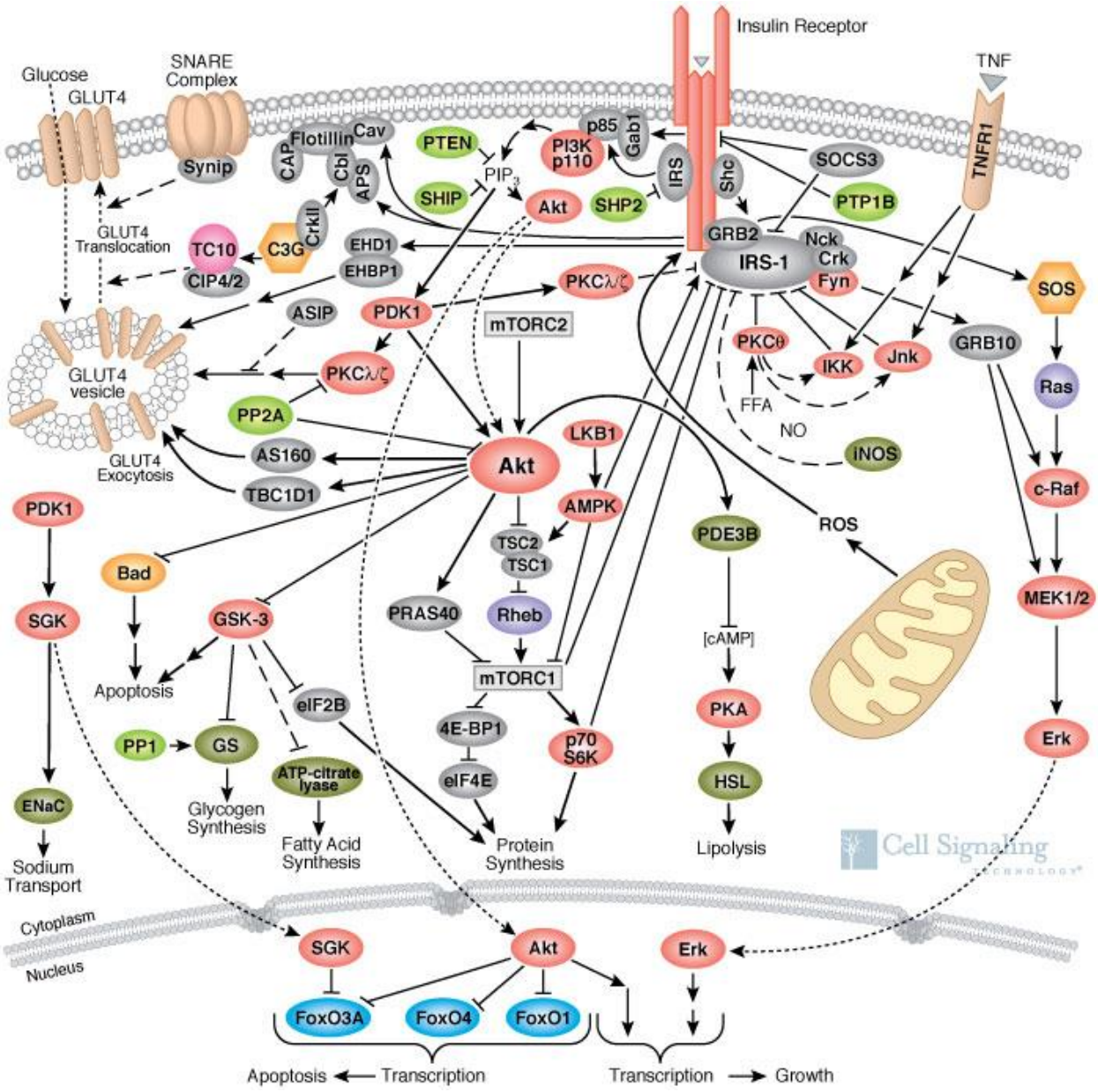


Figure 4: Molecular basis of insulin resistance

In their study, Stout and Vallance-Owen emphasized on the possibility that cell proliferation might be enhanced by insulin signaling. Similarly many studies reported that tumor growth is dependent on insulin for its growth. Since then, scientists have been exploring mechanisms that explain the role of insulin in promoting cell survival and proliferation.

Insulin signaling begins when it binds to Insulin receptor, a receptor tyrosin kinase. This receptor carries out autophosphorylation of the tyrosine residues present in the intracellular domain. This leads to IRS-1 (insulin receptor substrate-1) through phosphorylation. Insulin signal is transmitted by two main phosphorylation cascades. These two cascades are differentiated via their prime mediators namely mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K).

2.6 PI3K/ AKT PATHWAY IN CANCER

Since 1980's ,the lipid kinases named phosphoinositide 3-kinases (PI3Ks) have been studied for playing a crucial role in regulating essential cellular processes including cell proliferation, survival and differentiation. PI3Ks are the major downstream effectors of G protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). They are responsible for transduction of signals from cytokines and growth factors into intracellular signaling leading to activation of Akt and other downstream pathways. The central negative regulator of PI3K signaling is a tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10).

Genomic studies on human cancer have exposed that several PI3K pathway components are targeted by mutations (somatic and germline) frequently in a broad range of human cancers.

In terms of therapeutic mediation in cancer, PI3K patway is a very attractive target because of the fact that components of PI3K pathway are well suited for pharmacological intervention.

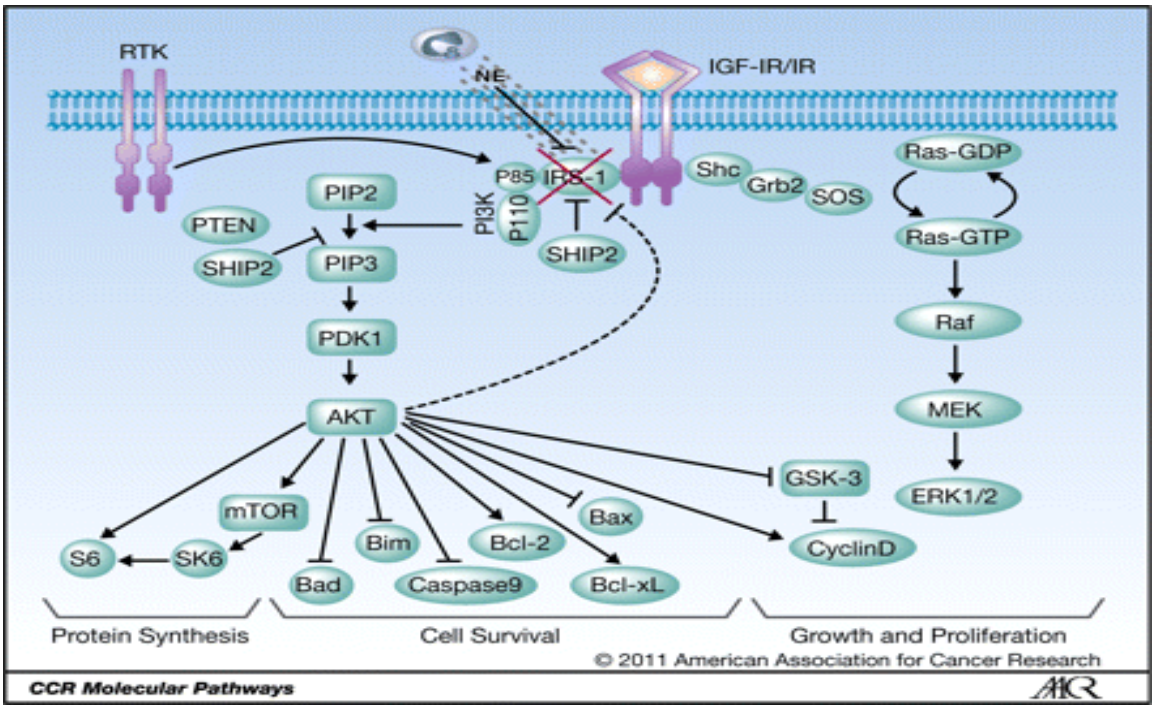


Figure 5: Involvement of PI3K/Akt pathway in cancer

2.7 ALTERED ENERGY METABOLISM IN DIABETES AND BREAST CANCER

On average 3-5% of glucose entering into the cell leads to Hexosamine Biosynthesis Pathway (HBP). Hexosamine Biosynthesis Pathway is nutrient responsive as it incorporates all the nutrients including carbohydrate, amino acid, lipids and nucleotide. In this pathway, glucosamine-6-phosphate is formed from fructose-6-phosphate by an enzyme glutamine: fructose-6-phosphate-amidotransferase in the rate limiting step glucosamine-6-phosphate is further converted to form uridine-5-diphosphate-N-acetylglucosamine (UDP-GlucNAc) (Figure 6). UDP-GlucNAc, also called the biosensor of metabolic changes, is used as a substrate by an enzyme O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) to carry out O- GlcNAcylation (Figure 7)

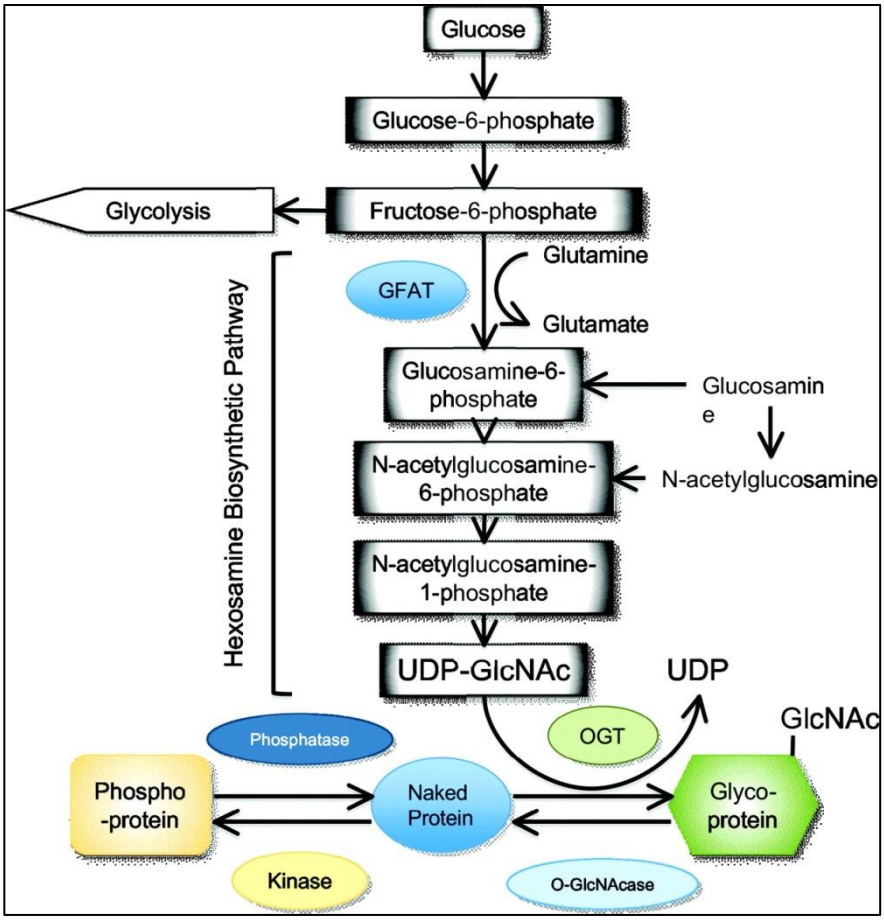


Figure 6: Hexoseamine Biosynthetic Pathway

Hexoseamine Biosynthetic Pathway incorporates all the nutrients resulting in the production of the biosensor of metabolism "UDP-GlcNAc". OGT uses UDP-GlcNAc as a substrate to carry out O- GlcNAcylation.

Hyperinsulineamia further aggravates glucose concentration in blood leading to hyperglycemia, an abnormal state in which excess glucose concentration is present in blood [14]. Studies suggest that hyperglycemia activates several signaling pathways that are involved in regulating cancer cell proliferation, metastasis and apoptosis for example the PI3K/Akt pathway. [15-17].

2.8 ROLE OF OGT IN REGULATION OF PI3K PATHWAY

O-GlcNAcylation is the recently characterized post-translation modification process. Alterations in this process have shown to play a vital role in development of several diseases including diabetes and cancer[18].Caldwell *et al.,* reported that the OGT gene expression and O-GlycNAcylation was increased in breast cancer cell lines compared to normal cell lines. Increased O-GlycNAcylation enhances the invasiveness of breast cancer cells in vivo [19]. Moreover, an increase in OGT gene expression has been observed with the increase in tumor grade implicating that OGT may play a role in tumor progression and metastasis [20]

Diabetes and diabetic complications occur due to hyper O-GlycNAcylation of many proteins involved in PI3K pathway[21]. An association between OGT and PI3 Kinase has been found by Yang *et al*., in 2008 while studying insulin resistance. OGT is involved in regulating insulin signaling pathway as it dampens the effect of insulin responsive genes. Moreover, nutrient excess leads to aberrant elevation in O GlcNac levels [22]. Therefore, increased O-GlycNAcylation of insulin signaling pathway causes insulin resistance.

Moreover, Caldwell *et al*., reported that the OGT gene expression and O-GlycNAcylation was increased in cancer cell lines compared to normal cell lines and increased O-GlycNAcylation enhances the invasive properties of breast cancer cells in vivo [19]. Furthermore, an increase in OGT gene expression has been observed with the increase in tumor grade implicating that OGT may play a role in tumor progression and metastasis [23].

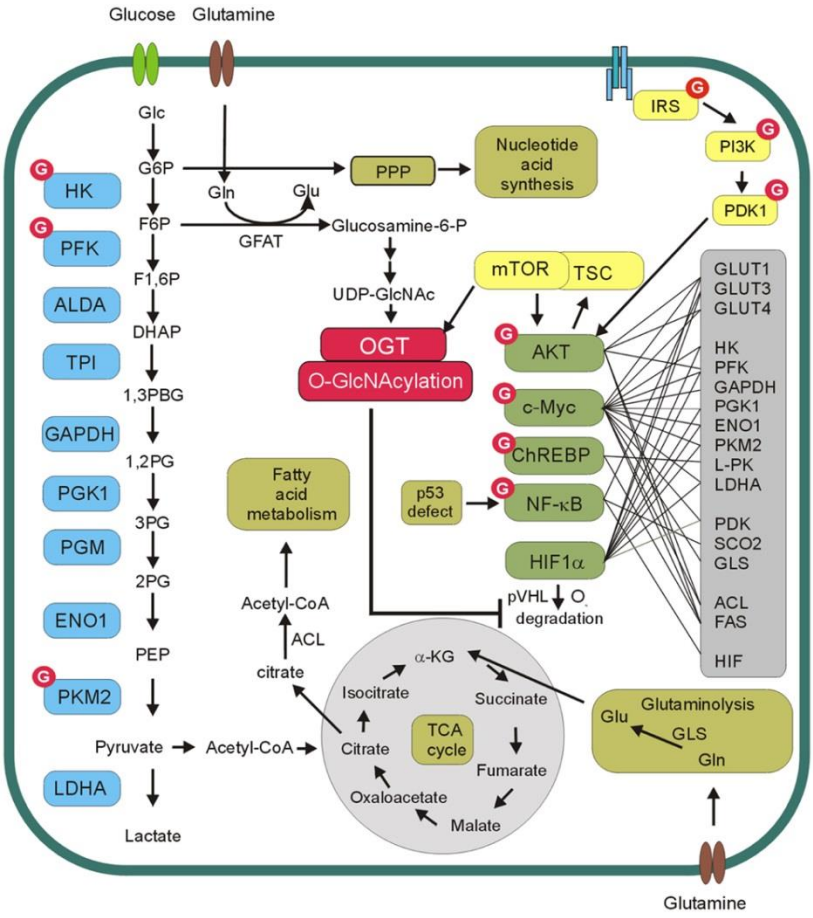


Figure 7: Transcriptional regulation of cancer metabolism by OGT

2.8.1 PROTEIN STURUCTURE OF OGT

OGT is an important enzyme encoded by OGT gene with the chromosomal location of Xq13. The human OGT gene is a heterotrimer containing one 78 kDa subunit and two 110 kDa subunits. Its functional domains include a catalytic domain, a linker domain and an N- terminal domain with 9-64 12 TPR repeats. The N-terminal is responsible for substrate recognition, linker domain for nuclear localization signaling and C-terminal for catalysis or break down of UDP (Uridine diphosphate). The structure of OGT is given in Figure 8:

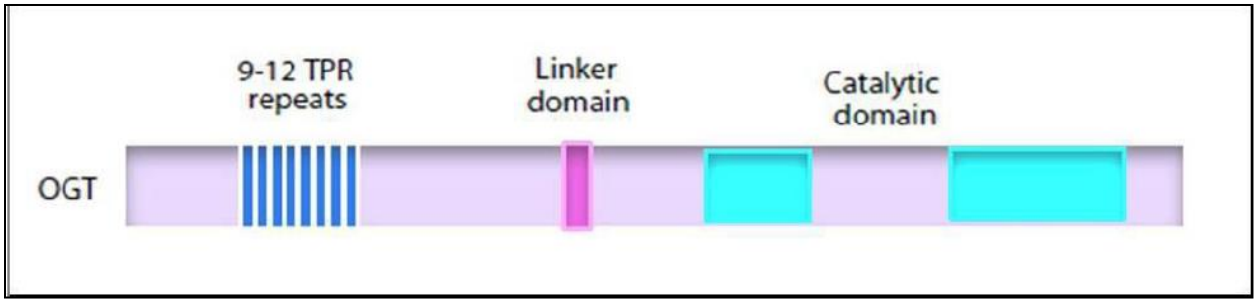


Figure 8: Protein structure of OGT[24]

2.8.2 MECHANISM OF ACTION

O-GlcNAc Transferase enzyme catalyzes the addition of the O-GlcNAc moiety to the hydroxyl group of serine or threonine residues on both cytoplasmic and nuclear proteins [25]

OGT is antagonized by another enzyme, O-linked N-acetylglucosaminase (OGA) which removes the O-GlcNAc from proteins as shown in the Figure 9:

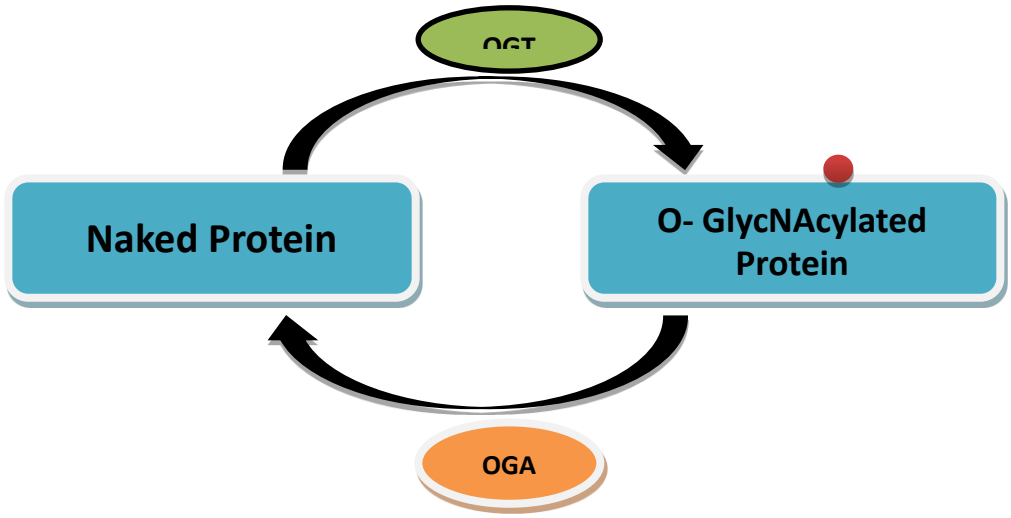


Figure 9: O-GlcNacylation

O-GlcNAc Transferase enzyme modifies naked protein by adding O-GlcNAc moiety at Serine or Threonine residues whereas O-GlcNAcase enzyme reciprocates this modification by removing the O-GlcNAc moiety from proteins.

2.8.3 OGT IN DIABETES AND BREAST CANCER

This process is very crucial to biological processes like transcription, translation, apoptosis, proteasomal degradation, cytoskeletal reorganization and nuclear transport [26]. However, increased activity of O-GlcNAc can rather be very harmful for healthy as well as diseased.

An aberrant expression of OGT is observed both in diabetes and cancer leading to a disturbed Akt signaling pathway[18]. However, the direct effects of increased OGT on downstream proteins of the Akt pathway yet remain unexplored. A deeper understanding of the role of dysregulated OGT in aggravating diabetes and cancer can help us find biological link between the diabetes and cancer.

One of the possible mechanisms that associate Type II diabetes and breast cancer is activation of PI3K/ Akt signaling pathway. PI3K/Akt pathway involves a number of downstream proteins forming a complex signalling network as it integrates signals for metabolism and cell growth/survival. When perturbed, it can produce dramatic consequences. Although a numerous mutations have been reported associating PI3K pathway to diabetes and breast cancer, our focus was on activation of the pathway, involvement of O-GlcNAc signaling and effect of hyperglycemia in insulin resistant adipocyte and breast cancer cell. O-GlcNAc signaling is one potential link among these diseases.

Akt, also called protein kinase B, is a threonine/serine kinase. It has three isoforms namely AKT1, AKT2 and AKT3 encoded by PKBα, PKBβ, and PKBγ genes respectively. These isoforms share a common protein structure consisting of PH domain on N-terminal, a central catalytic domain and a regulatory domain on C-terminal. After Akt is translocated to cell membrane it is activated as the PH domain of Akt is docked to PIP3 on the cell membrane, resulting in a conformational change. As a consequence two key amino acid residues (T308 and S473) are exposed which are phosphorylated by PDK1 and mTORC2 respectively to cause complete activation of Akt.

Activated Akt further phosphorylates various downstream proteins like FOXOs (forkhead family of transcription factors) and GSK3 (glycogen synthase kinase 3). Thereby, a broad range of cellular processes like cell survival, metabolism, proliferation and protein synthesis are regulated via Akt activation

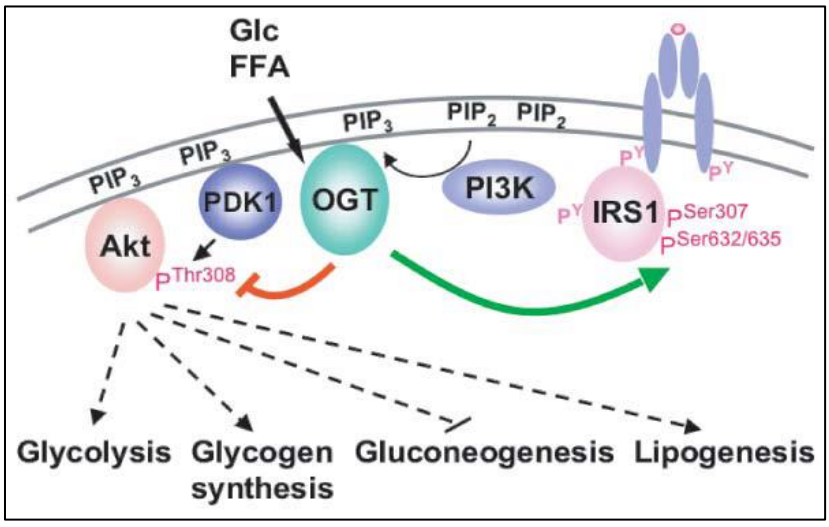


Figure 10: OGT interaction with downstream proteins of PI3K/Akt pathway

2.9 THE EFFECT OF OGT INHIBITION IN DIABETES AND BREAST CANCER

The biological role of OGT has been primarily explored through specific OGT inhibitors in eukaryotes. In previous years, numerous compounds have been employed leading to important advancements in science related to O-GlcNAcylation. Nonetheless, these inhibitors require further attention as off-target effects, limited cell permeability, lacks of specificity still offer hindrances that need to be reduced in the future.

2.9.1 OGT INHIBITORS

The biological role of O-GlcNAcylation has been explored using several OGT inhibitors in eukaryotes. O-GlcNAc field has advanced swiftly in the last few years as more and more inhibitory compounds have been introduced to study OGT function. One of high-throughput screening -derived OGT inhibitors namely BZX (4-methoxyphenyl 6-acetyl-2-oxobenzo[*d*]oxazole-3(2*H*)-carboxylate) was proposed as a neutral pyrophosphate mimic and has been applied to breast cancer cell. BZX was recognized as an cell permeable irreversible inhibitor of human OGT [27]. BZX cross links the active site residues Cys^{917} and Lys^{842} with S-thiocarbamate bond [28]. Treatment of BZX showed anti-invasion and antigrowth effects on breast cancer cell as it modulated transcription factor FoxM1 [19].

2.10 THE EFFECT OF ANTI DIABETIC DRUGS IN BREAST CANCER

Furthermore, population based studies have shown that the use of anti-diabetic drugs such as Metformin decreases the cancer risk in diabetic patients [29]. A study conducted by Bonanni and colleagues showed that the breast cancer patients who were given Metformin presented decreased level of phosphorylated Akt as compared to control group[30].

2.11 COMPUTATIONAL APPROACHES FOR STUDYING BIOLOGICAL SIGNALING PATHWAYS

Coordination among cells and basic activities are governed by an intricate communication system called cell signaling. The flow of information in a cell is achieved by processes like activation and deactivation of signaling proteins. The enzymes responsible for protein phosphorylations and de-phosphorylation are called kinases. Importantly, such signaling cascades do not involve substance flow but signal flow. These signaling cascades are multifaceted systems and understanding the exact signal flow through such complex system is a difficult task.

Diabetes and breast cancer system similarly produce a highly complex system. In these systems various interlinking signaling networks establish disease state when perturbed. There are a number of techniques in the field of systems biology that can be employed to study molecular interactions and signaling networks for example graphs or differential equations. Through computation of distinct behavior of cellular processes and components, the organization of designed cellular signaling network can be

studied in detail using graph based system biology methods. We can not only obtain a static view of signaling network but also find other pathways, cross-talks, hubs and junctions among the system. However, these methods do not elucidate the behavioral changes within a system. As biological processes are highly dynamic in nature, analysis of a dynamic system requires the use of tools like logical modeling and Petri nets.

Petri nets provide continuous, discrete or hybrid estimates of a system. Petri nets have an advantage over logical modeling as it can employ a huge number of entities in a particular system while logical system might undergo state space explosion. A state space explosion results as the complexity of a system rises due to addition of more entities. The cell signaling system that was modeled consisted of 20 entities so we chose Petri net modeling approach to carry out the analysis of diabetes and breast cancer system as it is one of the most effective and efficient technique to study system behaviors.

2.12 Types of Petri nets:

The following figure shows the hierarchy of Net classes that can be drawn in Snoopy:

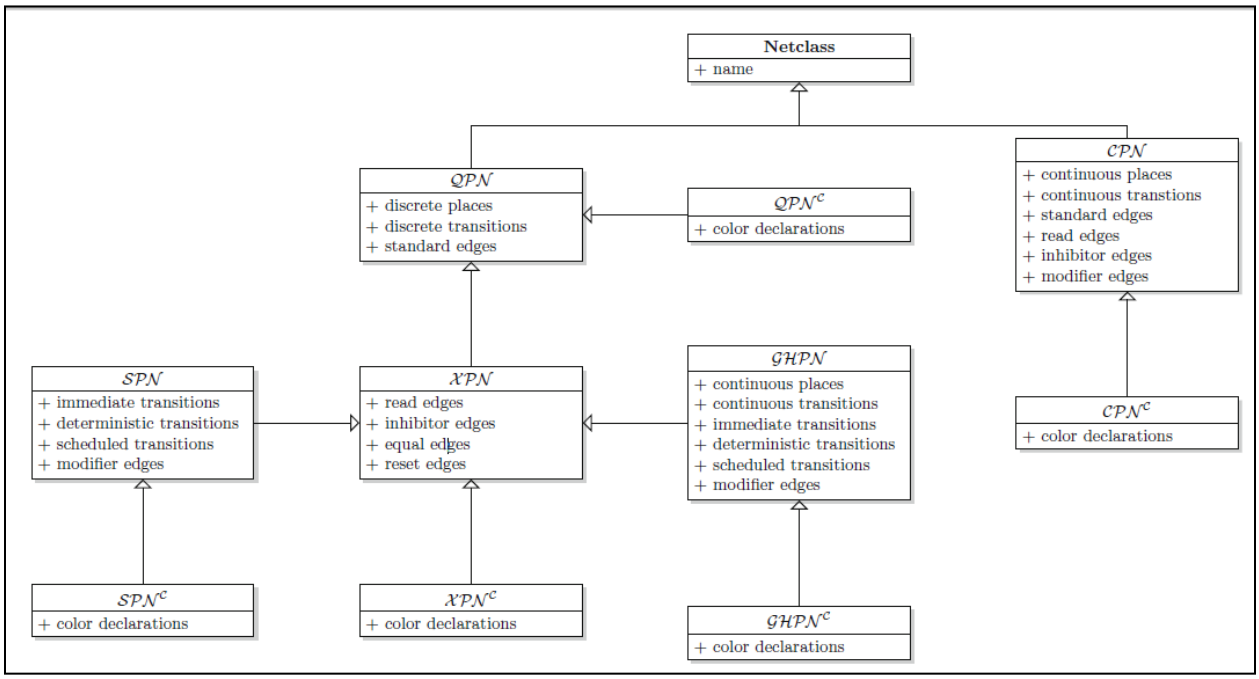


Figure 11: Types of Petri

2.12.1 Qualitative Petri nets (QPN)

A Qualitative Petri net utilizes standard Place/Transition nets and extended Petri nets (XPN). It does not involve any time feature; therefore, it allows purely qualitative modeling for example biological networks. Tokens used in such a Petri net may represent number of molecules or concentration[31].

Extended Petri net enhances standard Petri net by special edge types; inhibitor edge, read edge, reset edges and equal edges.

2.12.2 Stochastic Petri nets (SPN)

This net class is an extension of Qualitative Petri net as the transitions are assigned exponentially distributed waiting times that are specified by firing rate functions. A rate function is a state dependent property and can be an arbitrary arithmetic function that deploys the pre-places of a transition as user-defined, integer variables and real values- constants. Special modifier edges associate pre-places with transitions[32]. The firing rate of a transition maybe modified by using these edges, however, they do not effect enabledness of a transition.

Stochastic Petri nets also include mass action semantics. Several parameter lists, rate function lists and multiple initial marking can be maintained allowing flexible systematic evaluation through computational experiments.

2.12.3 Continuous Petri nets (CPN).

Systems of ordinary differential equations (ODEs) are unambiguously specified in a graphical form as Continuous Petri nets[33].

The continuous rate functions obey same rules as SPN. Similarly, the parameter lists, function lists and initial marking lists are also applied in a Continuous Petri net. The tokens may represent concentrations. The underlying systems of ordinary differential equations are automatically generated by Snoopy.

2.12.4 Generalized hybrid Petri nets (GHPN).

A Generalized hybrid Petri net is generated in Snoopy by integrating functionalities of both continuous and stochastic Petri nets. GHPN are specifically used to model systems that show interplay between continuous and stochastic behavior. Typically, a GHPN includes a hybrid representation of a biological reaction in which fast reactions are demonstrated by continuous reactions where as slow reactions are modeled by using stochastic transitions.

We constructed a hybrid Petri net (HPN) because it helps design continuous modeling with real numbers. Using this tool we were able to express the relationship between discrete and continuous values to analyze various cellular functions.

Construction of the model was followed by adjusting mass functions and arc weights according to pathway requirement, based on experimental evidences.

In this study we will focus on the effect of overexpression of OGT enzyme in insulin resistance and breast cancer. We will also look into the effect of hyperglycaemia on PI3K/Akt pathway and analyse the effect of OGT inhibitors and Metformin on cellular functions.

MATERIAL AND METHODS

CHAPTER 3: MATERIAL AND METHODS

3.1 PETRI NET MODELING

Petri Nets (PNs) were developed by Carl Adam Petri in 1962 for the analysis of technical systems, in particular the concurrent processes occurring in such systems, and were introduced as a part of his dissertation in 1962 [34] . The framework itself is simple and flexible enough, though, that it has been successfully applied in other domains and studies as well, such as biochemical processes, industrial mechanisms, software analysis etc. [35].

Numerous regulatory pathways and networks have been modeled using Petri Nets to analyze and predict system behaviors. The modularity and flexibility are a major advantage of Petri Nets that allow the user to model a single or a combination of systems to analyze a variety of networks including epigenetic, metabolic, transcriptional and protein-protein interaction etc.

3.1.1 Standard Petri Nets

Definition:

A Petri net is a bipartite graph consisting of two sets of vertices, places and transitions. Circles represent places where as boxes or bars represent transitions. Usually, a place describes a resource or an entity (for example proteins, DNA, RNA etc.) and its state (number of entity present, relative level, cellular concentration etc.). In comparison, a transition describes any process occurring in the system.

In a Petri net, the edges or arcs always connect vertices from two distinct sets only, i.e. places connect to transitions and vice versa. The weight of an arc is

equal to 1 by default and it represents the arc multiplicity. An arc never connects two transitions or two places. An arc with a hollow dot at its head (———○) represents an 'inhibitory arc'. The function of an inhibitory arc is suppression of token flow as it stops the firing of a transition. Places present before transitions are called "input places" whereas places after transitions are "output places" for that specific event. 'Tokens' are denoted as numbers or dots within a place in a Petri net. They are variable and represent states of entities [36]. Tokens, in particular, signify relative concentration levels of entities like RNA, proteins, ions, organic and inorganic molecules in a biological system [37]. 'Marking' represents the state of the system based on the presence of tokens in a particular place at that instance. In a dynamic system, marking evolves with time as the tokens flow in the model. All the input places must have tokens to fire a transition. In accordance with respective arc multiplicities, the number of tokens are withdrawn from input places and deposited to output place after a transition has been fired [38].

Formally, a Petri net consists of a 3-tuple (P, T, W), where:

- P is a finite set of places;

- T is a finite set of transitions;

- W is a set of arcs that connect a transition to a place or vice versa.

The two sets of vertices are:

1- Places : P= {P1, P2, P3….Pn}

2- Transitions: T= {T1,T2, T3 ….Tn}

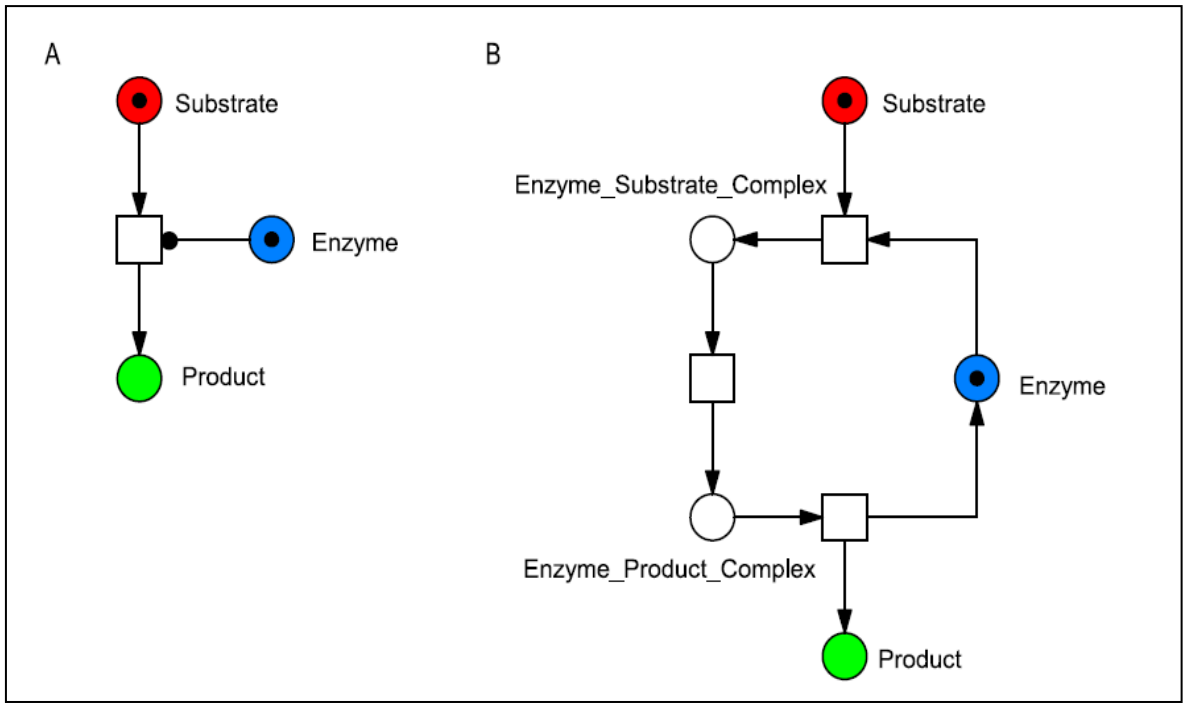


Figure 12: Representation of Enzymatic Reaction:

Figure (A) shows a Petri net of a simple enzymatic reaction. The enzyme is connected with a read edge depicting that the enzyme remains unconsumed. Figure (B) shows a Petri net describing steps of enzymatic reaction. The enzyme is consumed temporarily and released by the end of the reaction. [36]

3.1.2 Semantics and Properties of Petri Nets

Firing enabled transitions define how the behavior of a system undergoes evolution with time. The behavior and evolution of a Petri Net is defined by the firing of enabled transitions. A transition is an enabled or live transition when the arc weights amidst pre places and transition are satisfied with the marking of its pre-places. As the transition is fired, the weighted markings of pre-places are subtracted from it and deposited in the post- places. This property is called firing rule.

In the present study, petri net model generation and simulations were run using Snoopy version 2.0 [39]. Snoopy offers a variety of Petri net modeling options. Normally modeling tools do not offer much variety in net classes that can be used to design a model. However, Snoopy includes a number of net classes for example continuous, hybrid, stochastic, time-free petri nets and their colored extensions. It also includes a number of analysis tools such as, simulation, built-in animations and export to other tools for analysis [39] Snoopy offers a unified environment for modeling of Petri nets. Snoopy provides the following modeling functions:

- Addition of graphical elements (Places, transitions, edges)
- Edit or modify the properties of nodes (e.g. initial marking, name) and edges (multiplicity).
- Define declarations (in colored Petri nets)

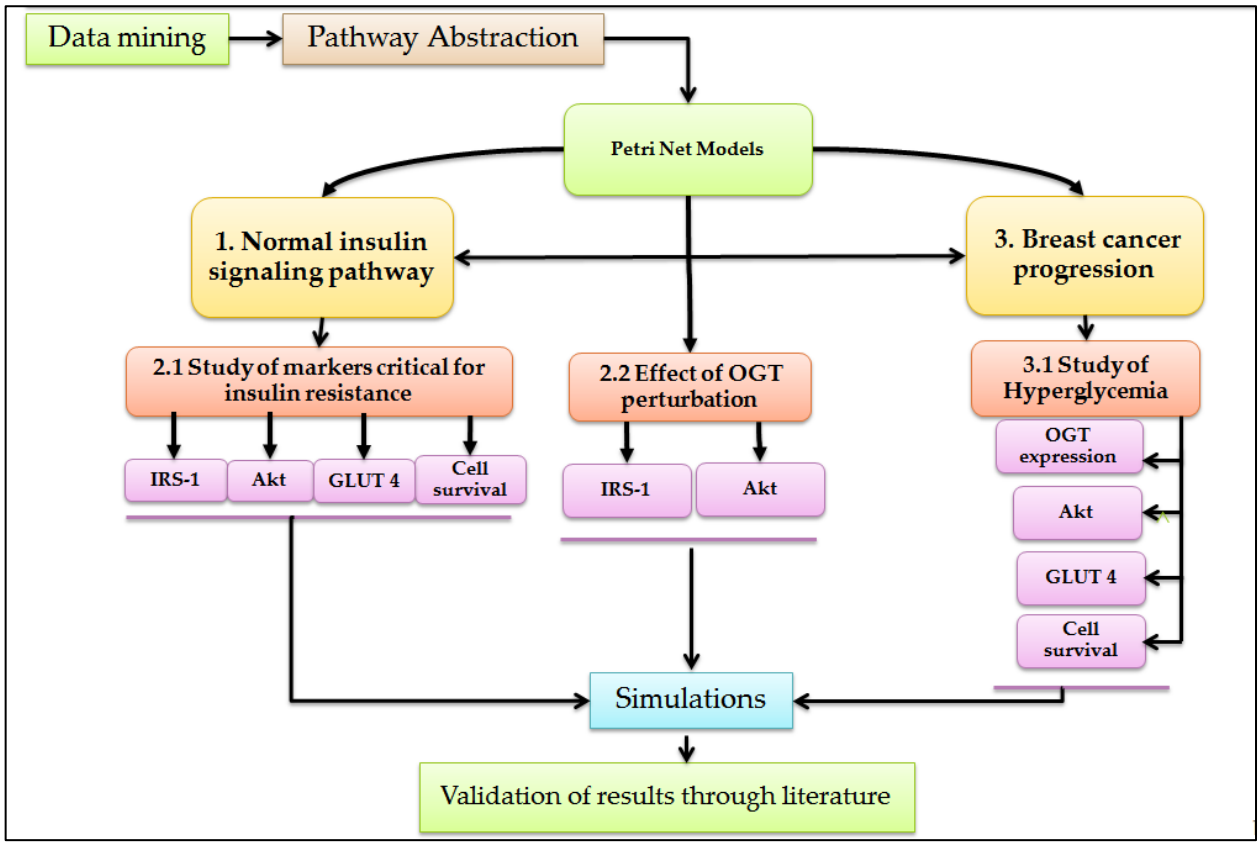


Figure 13: Flowchart explaining Model based study design

3.2 NON-PARAMETRIC STRATEGY FOR PETRI NET MODELING

Various studies have reported studies employing Petri net approaches for modeling cellular signaling cascades and gene regulatory networks [40]. This formalism has been extended by various groups [41] [37] [42] to comprehend the intricacy of the cellular environment. Our study is based on the non-parametric strategy devised by Ruths *et al.*,[43] for studying the dynamics of cell specific signaling pathways employing Petri net approaches. The PN model is based on the assumption that the signaling network connectivity is the most significant determinant of signal propagation [40]. Therefore, changes in the activity levels of the proteins within a particular signaling pathway correlated with their abstract quantities are represented in the PN model by token number [43].

3.2.1 Construction of the Petri net

The rules of a chemical reaction may not always be applied to a signaling pathway. A cell signaling pathway is activated as a foreign particle interacts with receptors present on cell surface. in turn, it activates downstream proteins through modifications like phosphorylation, de-phosphorylation or interaction with proteins. Modeling such a complex and dynamic pathway a modeling strategy was formulated that well-suited the network topology of the disease model (diabetes and breast cancer). In the designed the Petri net model (Fig. 16 to 26), places represent the proteins and genes (e.g. insulin receptor, ligands, enzymes, transport proteins, genes etc.) involved in the PI3K/Akt pathway while the transitions represent the processes like interactions or reactions occurring among the places (e.g. formation of complex, chemical reactions, post translational modification, transport processes etc.). The markings of continuous places are real numbers and the firing of transitions is a continuous process. All the arcs have weight equal to 1 except for those mentioned otherwise. Moreover, inhibitory arcs are used to show inhibitory effects of anti-diabetic drug (Metformin) on cellular processes. Our model depicts source transitions as the availability or synthesis of proteins/ drugs in the pathway while sink transitions represent the decay or dissociation of entities exiting the system.

In our study, Hybrid Petri Net was designed to understand relative activity change (up-regulation/down-regulation) and not the exact measurement of the protein concentration/parameters within the PI3/Akt signaling pathway.

A Hybrid Petri net does not require an exhaustive state space and can define a finite system with an infinite state space. It offers features that allow representation of modules in the designed model and analysis of network can be done through linear algebraic methods.

Various Hybrid Petri nets have been proposed in the literature, but there is so far no widely accepted classification of such models.

When studying biological systems, the concentration level or a discrete number of an entity (protein, DNA, RNA etc.) is represented by tokens. In order to perform simulations, it is important to indicate the availability of entities in a biological system. Therefore, initial values were assigned as tokens in the PI3K/Akt pathway that corresponded to an initial state of entities in the cell

Major steps taken to generate the Petri net model included; survey of literature

The steps involved in the PN model generation include: literature survey to extract the critical factors involved in insulin resistance and breast cancer with and without hyperglycemia; pathway abstraction; generation of Petri net model and its analysis.

3.3 PATHWAY ABSTRACTION

Due to limited analytical resources it is difficult to analyze kinetic parameters of each and every biological reaction in a system.[44]. During the analysis of complex biological signaling pathways the occurrence of state space explosion is common [45] . Therefore, the complex pathway taken from KEGG database with the pathway ID hsa04910 [46] we analyzed and isolated key proteins (IRS-1, Akt and GLUT-4) that play an central part in diabetes and breast cancer (Fig. 14). Data from literature and thorough analysis exposed the significance of OGT- Akt protein interaction in instigating diabetic and breast cancer complications.

After restricting the Petri net to this pathway, we applied the strategy to carry out abstraction of PI3K/Akt pathway as explained by Paracha *et al*. [47]. In short, an example indicated that

if an entity B is activated by an entity A and B activates another entity C which plays a role in another pathway, consequently B can be removed and the relation can be described as C is activated by A.

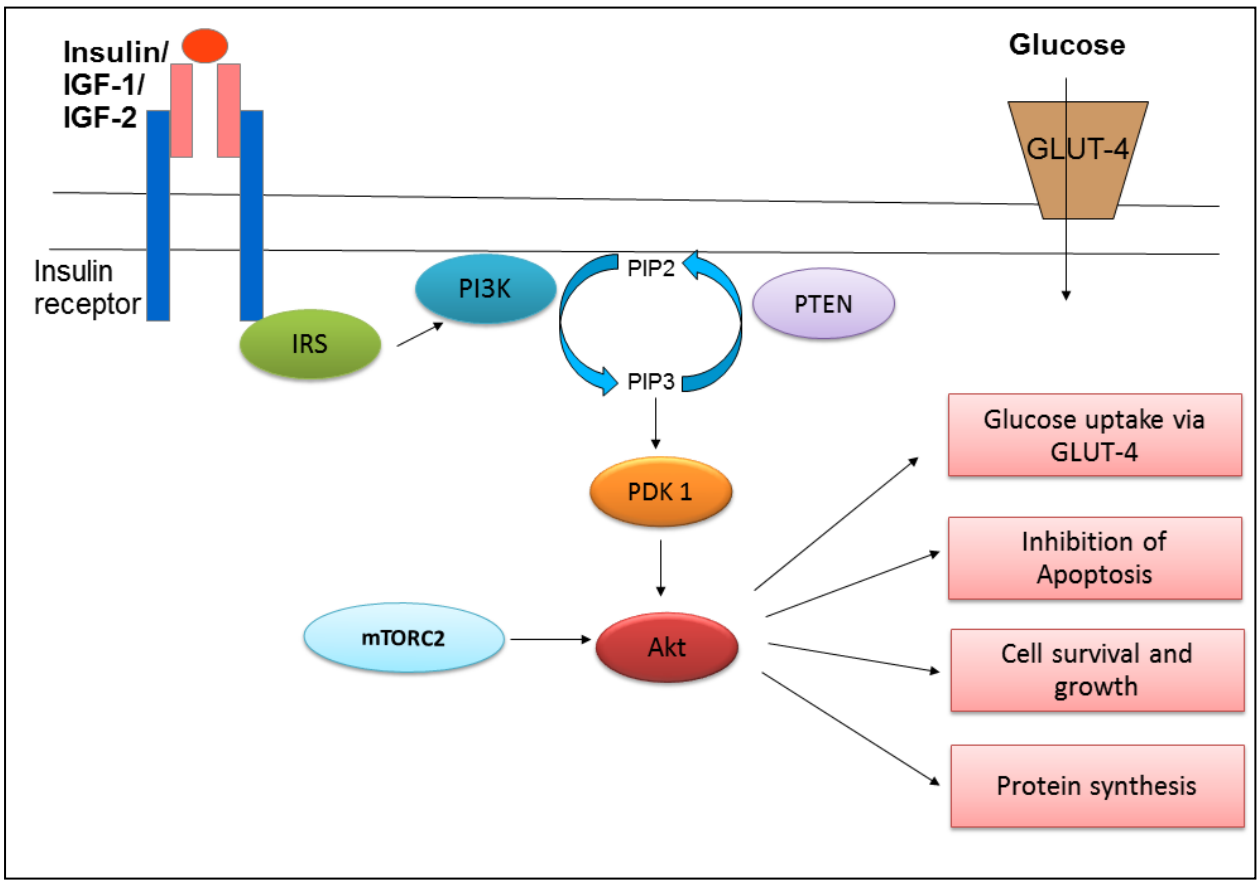


Figure 14: Abstracted pathway of PI3K/Akt signaling process as presented in (Figure 16)

3.4 UNDERSTANDING THE CROSS-TALK BETWEEN DIABETES AND BREAST CANCER

Epidemiological evidence shows that individuals with diabetes have significantly higher likelihood of developing multiple types of cancer, especially breast cancer [48]. The mechanisms driving cancer progression in diabetic patients were studied in detail and following theories were focused on.

In early stages of diabetes, pancreatic β-cells produce excess amount of insulin, resulting in hyperinsulinemia. While insulin-target organs are resistant to the actions of insulin in diabetes, hypeinsulinemia may have pro-growth effects on a nascent tumor by allowing the tumor to overcome an important early barrier in tumorigenesis, that is, lack of growth factor signaling. There is epidemiological data to suggest that insulin secretion rate influences cancer risk and/or cancer progression [49]. Insulin stimulates the proliferation of tumor cells *in vitro* [50] and promote glucose uptake in the subset of tumors that are insulin-dependent [51].

Recent research studies indicate that hyperinsulinemia or administration of synthetic insulin in diabetes may enhance growth factor signaling and promote glucose usage to promote tumor growth (Figure 15). As many tumors alter energy metabolism, increased glucose intake by tumor enhances its invasiveness. Moreover, insulin serves as the spark to initiate cancer development at early stages when self-sufficiency of growth factors has not yet been established. Hyperglycemia, another characterizing feature of diabetes, may also contribute to enhanced cancer risk [75]. Moreover, OGT is highly abundant in β cells in the islet [52] and O-GlcNAc levels in β cells are sensitive to glucose [53] , implying that O-GlcNAc may function as a glucose sensor to regulate insulin secretion. O-GlcNAc regulates insulin signaling in response to glucose flux, hyperglycemic condition leads to elevated O-GlcNAc modifications in response to increased flux through the HBP.

Furthermore, abnormal levels of O-GlcNAc in cancer cells may contribute to deregulated posttranslational control of protein function linked to oncogenic phenotypes. Interestingly, overexpression of OGT is observed in diabetes and cancer. At the systemic level in diabetes, the excess availability of glucose contributes to tumorigenesis Thus; diabetes associated hyperglycemia and can promote tumorigenesis by inducing hyperinsulineamia. However, the generality of this mechanism for tumors that exist in regions with high levels of adipocytes (e.g., breast) and the role of OGT in implicating diabetes and breast cancer need further elucidation.

These possibilities have been summarized in Figure 15.

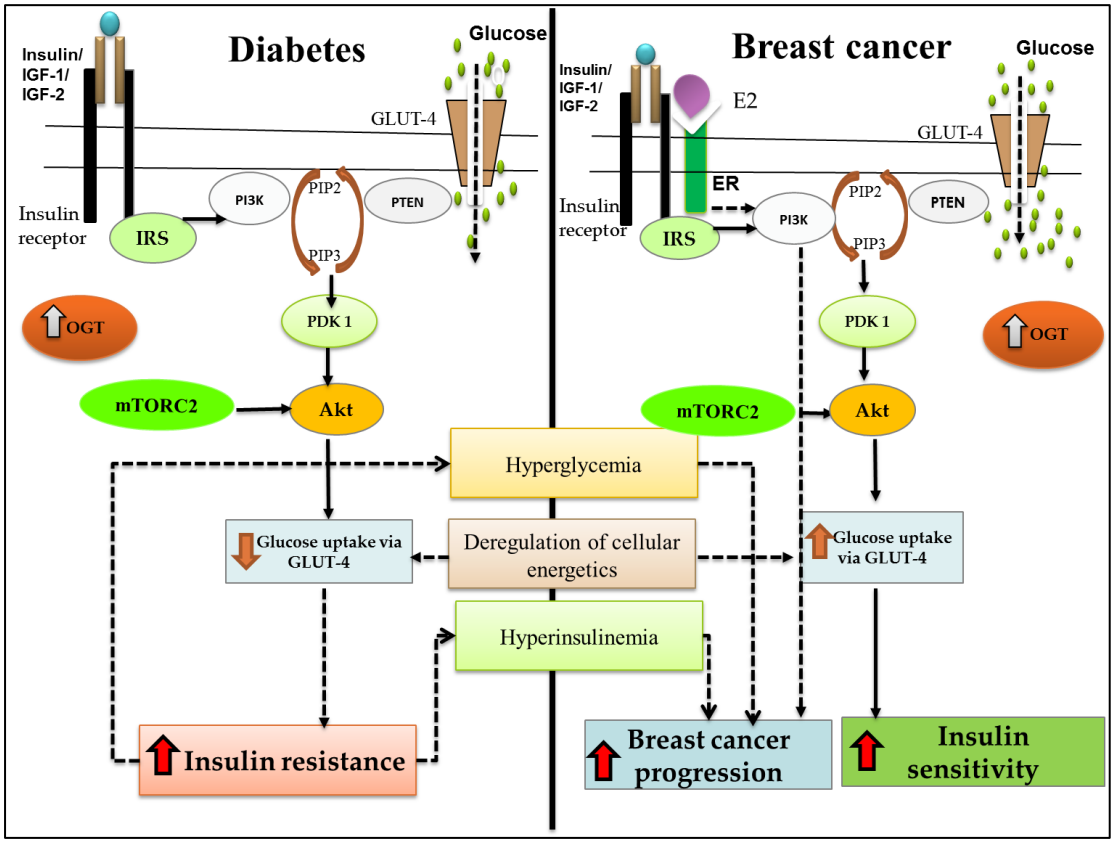


Figure 15: Crosstalk between Diabetes and Breast cancer.

PI3k/Akt pathway forms the junction for crosstalk between diabetes and breast cancer. As shown in the figure, increased insulin resistance leads to

hyperglycemia and hyperinsulinimea which further leads to breast cancer progression. OGT is overexpressed in both the systems and deregulation of cellular energetics effects GLUT-4 expression causing either insulin resistance (diabetes) or increased insulin sensitivity (breast cancer).

3.5 MODEL VERIFICATION THROUGH SIMULATIONS

A model can display all sorts of behavior which constitutes the state space of the model[54]. Analysis of distinct behaviors can be studied using discrete model. Contrastingly, a very dense and a frequent infinite state space is studied through continuous model. It is typically approximated to hybrid or discrete equivalents [55].

The state space information is used by model checking methods to check the presence of various behaviors and properties in the system[56]. A Petri net carries out simulations of place/ transition network with token flow. It can help us predict the dynamics of model with time. Analysis of a state space subset or all the possible state spaces can be done through simulation runs. Increasing the simulation runs contributes to increased precision of average time. With the passage of time, all simulation runs fluctuate and depict system behavior. Therefore, a model that has either infinite or infeasible state space can be verified through model checking. To validate the designed pathway, our methodology utilized the simulative property of Petri net.

For model verification, we studies on some of the critical properties (over expression of receptors and proteins) and OGT involvement in the PI3K/Akt pathway (Table 1) which have been validated experimentally.

When the simulation results were compared to already proven experimental data regarding protein expression in PI3K/Akt signaling pathway, the prior findings found in literature were verified.

In crux, our method computed the tokenized activity levels as abstract measures in which the changes over the passage of time depicted changes in active protein concentrations. Therefore, achieving similar system behavior relative to experimental data our model was validated. Henceforth, various biological insights can be driven through extending this model and this signaling pathway can be better understood. The details have been given in Table:1.

RESULTS

CHAPTER 4: RESULTS

4.1 Normal PI3K/Akt pathway:

The normal PI3K/Akt signaling pathway (Figure 16) was subjected to Petri net modeling. In the generated model, we assumed that when glucose intake occurs, insulin is immediately released in the bloodstream. Meanwhile, insulin receptors are readily available on cell membrane to initiate the PI3K/Akt pathway via auto-phosphorylation of the receptors and are referred as complex 1 in the proposed model (Figure 16) [18, 57].

Figure 16 shows the Petri net model of Normal PI3K/Akt signaling pathway.

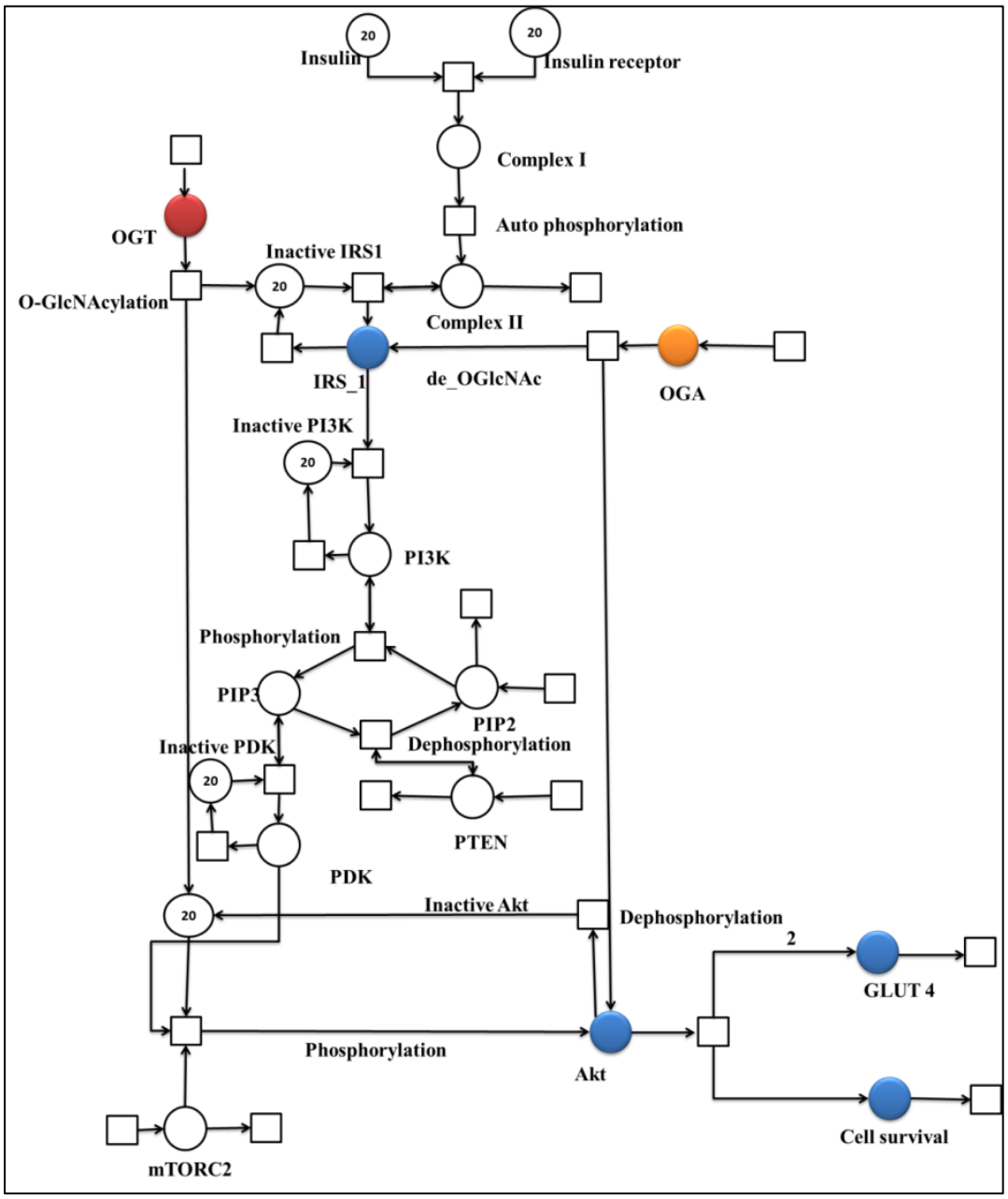


Figure 16: Illustration of the Petri net model representing normal PI3K/Akt pathway.

A standard place is illustrated as a circle ⬭ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation etc. A directed arc connects a place with a transition and vice versa. Blue places represent important proteins of PI3K/Akt pathway selected for this study in particular. Other colored places include (Red= OGT and Orange = OGA).

The model shows that as soon as the insulin receptor is activated, it recruits and activates the adaptor protein called IRS-1 via phosphorylation. Activated IRS protein displays binding sites for a variety of proteins for further signal transduction. Among them, a major player in insulin function is PI3K protein that further leads to Akt activation. Activated Akt has numerous functions, among which promoting cell survival through inhibition of pro-apoptotic proteins and regulation of glucose metabolism have been the focus of this study. The overall behavior of the system under normal insulin signaling can be observed as shown in Figure 16.1. According to `translocation hypothesis' glucose transporter proteins are present within the cell in the latent state. Upon activation the number of glucose transporters on the cell membrane is increased and the rate of glucose uptake by the cell is enhanced Figure 16.2 (C) [58]. PI3K activity also increases the cell survival rate, as shown in Figure 16.2 (D).

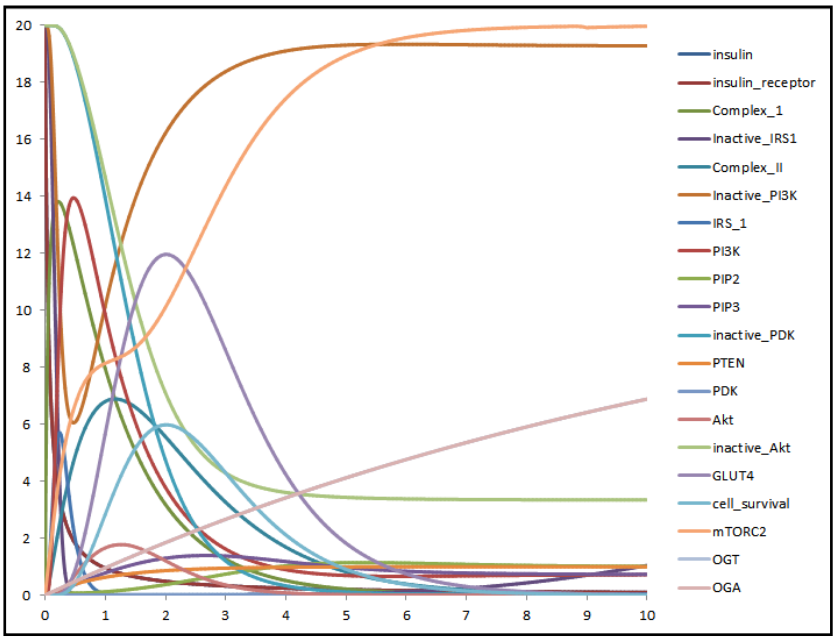
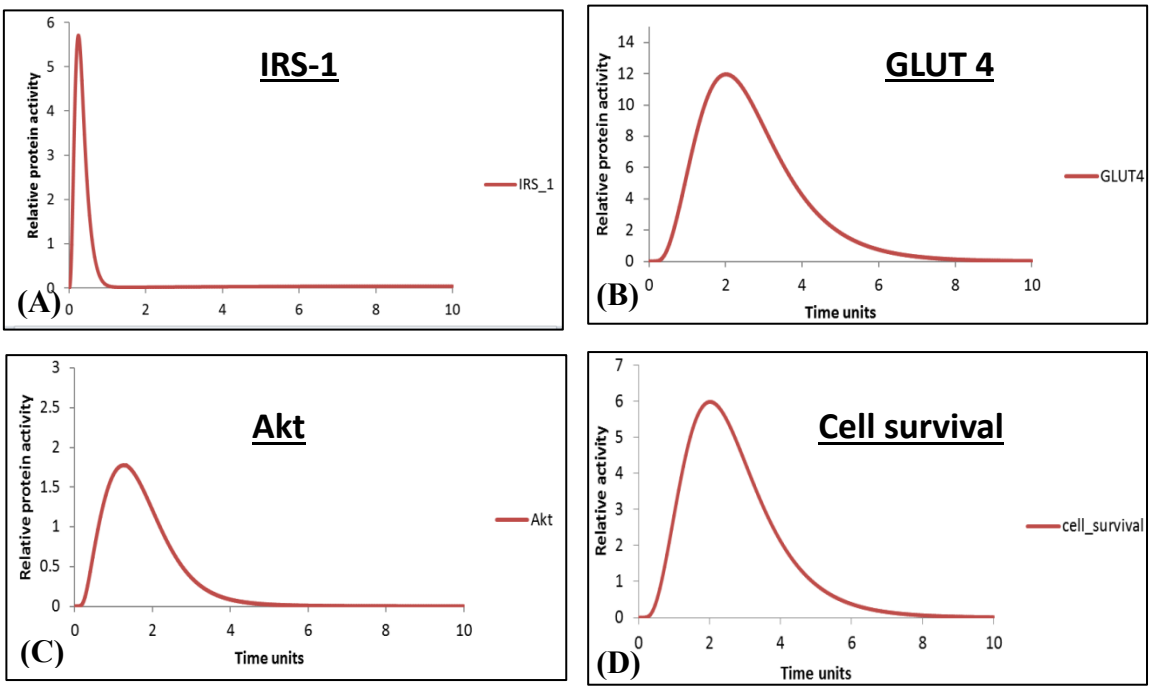


Figure: 16.1. Normal behavior of PI3K/Akt pathway. The graph shows collective simulation of 20 entities in the PI3K/Akt pathway (Figure 16) under normal condition. The relative activity change in protein levels is measured in response to insulin stimulation of PI3K/Akt pathway.

The overall simulation can help us evaluate the system as a whole as it shows how the proteins behave upon insulin stimulation under normal conditions. Each entity can be exclusively studied through simulations in relation to insulin stimulation.

It is observed that as soon as insulin binds to insulin receptor there is an increase in activation of downstream proteins. The insulin receptor stays constant while it initiates the downstream processes by activation of signaling protein. Interestingly, the dynamics of IRS-1, PI3K and PDK1 is depicted by an increase in their activity as insulin gets consumed. These proteins play a key role in insulin signaling pathway thus the individual and collective roles of IRS-1 and Akt and their correlation to OGT protein, have been studied in detail in Section 4.3. Moreover, the difference in cell survival rate and GLUT-4 expression has also been focused among normal and aberrant insulin signaling (Figure 16.2).



16.2 Relative changes in the activity levels of entities in Normal insulin signaling. (A) Activation of IRS1 after insulin stimulation. **B)** Increased expression of GLUT4 on cell surface after insulin stimulation. **(C)** Activation of Akt after insulin stimulation **(D)** Increased cell survival after insulin stimulation.

4.2. Altered PI3K/Akt pathway in adipocyte:

Generally, the magnitude of intracellular protein O-GlcNAc modification varies with extracellular glucose concentration. A study conducted by Yang *et al*, showed that the approaches used to increase O-GlcNAcylation (Adding OGA inhibitor and increasing glucose concentration) inhibited insulin stimulated phosphorylation of Akt in 3T3-L1 adipocytes.

The pathway formulated for the insulin resistant adipocyte is given in Figure 17 [22, 59].

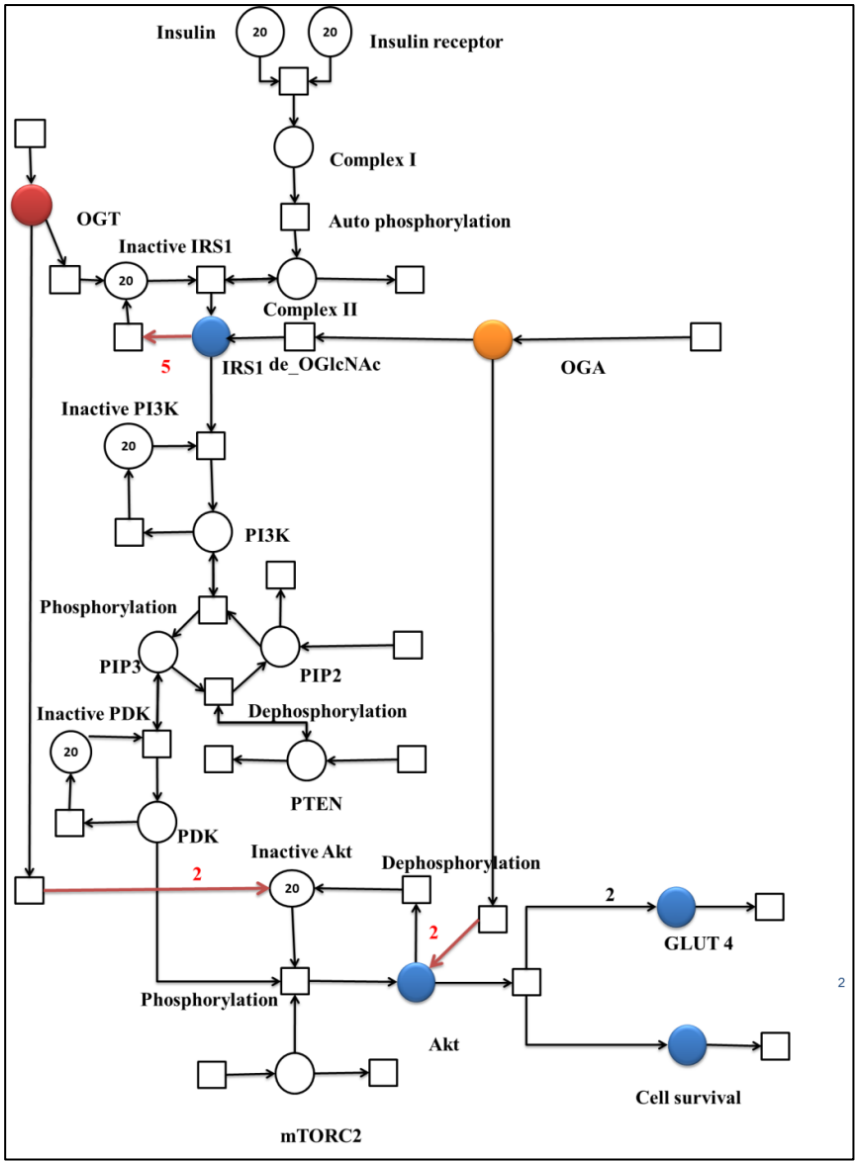


Figure 17: Illustration of the Petri net model representing altered PI3K/Akt pathway in adipocyte. A standard place is illustrated as a circle ◯ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc connects a place with a transition and vice versa. Blue places represent important proteins of PI3K pathway selected for this study in particular. Red arcs represent the changes in Petri net model as compared to normal signaling pathway.

As reported by earlier studies, exposing the cells to various glucose concentrations does not affect the global concentration of O-GlcNAc but rather the change is protein specific [22]. It can be seen in the graph that important proteins such as

IRS-1, PI3K, Akt and GLUT-4 show a drop in activity due to increased O-GlcNAcylation, confirming that insulin signaling is dampened.

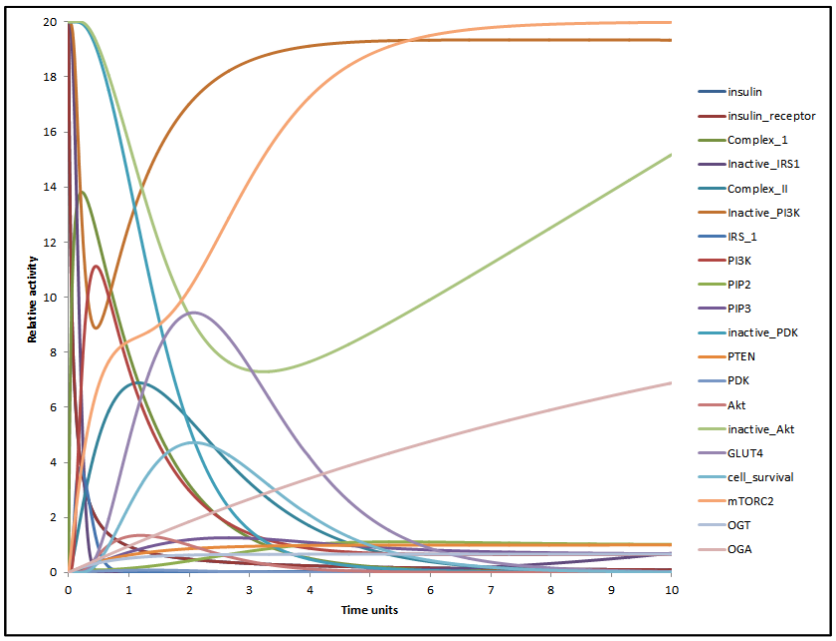


Figure 17.1 1: Altered behavior of PI3K/Akt pathway. The graph shows collective simulation of 20 entities in the PI3K/Akt pathway under altered PI3K/ Akt pathway. The shift in relative activity of proteins levels is represented as the cell becomes insulin resistant.

As seen in the overall simulation of the altered PI3K/Akt pathway model (Figure 17.1), the curves depicting inactive proteins such as inactive IRS-1, PI3K, PDK and Akt show a marked increase with time, representing that with the passage of time inactivity of downstream proteins is increasing and the cell switches from an insulin responsive-cell towards an insulin-resistant phenotype. Moreover, OGT overexpression lowers the activation of IRS-1 and Akt, therefore, inhibiting insulin signaling in insulin resistant adipocyte (Figure 17).

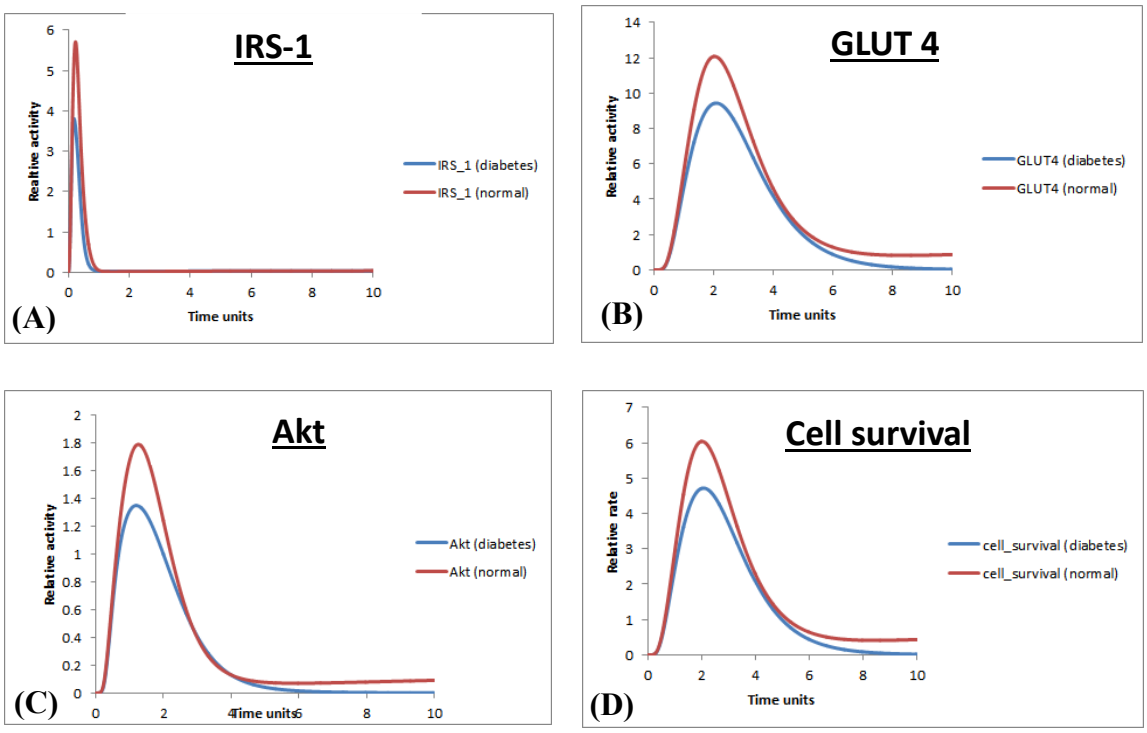


Figure 17.2: Relative changes in the activity levels of entities in altered PI3K pathway in insulin resistant adipocyte.(A) Reduced activation of IRS1 after insulin stimulation. **(B)** Reduced expression of GLUT4 on cell surface after insulin stimulation. **(C)** Decreased activity of Akt after insulin stimulation **(D)** Decreased cell survival after insulin stimulation.

The graphs in Figure 17.2 explain how the relative activities of proteins (IRS-1, GLUT-4 and Akt) as well as cell survival decrease with the passage of time.

4.3 Comparison of relative changes in the activity levels of IRS-1 and Akt:

O-GlcNAc transferase carries out regulation of insulin signaling through modifying proteins and altering their activity. In later phase as the adipocyte becomes insulin resistant due to increase in OGT expression, the values of IRS-1 and Akt drop significantly as increased O-GlcNAcylation inhibits the activation of these proteins.

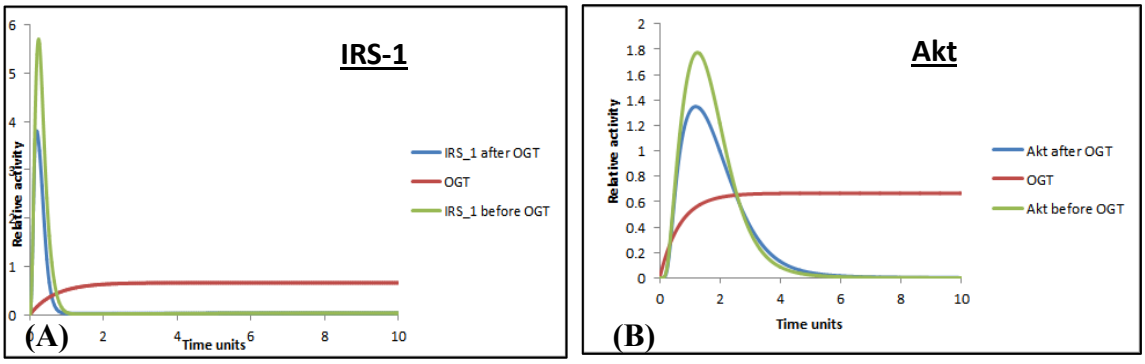


Figure **17.3 1: OGT reduces IRS-1 and Akt activation via O-GlcNAcylation in insulin resistant cells**

(A) Decrease in IRS-1 activity due to O-GlcNAcylation in adipocytes. The X-axis shows time units while the Y-axis shows the relative IRS-1 activity. The blue line represents IRS-1 activity before OGT and the red line presents IRS-1activity after OGT. The graph explains that IRS-1 activity is significantly reduced as OGT activity increases. **(B)** Akt inhibition by OGT in adipocytes The X-axis represents time units while the Y-axis represents the relative Akt activity. The blue line shows Akt activity before OGT and the red line shows the Akt activity after OGT expression. According to the graph, Akt activity shows a sudden decrease as OGT activity increases.

4.4 Insulin signaling in breast cancer

Insulin is a mild mitogen and has been shown to potentiate mitogenic influence of other growth factors. Because hyperinsulinemia and/or overexpression of insulin receptors have been linked to development, progression, and outcome of breast cancer, we attempted to evaluate the mechanism of these associations.

Insulin plays a crucial role in carcinogenesis as hyperinsulinemia induces cell proliferation and increases breast cancer. Insulin exerts a significant mitogenic action in normal mammary tissue and breast cancer cells in culture [60] . Many human breast cancers overexpress the insulin receptor [61]. Additionally, breast cancer cells fail to downregulate the insulin receptor in the presence of hyperinsulinemia [62]. The Petri net generated for breast cancer under hyperinsulineamia condition is given in Figure 18. This Petri net also involves Hexoseamine Biosynthetic Pathway as we wanted to compare how an increase in the glucose concentration effects OGT expression in invasive breast cancer.

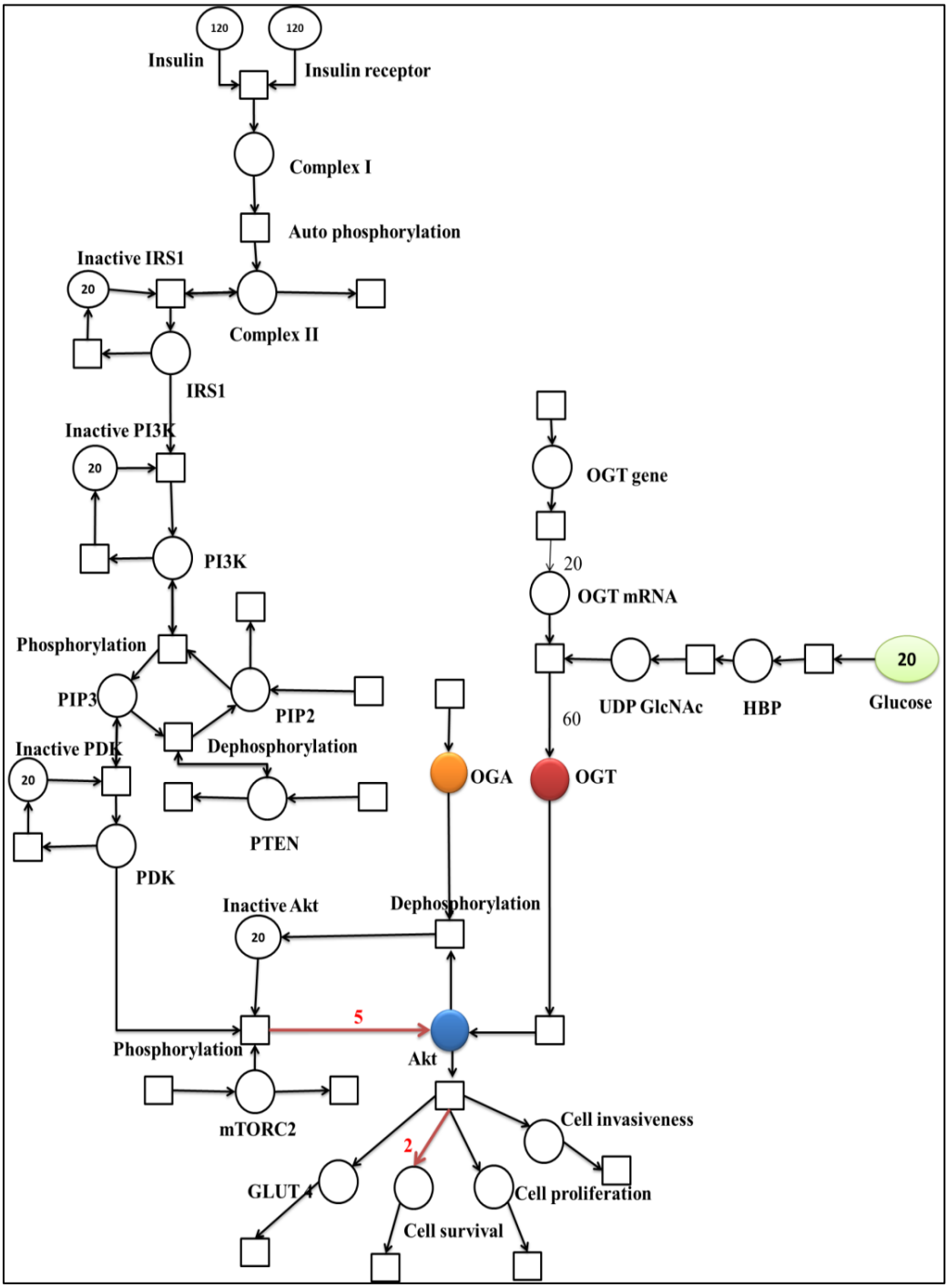


Figure 18: Illustration of the petri net model representing increased insulin signaling in breast cancer. A standard place is illustrated as a circle ○ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc → connects a place with a transition and vice versa. Blue places represent important proteins of PI3K pathway selected for this study in particular. Colored places include (Light green = Glucose molecules, Red = OGT and Orange = OGA). Red arcs represent the changes in Petri net model as compared to normal PI3K/Akt signaling pathway.

The variations among protein expression and cell survival is quiet significant as compared to normal insulin signaling. The graphs in Figure 18.1 show the relative increase in protein expression of OGT, GLUT 4, Akt and breast cancer cell survival.

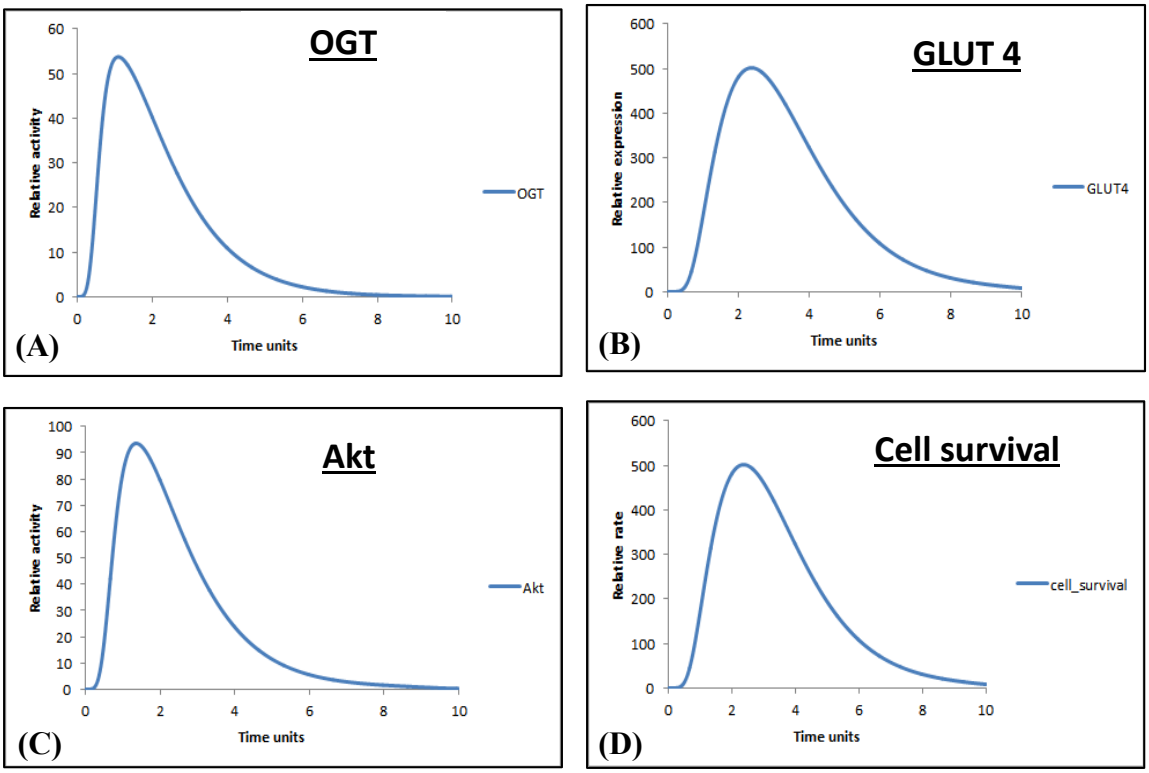


Figure 18. 1: Relative changes in the activity levels of entities in Breast cancer.

(A) Increased activity of OGT in invasive breast cancer. **(B)** Increase in GLUT4 expression due to increased PI3K activity. **(C)** Increase in Akt activity in breast cancer. **(D)** Increase in cell survival due to increased activation of PI3K activity.

As shown in the graphs above, increase in insulin receptor expression by 6 folds enhances the activity of downstream proteins of the signaling cascade such as Akt and GLUT-4. Similarly, the cell survival also increases as compared to normal state.

Moreover OGT expression is observed to be enhanced in breast cancer. This is a confirmation that glucose metabolism effects OGT expression i.e. influx of glucose in Hexosamine Biosynthetic Pathway leads to production of substrate of OGT, UDP-GlcNAc, causing increased activity of OGT.

4.5 Effect of hyperglycemia on cancer

Hyperglycemia contributes to malignant cancer cell phenotypes. There is increasing evidence suggesting that there is a link between cancer and diabetes. Regardless of other shared metabolic factors, hyperglycemia, the most typical characteristic of diabetes, may be one reason to explain the prevalence of cancer incidence in patients with diabetes. Research shows that hyperglycemia may contribute to an enhanced proliferation ability, apoptosis inhibition, metastasis, perineural invasion, chemotherapy resistance and chemotherapy intolerance [63]

Glucose is specifically required to meet the metabolic demands of the fast proliferation cancer cells. It has been known that glucose is a primary driving force for the growth of tumor cells for more than two decades [64]. The significant role of hyperglycemia in cancer proliferation is clearly understood. Hyperglycemia is often accompanied with hyperinsulinemia in people with diabetes. Moreover, recent studies showed that insulin promote cancer progression by enhancing metabolic capacities of cancer cells [65]

When we analyzed the effect of hyperglycemia in breast cancer, it was observed that there was an approximately 4 fold increase in OGT and a 10 fold increase in cell proliferation (Table 2).

The graphs in Figure 19 depict the exponential increase in protein expression and cell survival under hyperglycemic condition. As hyperglycemia induces increased OGT expression, it results in hyper-activation of PI3K/Akt pathway via O-GlcNAcylation in breast cancer. Moreover, there is a significant increase in the

GLUT 4 expression and cell survival rate of breast cancer cell as shown in Figure 19.

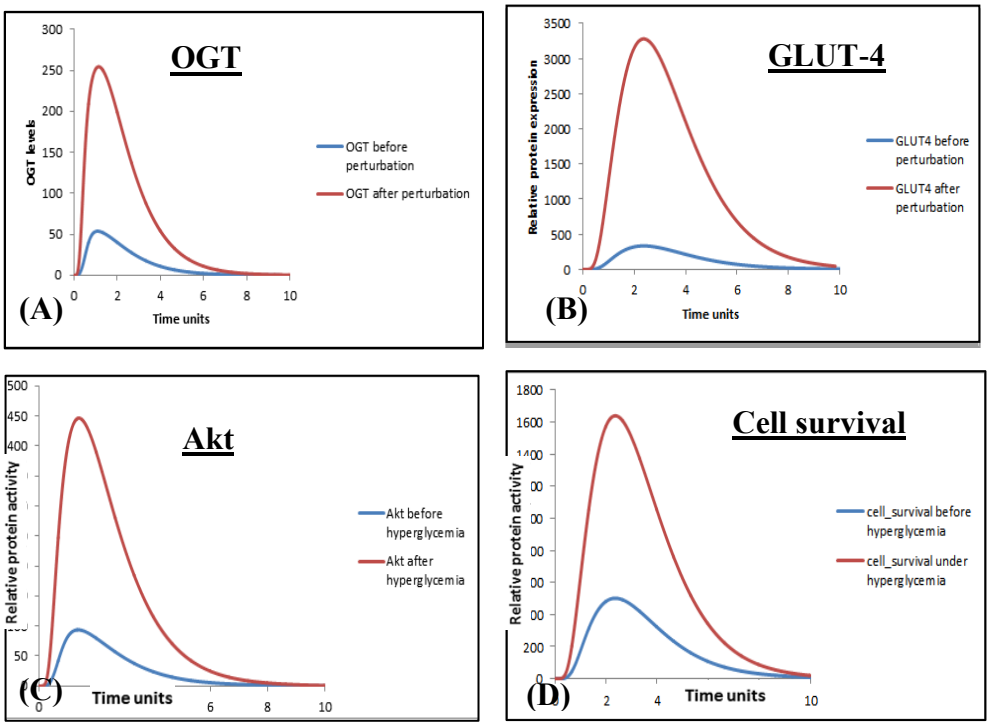


Figure 19: Relative changes in the activity levels of entities in Breast cancer under hyperglycemia.

(A) Increased activity of OGT in invasive breast cancer. **(B)** Increase in GLUT4 expression due to increased PI3K activity. **(C)** Akt over activation in breast cancer cell under hyperglycemic condition **(D)** Increase in cell survival due to increased activation of PI3K activity.

4.6. Comparison of insulin resistant adipocyte and invasive breast cancer cell

4.6.1 Differential role of OGT

The designed Petri net model represents how increased feedback inhibition of the PI3K/Akt pathway due to dysregulated O-GlcNacylation of Akt in adipocytes causes the cell to become resistant to insulin. This results in reduced insulin utilization and causes hyperinsulineamia. Consequently, PI3K/Akt pathway stimulation is decreased. These cells then reduce the expression of GLUT 4 receptors leading to reduced glucose uptake. In turn, hyperglycemic condition prevails in the blood stream.

On the other hand, OGT over activates the PI3K/Akt pathway in the breast cancer cell by increased O-GlcNacylation of Akt leading to increased glucose uptake by the cancer cell through GLUT4 expression as well as increased cell survival. The graph comparing the Akt activation in adipocytes and breast cancer cell has been shown in Figure 20.

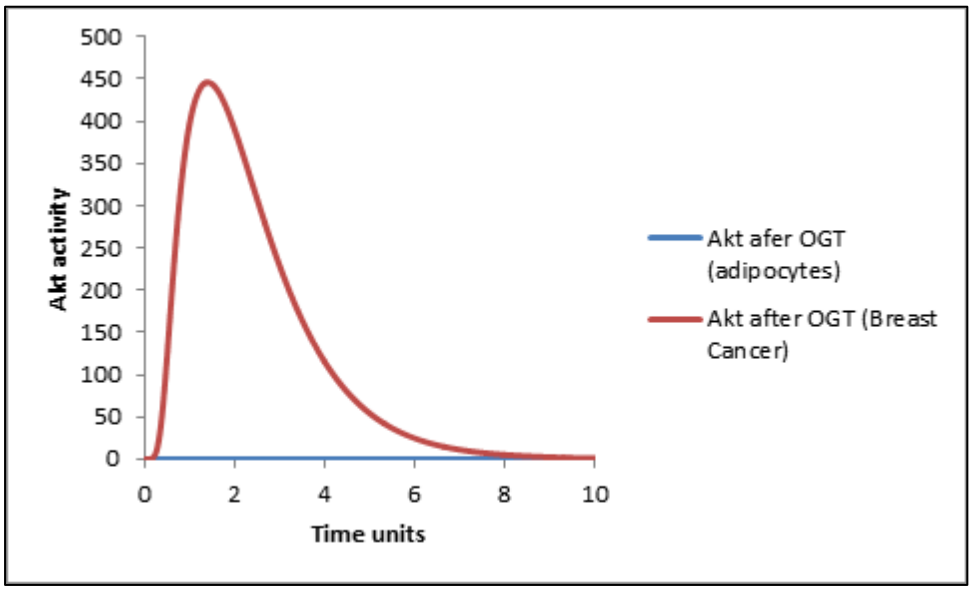


Figure 20: Differential role of OGT. The X-axis shows time units while Y-axis shows relative activity of Akt. The red line represents Akt activity after OGT in breast cancer cell whereas the blue line represents Akt activity after OGT in adipocytes. The graph shows that there is an exponential increase in Akt activity as aberrant O- GlcNacylation enhances Akt in the breast cancer cell as compared to its concentration in adipocyte.

Since hyperactivity of OGT induces cancer cell proliferation and survival, controlling OGT at the appropriate level would be beneficial in breast cancer patients bearing diabetes. For this we designed perturbation experiments to inhibit OGT through RNA and protein inhibitors.

Following a similar methodology, biologists can estimate and explore the interactions among groups of entities in a cell, supported by their respective properties and biological functions, as well as system-level perspective for various diseases prior to spending time and resources in wet lab experiments.

Observations	Findings				Citations
	Experimental		Model simulation		
	Diabetes	Breast cancer	Diabetes	Breast cancer	
Effect of OGT on IRS-1 Activity	—	-	—	-	[66]
Effect of OGT on Akt activity	—	+	—	+	[67] [66]
GLUT-4 expression	—	+	—	+	[68]
Effect of OGT on cell survival	—	+	—	+	[19]

Table 1 Summary of the observations as reported by experimental studies and their comparison with our simulations. Symbols represent changes in expression levels of observed proteins in the PI3K/Akt pathway. + represents the up-regulation while — represents down-regulation of the entities/proteins. – represents non-availability of data

4.7 Perturbation experiments:

Perturbation biology is a useful tool to study effects of therapeutic drugs or protein inhibitors for further clinical research. If successful, they can be implemented in clinical setting and can also be followed by clinical trials.

Based on data found in the literature, we constructed a cell-type specific signaling model that linked drug perturbation and cellular mechanisms. Perturbation experiments were designed to study RNA interference by shRNA and protein inhibition by OGT inhibitor (BZX) of OGT.

Individual effect of anti-diabetic drug—Metformin was studied on breast cancer cell proliferation. Moreover, we also investigated two combinatorial strategies for ameliorating breasts cancer i.e. (shRNA + BZX) and (BZX + Metformin). To enhance the model accuracy and narrow down the parameter search space, prior pathway information was extracted from signaling databases. The Petri net

models (Figure 24 and 25) capture the signaling events and provide responses to combinatorial interventions that are previously untested.

The steps have been summarized in the Figure 21:

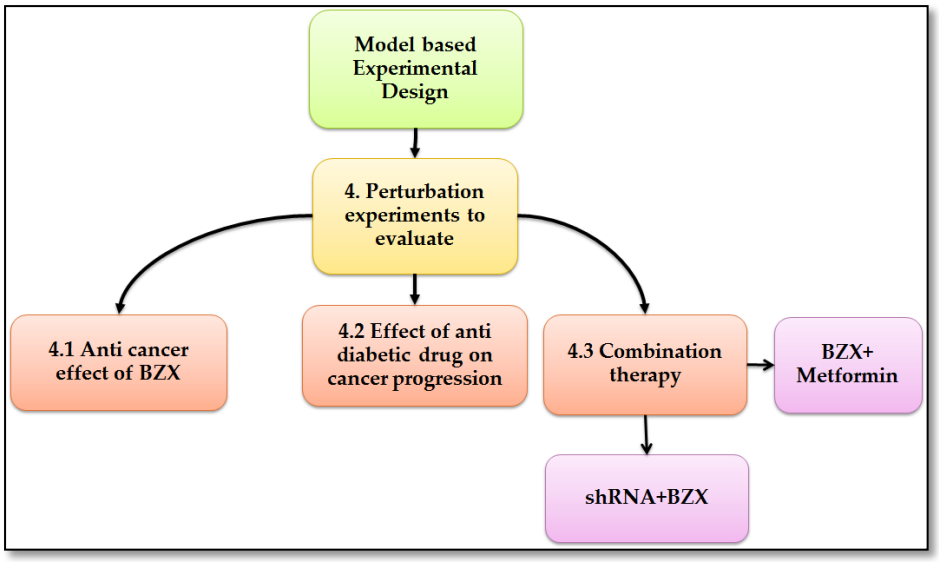


Figure 21: Summary of perturbation experiments

4.7.1 CASE 1: Effect of shRNA interference on OGT

An artificial RNA molecule with a hairpin structure that is used to carry out RNA interference through targeting gene expression is called a short hairpin or small hairpin RNA (shRNA). shRNA expression is achieved in cells through plasmid delivery or vectors. As shRNA has a low turnover and degradation rate it is an effective mediator of RNA interference. The data of shRNA used for this experiment was taken from a study conducted by Caldwell *et. al*, (2010) [19]

The Petri net model (Figure 22) designed to study the effect of shRNA interference of OGT depicts the interaction of proteins in breast cancer.

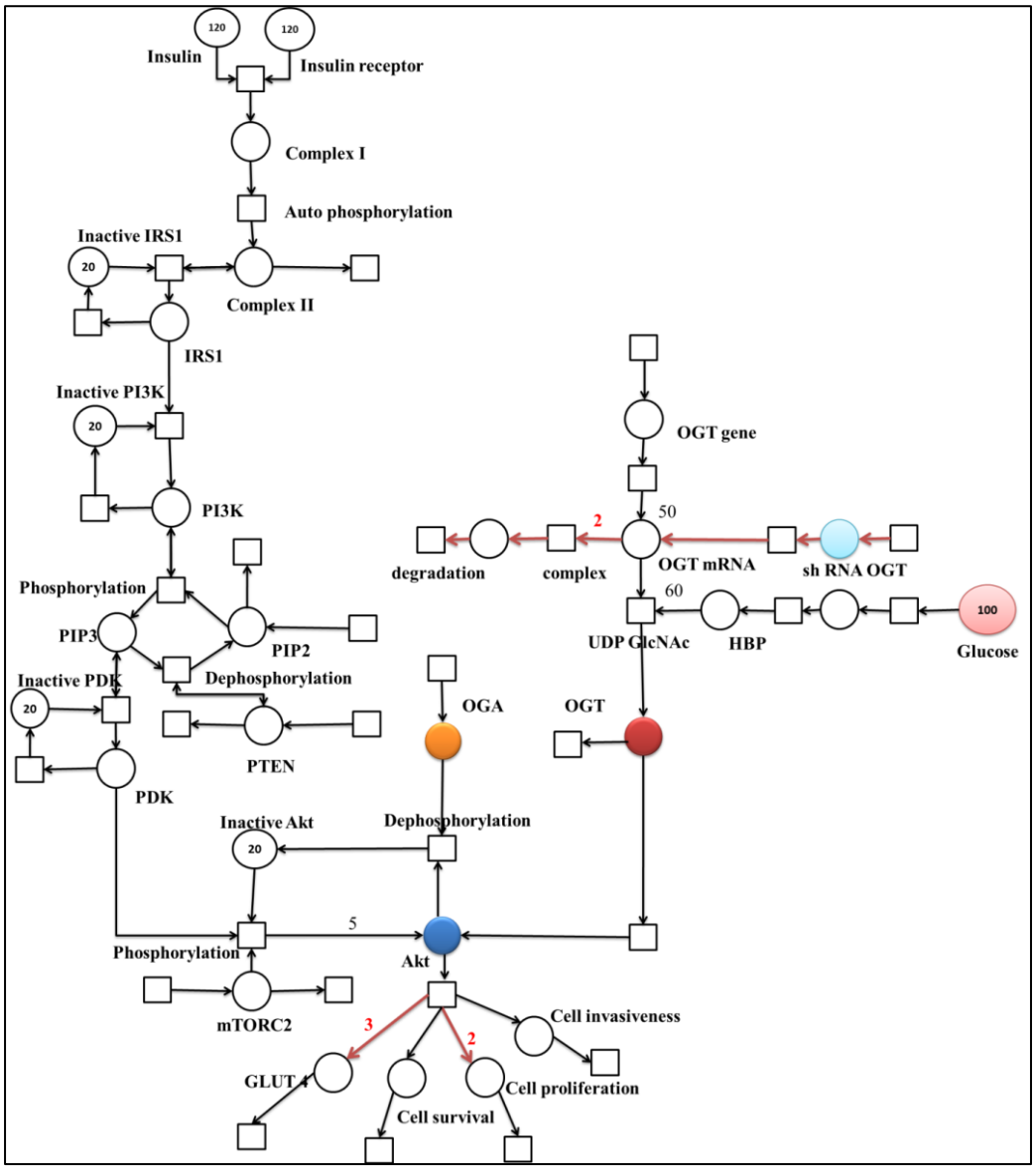
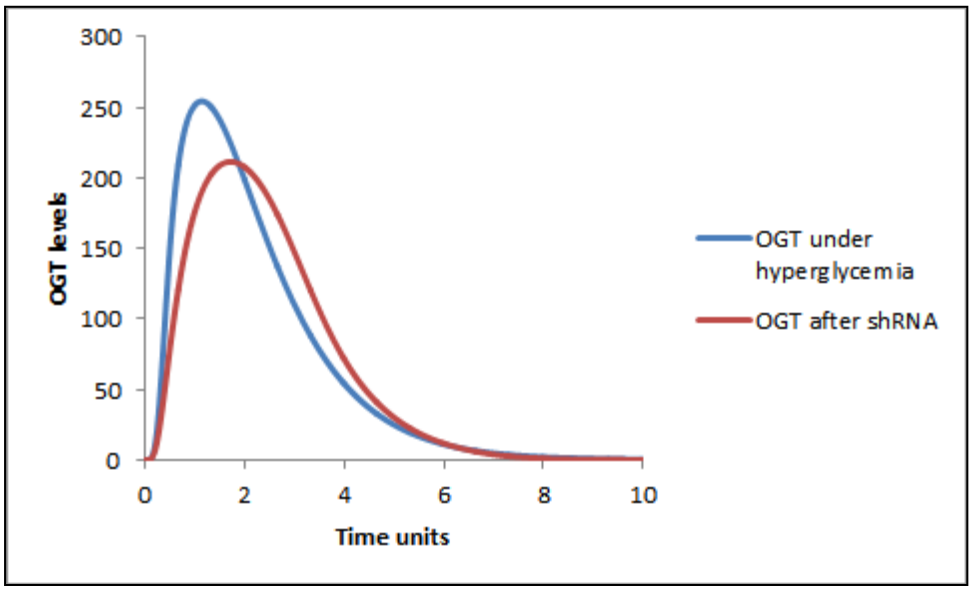


Figure 22: Illustration of the Petri net model representing intervention in Hexoseamine Biosynthetic pathway through shRNA in breast cancer cell under hyperglycemia.

A standard place is illustrated as a circle ⬭ representing proteins involved in the pathway. A continuous transition is depicted as ▭ representing cellular processes including phosphorylation and dephosphorylation. A directed arc connects a place with a transition and vice versa. Red arcs represent changes in Petri net as compared to normal PI3/Akt pathway. Colored places include (Blue = Akt, Light blue = shRNA OGT, Red= OGT and Orange = OGA and Pink= increased glucose molecules)

As shown in the Figure 22, the transcription of OGT is also considered. The model describes that addition of shRNA against OGT RNA binds and degrades the OGT mRNA.



22. 1: OGT protein inhibition by shRNA drops the OGT activity in breast cancer cell. The X-axis shows time units while the Y-axis shows relative OGT activity. The blue line represents OGT activity under hyperglycemia whereas the red line represents OGT activity after shRNA intervention.

As seen in Figure 22.1, with the increase in time, OGT activity decreases. Based on our calculation there was a decrease up to 0.2 folds in OGT activity (Table 2). This can be significant as temporary inhibition of OGT via RNA interference can be further tested in animal models to validate the efficacy of this strategy.

4.7.2 CASE 2: Effect of BZX on OGT expression

Over the years, high-throughput screening methods against a huge library of drug-like composites have produced a number of OGT inhibitors . the application of This screening method has detected a compound called 4-methoxyphenyl 6-acetyl-2-oxobenzo[d]oxazole-3(2H)-carboxylate also denoted as BZX that competes with the binding of sugar nucleaotide.

It is a suicide inhibitor that cross-links the active site of OGT (Lys842 and Cys917) through a double-displacement mechanism.

We chose BZX as it has successfully been used against breast cancer *in vitro*, promoting anti-invasion and antigrowth effects as it modulated FoxM1 transcription [19]. Recent studies revealed that the use of BZX against OGT downregulated the expression of genes that are linked to cell cycle regulation, DNA replication and also destabilized c-Myc (a known oncogene) in human prostate cell lines[40]. This compound has further been used to study the significance of O-GlcNAcylation through inflection of Rho kinase pathway in vascular contractile response [41].

Using previous data generated through research, we applied BZX to our invasive breast cancer model to study its effect on OGT activity and breast cancer cell proliferation (Figure 23)

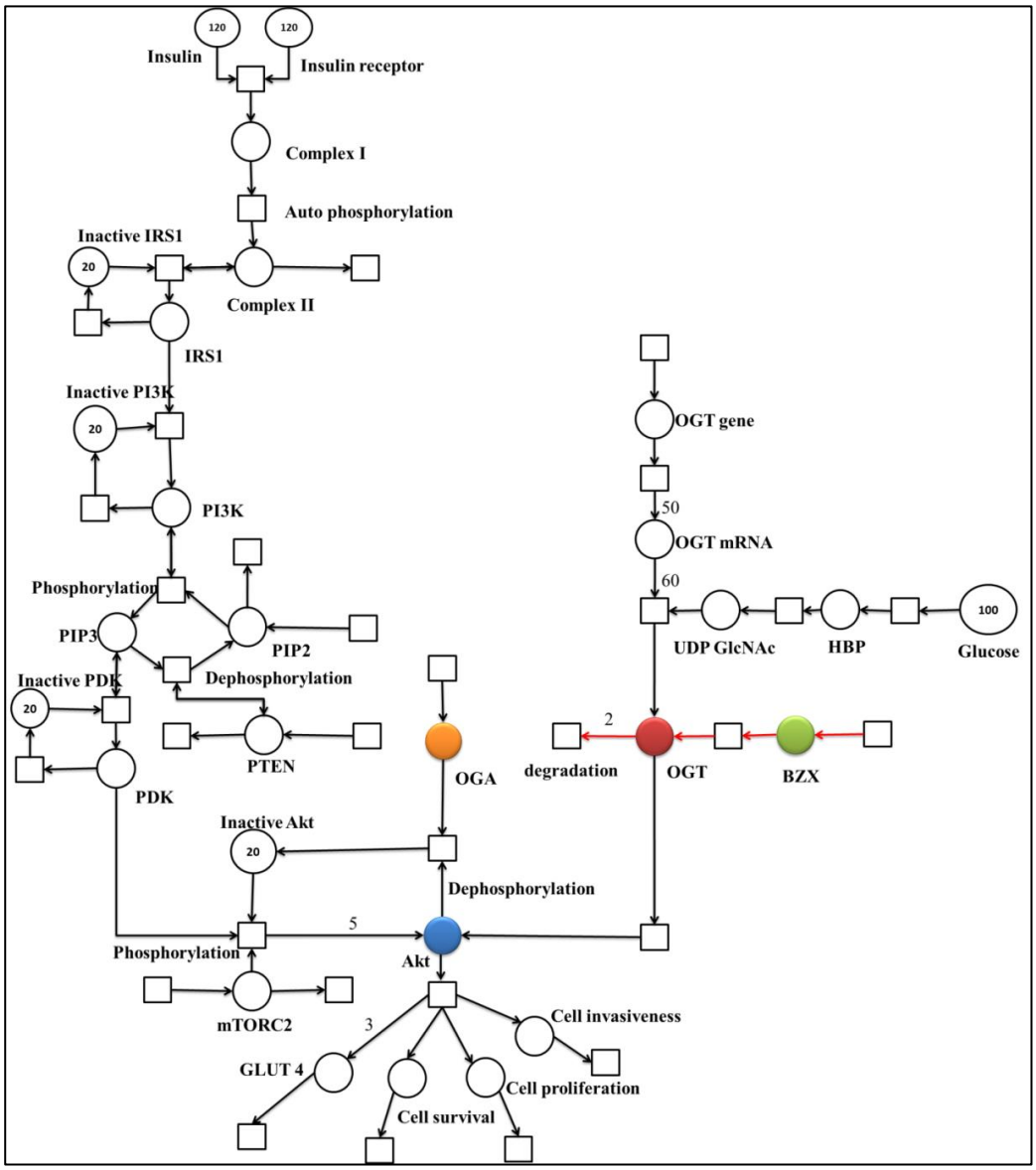
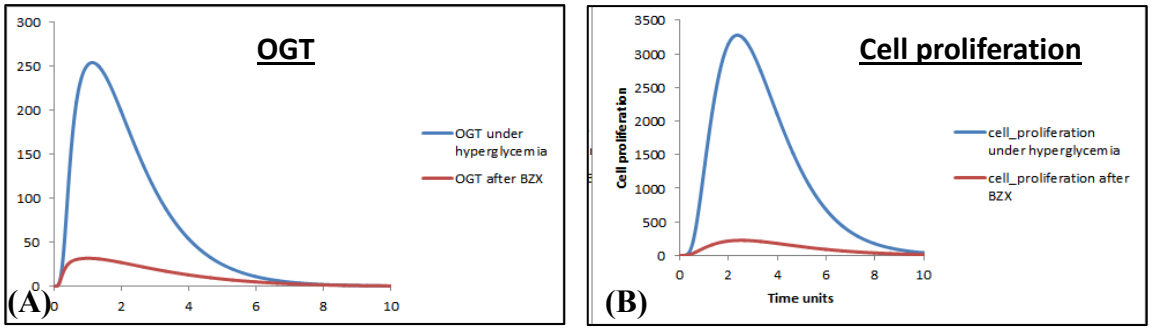


Figure 23: Illustration of the petri net model representing BZX inhibition of OGT.

A standard place is illustrated as a circle ⬭ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc ⟶ connects a place with a transition and vice versa. Red arcs represent the action of BZX on OGT. Colored places include (Blue = Akt, Green = BZX, Red= OGT and Orange = OGA).

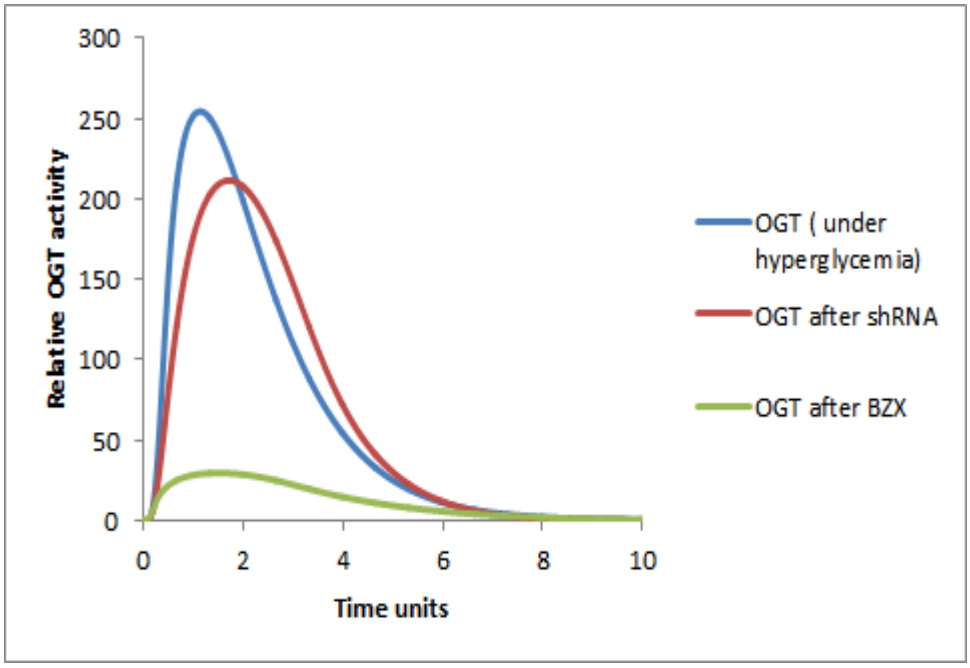


23. 1: Anti-cancer effect of BZX. (A) OGT activity reduces significantly by BZX. The figure shows that OGT levels drop drastically due to BZX inhibition. **(B)** Effect of OGT inhibitor BZX on breast cancer cell proliferation. The x-axis shows the time units while the y-axis represents the relative rate of breast cancer cell proliferation. The graph shows a drastic reduction in breast cancer cell proliferation by BZX.

The results show that BZX inhibition was more effective than shRNA interference as it reduced OGT activity up to 7 folds. Interestingly, OGT inhibition via BZX also decreased cell proliferation significantly i.e. up to 14 folds as given in Table 2. This finding confirms the role of OGT in regulating cell growth and invasion as studied in breast cancer cells by Caldwell *et al*., [19]

4.7.3. CASE 3: Comparison of shRNA and BZX

To compare the relative efficacy of RNA interference and protein inhibition of OGT we compared the effect of shRNA and BZX. Figure 23.2 describes the difference among the two strategies:



23. 2: Comparison of shRNA and BZX on OGT expression. The x-axis shows the time units while the y-axis represents the relative activity of OGT. The figure shows that the inhibition by BZX is more effective as compared to shRNA inhibition.

Based on our model, OGT inhibition by BZX produced better results as compared to shRNA interference as OGT activity dropped significantly through BZX inhibition.

4.7.4 CASE 4: Effect of Metformin on breast cancer cell proliferation:

A well-known anti-diabetic drug, Metformin has been used to study its anti-tumor activity in a variety of cancers, including breast cancer. This drug is used very commonly and has well- established safety profiles. Using data from literature, we carried out inhibition of breast cancer cell proliferation via Metformin. Figure 24 depicts the Petri net that was subjected to Metformin intervention.

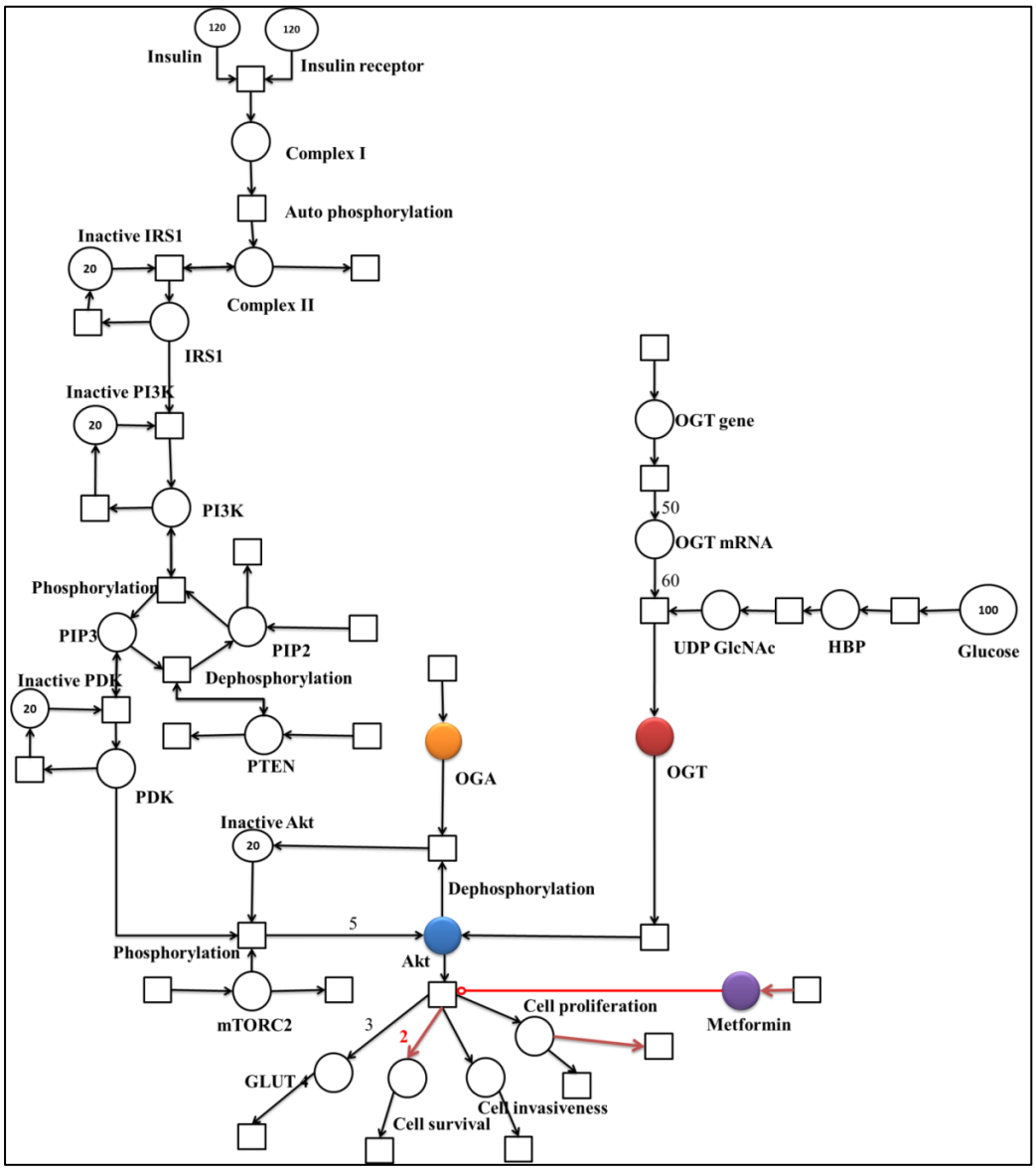
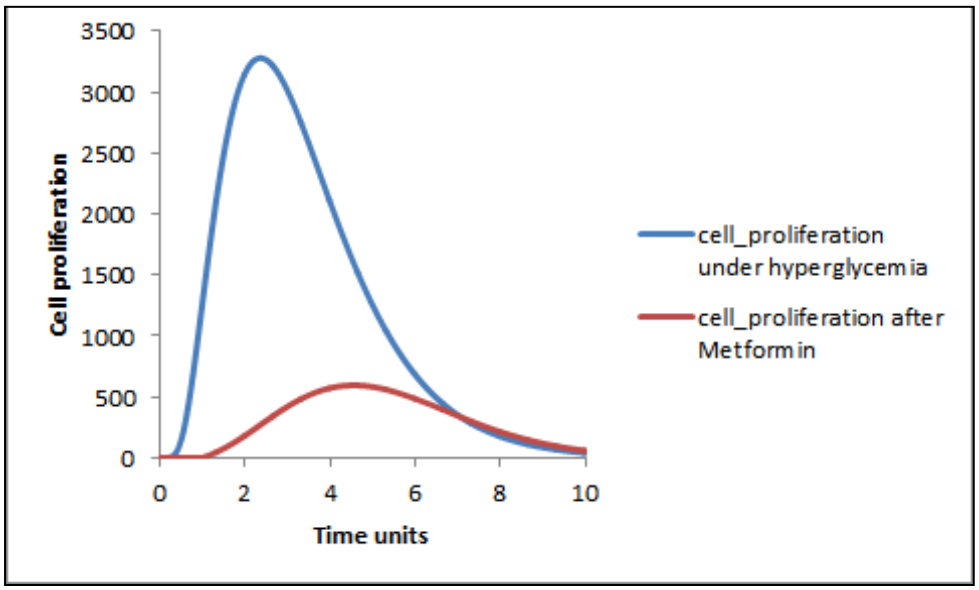


Figure 24: Illustration of the petri net model representing Metformin intervention. A standard place is illustrated as a circle ◯ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc ⟶ connects a place with a transition and vice versa. Inhibitory arc is represented by an arc with a hollow dot as its head in red color (⟶○). Colored places include (Blue = Akt, Purple = Metformin, Red= OGT and Orange = OGA).



24. 1: Effect of Metformin on breast cancer cell proliferation. The x-axis shows the time units while the y-axis represents the relative rate of breast cancer cell proliferation. The blue line represents cell proliferation under hyperglycemia whereas the red line represents cell proliferation after Metformin.

The graph shows that with the passage of time, Metformin reduces breast cancer cell proliferation up to approx. 5 folds. The explosive cell proliferation at the start is due to the immense increase in glucose flux into Hexose amine biosynthetic pathway. However, the rate of cell proliferation decreases as Metformin is applied showing indirect inhibition of cell proliferation over time.

4.7.5 CASE 5: Combination therapy (shRNA+ BZX)

A therapy consisting of more than one modality or medication to treat a single disease is known as a combination therapy. To analyze the combinatorial effect of OGT inhibition through shRNA and BZX, Petri net model was formulated (Figure 25)

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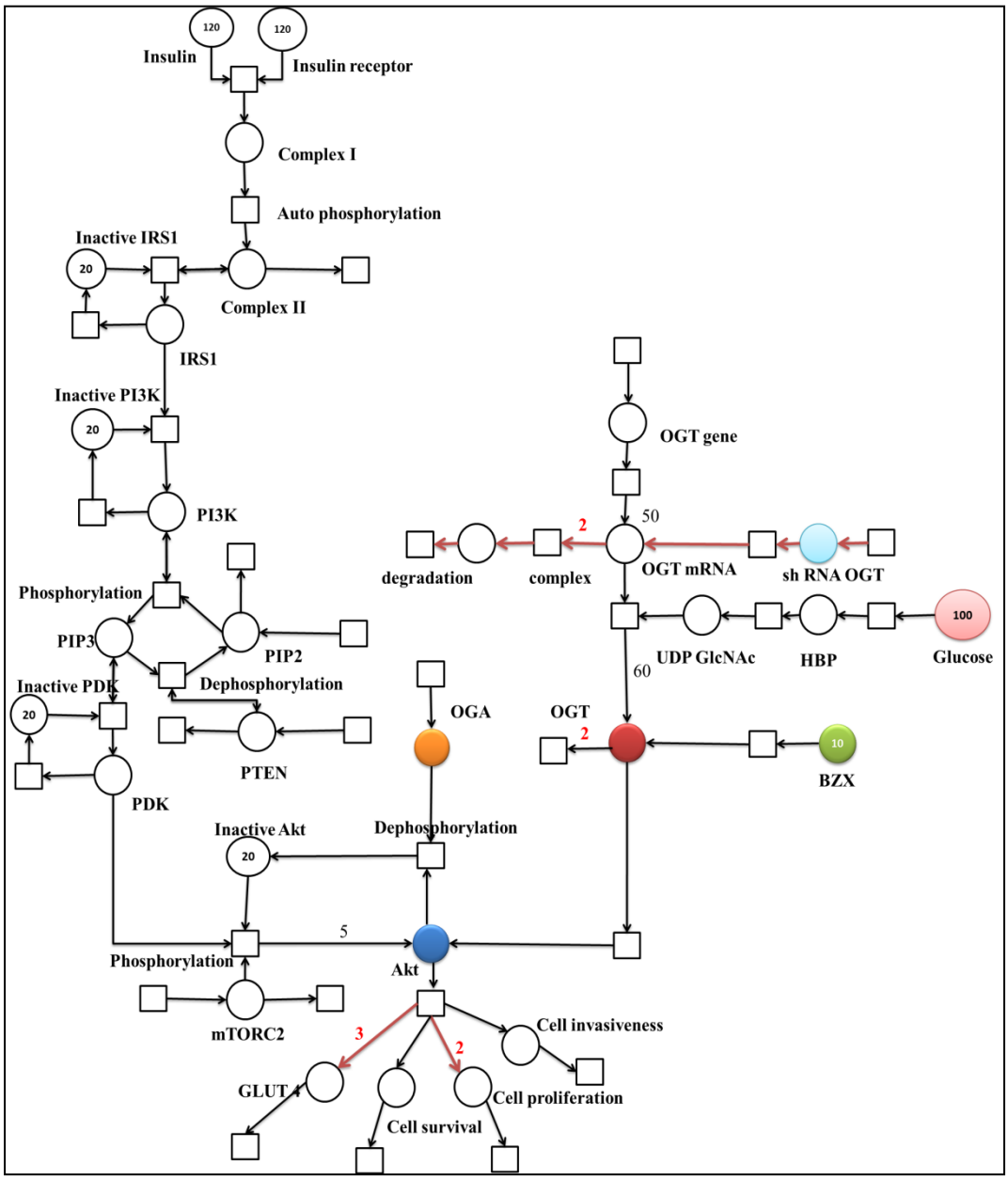

Figure 25: llustration of the Petri net model representing shRNA intervention.

A standard place is illustrated as a circle ⬭ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc ⟶ connects a place with a transition and vice versa. Colored places include (Green = BZX, Red= OGT and Orange = OGA, Pink = increased glucose molecules and Light blue = shRNA).

The effect of shRNA and BZX in combination on OGT activity is represented in Figure 25.1.

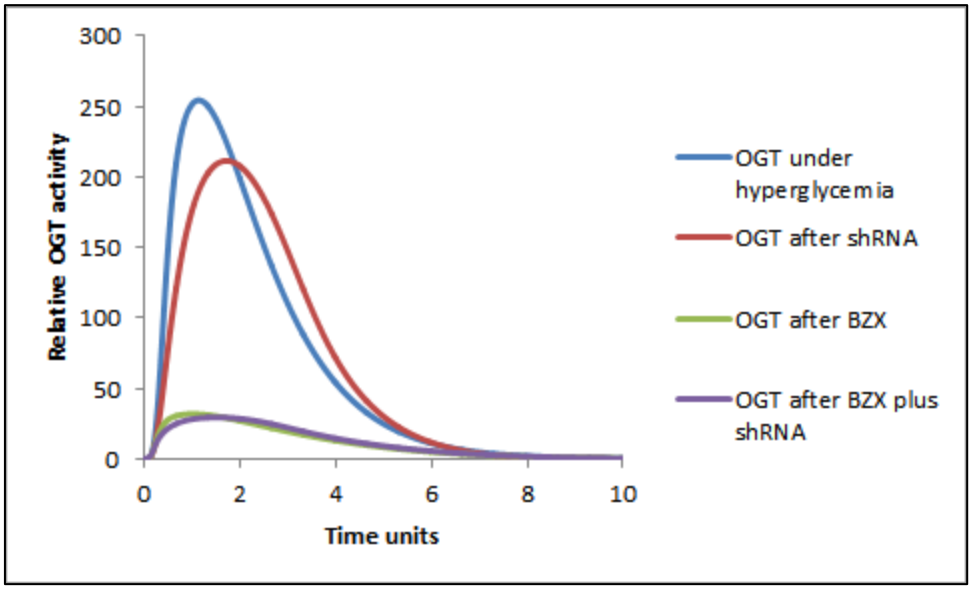

Figure 25.1: Combinatorial effect of shRNA and BZX on OGT expression

The x-axis shows the time units while the y-axis represents the relative activity of OGT.

The figure shows that the inhibition by a combination shRNA and BZX did not show a substantial difference; therefore we tested increase in dosage of BZX with time.

Increasing BZX dosage by 10 units with time, we observed a visible decline in OGT activity. We tested three values by giving BZX a token of 10, 20 and 30. The graph in Figure 25.2 explains the effect of increasing BZX dosage on OGT activity:

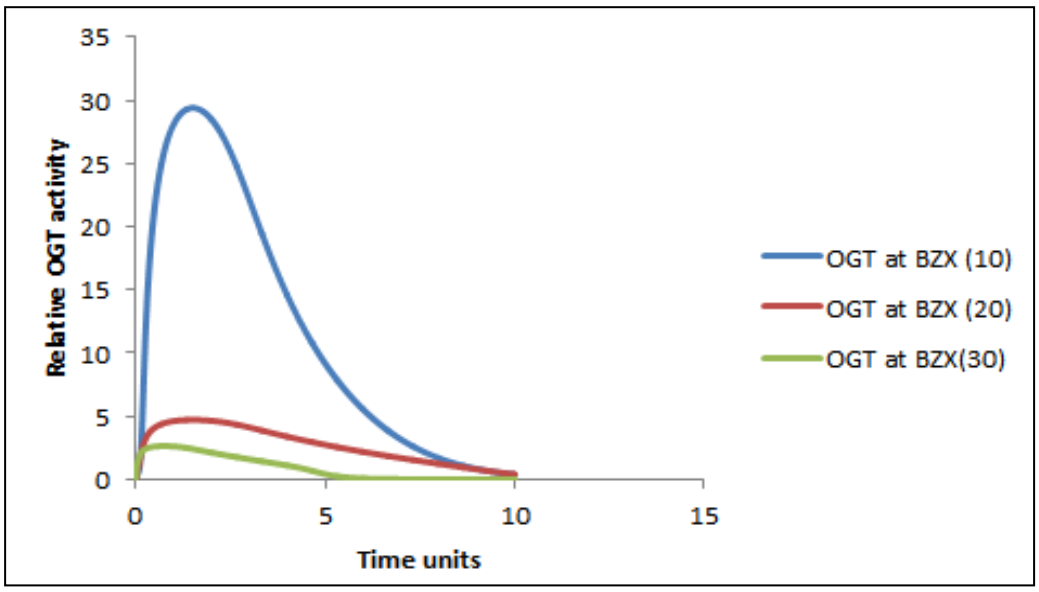

Figure 25.2: Increasing BZX dosage reduces OGT activity considerably

The x-axis shows the time units while the y-axis represents the relative activity of OGT. When BZX was increased to 30 units, OGT activity decreased completely, much earlier than that at BZX at 20 and 10.

4.7.6 CASE 6: Combination therapy (BZX+ Metformin)

To check the efficacy of combination therapy including OGT inhibitor BZX and Metformin on breast cancer cell proliferation another Petri net was designed (Figure 26).

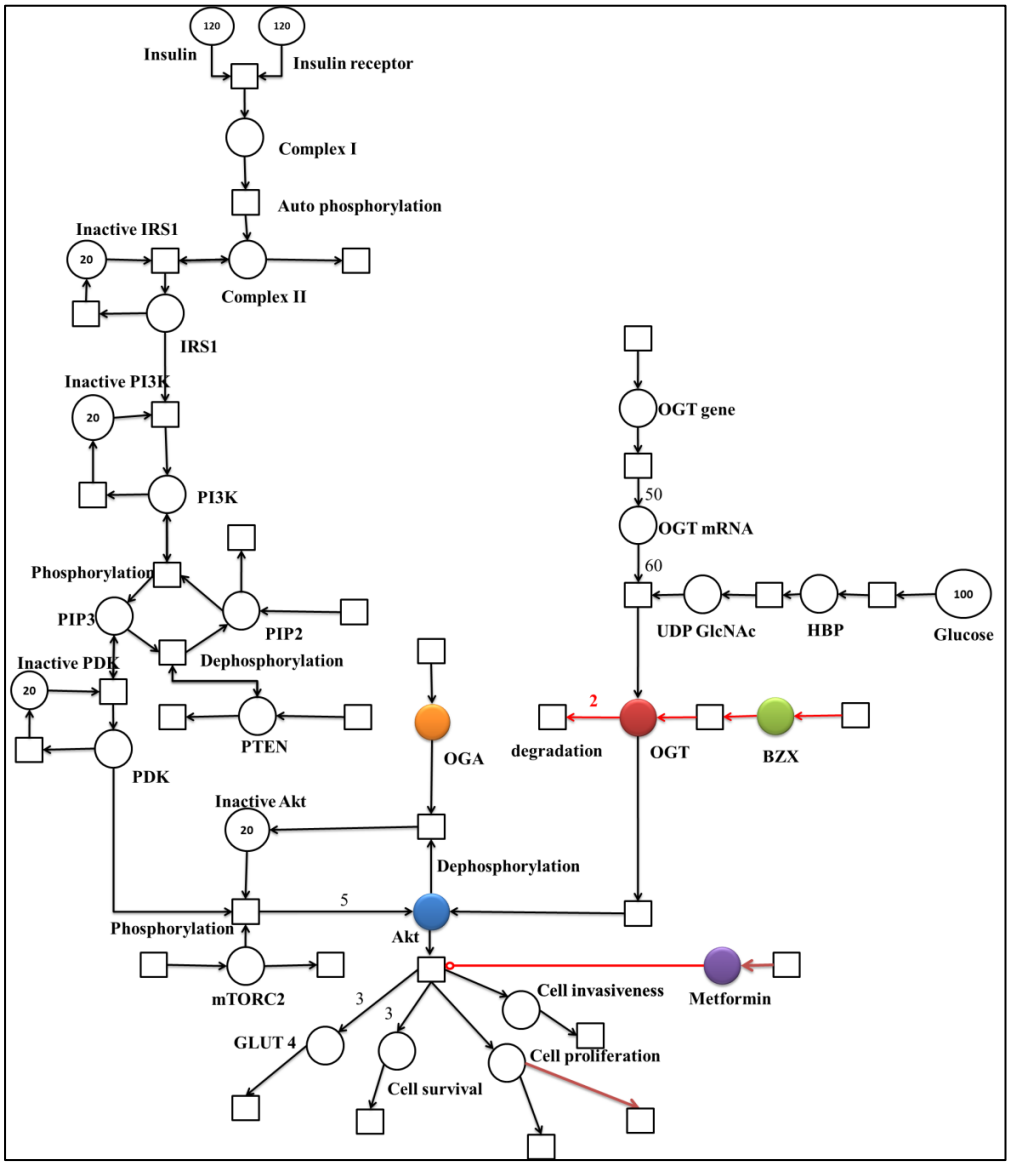

Figure 26: Illustration of the petri net model representing BZX and Metformin intervention in breast cancer cell under hyperglycemia. A standard place is illustrated as a circle ◯ representing proteins involved in the pathway. A continuous transition is depicted as □ representing cellular processes including phosphorylation and dephosphorylation. A directed arc ⟶ connects a place with a transition and vice versa. Inhibitory arc is represented by an arc with a hollow dot as its head in red color . Colored places include (Blue = Akt, Green = BZX, Red= OGT and Orange = OGA and Purple = Metformin)

We used this combination to check the whether OGT inhibition and blocking cell proliferation will impair breast cancer progression further. Also, interaction of Metformin with OGT has not yet been reported, therefore this combination targets two separate processes. Based on our calculations, up to 20 fold decrease in cell proliferation was observed (Table 2).

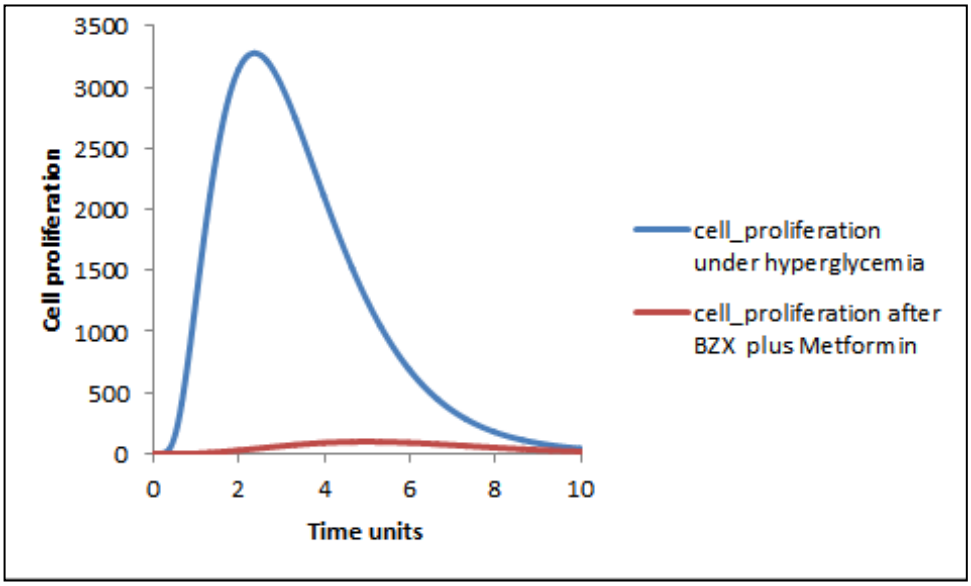

Figure 26.1 Effect of Metformin on breast cancer cell proliferation

The x-axis shows the time units while the y-axis represents the relative rate of breast cancer cell proliferation. According to the graph, substantial difference in cell proliferation was observed using this combination strategy.

Figure 26.1 depicts that breast cancer cell proliferation becomes almost negligible through this drug combination .This strategy can prove to be very beneficial as it not only decreases breast cancer cell proliferation but also inhibits OGT that aggravates cancer by modifying central player in cancer progression, Akt.

The observations of perturbation experiments in terms of fold changes have been summarized in Table 2:

Effect of:	OGT (relative units)	Cell proliferation (relative units)
Hyperglycemia	↑ 4 fold	↑ 10 fold
shRNA	↓ 0.2 fold	↓ 0.3 fold
BZX	↓ 7 fold	↓ 14 fold
shRNA+ BZX	Not significant	↓ 3 fold
Metformin	No change	↓ 5 fold
BZX+ Metformin	↓ 7 fold	↓ 20 fold

Table 2: Summary of the observations derived through simulated results

DISCUSSION

CHAPTER 5: DISCUSSION

Synergistic relationship between Diabetes Type II and breast cancer has been proposed through recent research. The precise mechanism or a common cell signaling pathway of this relationship is still uncertain, however, it is becoming clear that aberrations in PI3K pathway due to hyperinsulinemia and hyperglycemia increase breast cancer risk as well as treatment outcome in diabetic patients. PI3K pathway plays a significant role in regulating mechanisms crucial to tumor initiation, progression and consequences like cell growth, metabolism, proliferation, invasion, angiogenesis and survival[69]. Aberrations in this pathway lead to a number of implications in development and progression of breast cancer. Moreover, the nutritional environment greatly directs the growth and proliferation of cancer cells particularly the availability of glucose. Glucose is essential for biosynthesis of cellular components such as membranes, nucleic acids and proteins crucial for cell proliferation [70]. It can directly regulate signaling pathways and cellular processes through O-GlcNacylation of nucleic and cytosolic proteins. When glucose concentration in blood is high above the threshold value due to insulin resistance, it leads to hyperglycemia, which is an important risk factor for cancer. Increased glucose flux leads to amplified O-GlcNAcylation permitting dynamic changes in cancer cell. [71]. In this study we analyzed the role of OGT in insulin resistant adipocyte and breast cancer. Moreover, we studied the effect of hyperglycemia in breast cancer and the changes in the system behavior due to overexpression of OGT enzyme.

During early insulin mediated signaling and normal cellular conditions, O-GlcNAc transferase is present within the nucleus. As the insulin signaling is prolonged, OGT moves out of the nucleus and carries out O-GlcNAcylation of proteins in

order to regulate insulin signaling. OGT moves to plasma membrane where it performs its function. Key molecules in PI3K pathway such as IRS1 and Akt are O-GlcNAcylated. Increased O-GlcNAcylation of IRS1 and Akt, as a result of exposure to high glucose concentration, attenuates he PI3K pathway[22].

In PI3K pathway, there is a complex interplay between O- GlcNAcylation and phosphorylation. The increased O- GlcNAcylation of proteins in the PI3K pathway might antagonize activation of insulin signaling via phosphorylation. As shown in Figure 16, O-GlcNAc modification of IRS-1 reduces its activity. Moreover, Akt O-GlycNAcylation also leads to attenuation of insulin signaling (Figure 17). The overall effect of insulin pathway is dampened via negative feedback by OGT [18]. When the cells are chronically exposed to increased glucose concentration, this negative feedback may be exacerbated. Therefore, over expression and aberrant regulation of OGT in adipocytes leads to insulin resistance.

Breast cancer has been characterized by an aberrant activation of the PI3K/Akt pathway because of the mutations in oncogenes (p53, BRCA1) [59]. In the Petri net model designed for invasive breast cancer, it was assumed based on a study conducted by Millazo *et. al*, that there is a 6 fold increase in insulin receptor expression on the breast cancer cell membrane as compared normal breast cells [72]. Under hyperglycemia, the PI3K pathway was hyperactivated to increase the cell proliferation and invasiveness. Interestingly, in this pathway the role of OGT was opposite to that in adipocytes. The dual function of OGT explains that OGT function is cell specific. We assumed similar conditions in both the cells i.e. enhanced HBP activation, hyperglycemia and hyperinsulinemia, but the factor that

allows OGT to switch the PI3K pathway "on" or "off" is still unidentified. Rigorous studies to identify all possible sites and the effect of O-GlcNacylation on them can help us understand the differential activity of OGT.

The difference between breast cancer before and after hyperglycemia was analyzed and it was observed that hyperglycemia promotes OGT expression, Akt activity and cell processes such as cell proliferation, cell invasiveness as well as glucose transport.

Further, perturbation experiments were carried out to inhibit OGT in order to observe the significance of OGT in invasive breast cancer. It was observed that the OGT mRNA inhibition decreased the OGT expression by 0.2 fold and cell proliferation by 0.3 fold. The application of BZX inhibition showed that there was a 7 fold decrease in OGT activity and 14 fold decrease in cell proliferation, proving that the protein inhibition of OGT is more potent as compared to RNA inhibition and also effective in reducing cell proliferation. Moreover, the combined effect of shRNA and BZX was also studied. Based on simulations, we did not observe significant difference in OGT activity but as the concentration of BZX was increased by 10 units, the OGT activity reduced considerably.

Next, we analyzed the effect of Metformin on cell proliferation in invasive breast cancer. According to Phoenix *et. al*, Metformin reduces cell proliferation in breast carcinoma cells *in vitro*. Because Metformin does not interact directly with constituents of PI3K pathway, we applied a combination of BZX and Metformin to check if cell proliferation is further decreased. We observed that the combination of BZX and Metformin decreases the cell proliferation up to 20 folds. This model reveals that this combination therapy with BZX and Metfromin given simultaneously is far more effective than sequential therapy and can prove to be comparatively

better as it not only decreases OGT expression but also reduces breast cancer cell proliferation significantly.

CHAPTER 6: CONCLUSION AND FUTURE PROSPECTS

The Petri net model predicted that alterations in O-GlcNAc signaling affect both insulin resistance and breast cancer. Under hyperglycemia the breast cancer cell survival, growth and proliferation were greatly enhanced. Moreover, the combination therapy for breast cancer patients consisting of antidiabetic drugs such as Metformin along with OGT inhibitors for example BZX can produce better treatment regimens.

In future, the significance of O-GlcNAcylation in causing diabetic compications needs further elucidation. Moreover, it is expected that other therapeutic drugs and inhibitors that target GlcNAcylation can be tested for treatment of diabetes and breast cancer.

Furthermore, numerous studies show a correlation between increased Hexose amine biosynthetic flux and insulin resistance; however, the association between the two mechanisms has not been established. Although the affiliation between insulin resistance, Hexoseamine biosynthetic pathway and breast cancer progression may be quite complicated, it undoubtedly deserves additional studies so better treatments can be devised.

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