

EXAMINING THE EFFECTS OF SMALL MOLECULE TYROSINE KINASE INHIBITORS ON GLUCOSE METABOLISM IN SKELETAL MUSCLE AND LIVER CELL LINES *IN VITRO*

A thesis submitted in fulfilment of the requirements for the degree of
Master of Science (Pharmacy)

By

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RHODES UNIVERSITY
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ABSTRACT

Introduction: Diabetes mellitus is rising due to aging, sedentary lifestyles, obesity, and unhealthy diets, posing a global health threat. Due to increase in prevalence together with shortfalls associated with current treatment options, there is still a necessity for a continuous search of new pharmacotherapies. Small molecule tyrosine kinase inhibitors are drugs, used in cancer chemotherapy and have been shown to affect glycaemic control and metabolism variably. Studies have shown that tyrosine kinase inhibitors can alter glycaemic control and glucose metabolism, with some demonstrating hypoglycaemic activities whilst others showing hyperglycaemic properties. The mechanism by which tyrosine kinase inhibitors cause glycaemic dysregulation is not well understood, therefore, the clinical significance of these chemotherapeutic agents on glucose handling is also poorly documented.

Aims and objectives: This study aims to elucidate how small molecule tyrosine kinase inhibitors affect glucose metabolism in C2C12 and HepG2 cells *in vitro*, including their impact on glucose uptake, AKT, GLUT-4, and IL-6 expression, GLUT-4 translocation, and alpha-amylase and alpha-glucosidase activity.

Methods: In this study, C2C12 and HepG2 cells were seeded in well plates and the initial media glucose concentration was recorded. Cells were then treated with small molecule tyrosine kinase inhibitors; imatinib, dasatinib, axitinib, and erlotinib for 24 hours. Thereafter, the effect of the test drugs was assessed on cell viability, glucose uptake, expression of AKT GLUT-4 and IL-6, and translocation of GLUT-4. Furthermore, effects of the drugs were assessed on the activities of alpha amylase and glucosidase using calometric assays.

Results and Discussion: Cells treated with small molecule tyrosine kinase inhibitors were viable after 24 hours. A concentration-dependent increase in glucose uptake in C2C12 cells treated with imatinib was observed as the concentration of imatinib increased. Axitinib, dasatinib, and erlotinib demonstrated glucose uptake levels comparable to the control across all concentrations. Small molecule tyrosine kinase inhibitors demonstrated an increase in GLUT4 translocation in the absence of insulin. GLUT4 expression was comparable in cells treated with small molecule tyrosine kinase inhibitors and the control. Small molecule tyrosine kinase inhibitors showed an increase in AKT expression. C2C12 cells treated with small molecule tyrosine kinase inhibitors were observed to have elevated IL-6 expression compared to the control. The HepG2 cells treated with erlotinib and imatinib demonstrated elevated glucose uptake while cells treated with axitinib and dasatinib were observed to have a lower glucose uptake. Treatment with dasatinib led to a decrease in Akt expression as concentration increased. Small molecule tyrosine kinase inhibitors demonstrated inhibition of alpha-amylase, while only dasatinib and axitinib showed inhibition of alpha-glucosidase.

Conclusion: The results show that small molecule tyrosine kinase inhibitors impact glucose metabolism in C2C12 and HepG2 cells via their effect on GLUT-4 translocation and expression and AKT expression. Dasatinib showed promising potential with regard to antidiabetic capabilities. Further research is needed to better understand these mechanisms' effects on metabolic homeostasis and inform future therapeutic strategies.

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
DM	Diabetes mellitus
C2C12	Myoblast cell line
HepG2	Liver cell line
GLUT	Glucose transporter
AMPK	AMP-activated protein kinase
GABA	Gamma-Aminobutyric acid
ATP	Adenosine triphosphate
Akt	Protein kinase B
PI3K	Phosphatidylinositol-3 kinase
AS160	Akt substrate
Rab-GAP	GTPase-activating protein
PTP	Protein tyrosine phosphatases
DSP	Dual-specificity phosphatases
RTK	Receptor tyrosine kinases
FGFR	Fibroblast growth factor receptor
EGFR	Epidermal growth factor receptor
PDGFR	Platelet-derived growth factor receptor
IR	Insulin receptor
NRTK	Non-receptor tyrosine kinase
JAK	Janus kinase
μ	Micro
VEGFR	Vascular endothelial growth factor receptor
GIST	Gastrointestinal stromal tumor
HbA1c	Hemoglobin A1C
FDA	Food and Drug Administration
NF-KB	Nuclear factor-kappa B
ERK	Extracellular regulated kinase

RIPK2	Receptor-interacting protein kinase 2
FDG	Fluorodeoxyglucose
TNF- α	Tumour Necrosis Factor Alpha

Thesis Outline

Chapter 1: Introduction and Literature Review

- A detailed background on small molecule tyrosine kinase inhibitors (TKIs) and their relevance to glucose metabolism and insulin signalling
- Objectives of the study
- The specific gap in research this study aims to address
- The importance of this study to pharmacology, healthcare, and future studies

Chapter 2: Methodology

- The experimental setup and research approach
- Assays for measuring glucose uptake, GLUT4, Akt, and IL-6 expression and GLUT4 translocation
- Assays for measuring inhibitory effect on alpha amylase and glucosidase
- Data Analysis, statistical tools and software used for analysing results

Chapter 3: Results

- A detailed account of experimental results, supported by figures and graphs
- Description of the effects of small molecule tyrosine kinase inhibitors on glucose metabolism and insulin signalling
- Statistical Analysis

Chapter 4: Discussion

- The significance of the findings
- The possible biochemical or molecular mechanisms behind observed results
- Comparative Analysis
- Clinical or research implications

Chapter 5: Conclusion

- Summary of key findings
- The novel contributions of this study to pharmacology

Chapter 6: Limitations and Future studies

- Challenges or constraints faced during this study
- Propose directions for further studies

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterised by elevated blood glucose concentration due to insulin resistance, deficiency of insulin, or both (1). DM affects the metabolism of fats, carbohydrates, and proteins, resulting in chronic hyperglycaemia (1,2). The global prevalence of DM is on the rise. The increase in DM prevalence may be attributed to sedentary lifestyles and diets rich in refined carbohydrates (1–3). Diabetes can be classified into type 1, type 2, and gestational diabetes mellitus. Reports have estimated that 90% of all DM cases are type 2 (2–4). An estimated 171 million people were affected by DM before 2010, and this figure is predicted to increase to 300 million by 2025 (3). An estimated 5.2 million deaths globally per year have been attributed to DM, exerting pressure on health systems worldwide (5).

Many routinely prescribed pharmacological agents are believed to induce glucose intolerance and/or cause DM in non-diabetic individuals or they can negatively impact glycaemic control in diabetic patients (6,7). These effects on glycaemic control by pharmacological agents can be attributed to the induction of insulin resistance, inhibition of insulin secretion, and direct cytotoxic effects on pancreatic cells (8). Patients with diabetes often have other coexisting medical conditions which often leads to polypharmacy (9). The use of polypharmacy has the potential to worsen glucose tolerance (10). Several pharmacological agents influence glucose metabolism, including certain antihypertensives, antiretrovirals, atypical antipsychotics, antibiotics, and immunosuppressives (11). Literature indicates that patients being treated with β -blockers, protease inhibitors, thiazide diuretics, and atypical antipsychotic drugs are at a higher risk of developing DM as the prevalence of hyperglycaemia is higher with these agents (11).

Due to the heightened risks of developing DM to date, there is a need for more antidiabetic options to be available for physicians to utilize in the management of diabetes. Interestingly, anticancer agents known as small molecule kinase inhibitors have shown mixed observations, where some generations have been reported to be associated with blood glucose-lowering effects in patients with cancer, whilst others have been associated with hyperglycaemia. This, therefore, highlights the necessity for more research regarding these agents and their effect on glucose metabolism, thus allowing a deeper insight in terms of their mechanisms of action at both cellular and molecular levels. Where consensus and a solid understanding of these agents on glucose handling is achieved, clear insights on their role in worsening or managing hyperglycaemia can be realised.

Considering the burden exerted by DM, together with the time and challenges it takes to discover and develop new antidiabetics, there is merit for studies looking into leveraging on the existing hypoglycaemic effects demonstrated by small molecule tyrosine kinase inhibitors. With a defined mechanism of action, and structure activity relationship, small molecule tyrosine kinase inhibitors can potentially be repurposed for diabetes management, a route that could be time and cost-effective. Small molecule tyrosine kinase inhibitors are already in use and registered for the use of the treatment of cancers; therefore, safety and release profile studies are already available for these drugs. This study aimed to elucidate how small molecule

tyrosine kinase inhibitors affect glucose metabolism in C2C12 and HepG2 cells *in vitro*, including their impact on glucose uptake, AKT, GLUT-4, and IL-6 expression, GLUT-4 translocation, and alpha-amylase and alpha-glucosidase activity. For a better understanding of small molecule tyrosine kinase inhibitors on glucose metabolism, firstly we will start by briefly outlining key processes involved in glucose homeostasis.

1.2 Glucose metabolism

1.2.1 GIT (Gastrointestinal Tract) handling of glucose

Dietary carbohydrates are a diverse group of chemically defined substances. They are classified based on type of linkage (α or β), degree of polymerization (DP), and individual monomers (12). According to the Food and Agriculture Organization/World Health Organization Expert Consultation in 1997, there are 3 main groups of carbohydrates namely, polysaccharides (DP ≥ 10), oligosaccharides (short-chain carbohydrates) (DP 3-9), and sugars (DP 1-2) (12,13). Approximately 50% of the energy source in most human diets are carbohydrates (14). In the human body carbohydrates are an energy source, they also play a role in insulin metabolism, regulation of blood glucose, triglyceride metabolism, and cholesterol metabolism (15). Studies have shown that high carbohydrate diets are associated with obesity and related diseases namely diabetes and cardiovascular diseases (16,17). The majority of the carbohydrate intake is in the form of sucrose and starch (18). Starch is the major polysaccharide, composed of 85% amylopectin and 15% amylose (19). Amylopectin is a branched glucose polymer with a α -1,4-linked glucose and α -1,6-linked glucose backbone. Amylose is a straight-chain glucose polymer (α -1,4-linked) (20). Carbohydrate digestion is initiated in the oral cavity. The salivary gland serous cells produce and secrete α -amylase in the saliva during mastication. The α -amylase hydrolyses the α -1,4 bonds of starch to form dextrins (19). Salivary α -amylase breakdown of carbohydrates only accounts for 5% of carbohydrate digestion (21). Salivary α -amylase is deactivated by the acidic pH of the stomach (20). Carbohydrate digestion resumes in the small intestine, acinar cells in the pancreas secrete pancreatic α -amylase. Both pancreatic and salivary α -amylase are endosaccharidase that hydrolyse internal α -1,4 bonds (22). Pancreatic α -amylase further breaks down dextrin into α -limited dextrins, maltotriose, maltose, trisaccharides, and larger oligosaccharides (23). The small intestine is only able to absorb monosaccharides (24).

The gut houses both α -amylase and α -glucosidase for digestion of carbohydrates to allow for absorption into the intestinal epithelium (20). Alpha-glucosidase are membrane bound enzymes found in the brush border of the epithelium of the small intestine. They catalyse the breakdown of oligosaccharides and disaccharides into monosaccharides (simple sugars) that can be absorbed in the gut (25). These simple sugars are absorbed and enter the blood leading to an elevation in blood glucose concentration known as post-prandial hyperglycaemia (26). Postprandial hyperglycaemia is more pronounced in diabetic patients due to their limited or inability to regulate their blood glucose concentration. Hence inhibiting glucose uptake in the intestine through inhibition of α -glucosidase and α -amylase aids patients in the regulation of their blood glucose concentration in the postprandial period (27,28). Inhibition of α -amylase and α -glucosidase digestion subsequently reduces blood glucose concentration (29). Alpha amylase and alpha glucosidase inhibitors include acarbose, voglibose, and miglitol (30,31). Although effective in stabilizing postprandial blood glucose concentration, they are frequently associated with considerable gastrointestinal side effect including flatulence and diarrhoea

(32,33). Therefore limiting their use clinically. There is need to uncover more compounds that can be used to inhibit these carbohydrates hydrolysing enzymes, hence the inhibitory effect of small molecule tyrosine kinase inhibitors on α -amylase and α -glucosidase were investigated.

1.2.2 Insulin secretion

Insulin secretion is primarily glucose-induced, with other nutrients such as amino acids and fatty acids augmenting the glucose-induced secretion (34). Glucose-induced insulin secretion requires the intracellular uptake of glucose and its metabolic oxidation. The primary transporters of glucose in β -cells are glucose transporter 1 (GLUT1) and GLUT3 (35). Insulin is produced in the rough endoplasmic reticulum as prepro-insulin, a 110 amino acid precursor. It is then packed in the Golgi apparatus as proinsulin after the removal of 24 residue signal sequence from prepro-insulin. Insulin is stored in immature secretory granules (36). Studies of isolated islets with radioactive leucine showed that granules synthesised last are the most likely to be released when β -cells are stimulated (37). The breakdown of insulin is by crinophagia and the intracellular destruction of insulin is by lysosomes (38). The secretory granules containing insulin also contain approximately 50 polypeptides and low molecular weight compounds like GABA, glutamate, and serotonin. The granules also contain high concentrations of metal ions like Zn^{2+} and Ca^{2+} (39). The secretion of insulin is biphasic, with glucose providing a triggering action and generating triggering and amplifying signals in β -cells (40). β -cells are electrically excitable and utilize changes in their membrane potential to link changes in plasma glucose concentrations with the stimulation or inhibition of insulin secretion (34). β -cells have ATP-sensitive K^+ (K_{ATP}) channels which at low glucose concentrations maintain a negative membrane potential and close in response to glucose stimulation (41). Insulin secretion also involves ATP production, glucose metabolism, Ca^{2+} -dependent action potential firing, and membrane depolarization (42). β -cell electrical activity leads to elevation of Ca^{2+} level which triggers exocytosis of insulin granules. This is referred to as Ca^{2+} -dependent exocytosis of insulin granules (43). Plasma glucose concentration homeostasis is under the feedback loop control of insulin through alterations in K_{ATP} channel closure, β -cell metabolism, and electrical activity (42). Pharmacological agents that act by depolarization and Ca^{2+} influx into β -cells lead to desensitization of insulin secretion, which is a reversible state of decreased insulin secretion responsiveness of β -cells due to prolonged exposure to stimuli. Other stimuli include glucose and free fatty acids (44). Sulphonylureas, namely, glibenclamide and tolbutamide, cause recoverable impairment of insulin secretion (45). Studies have shown that prolonged sulphonylurea exposure of isolated islets reduced glucose-induced insulin secretion by approximately 50% (46). Calcineurin inhibitors including tacrolimus and cyclosporine are implicated as diabetogenic drugs. Calcineurin inhibitors have been linked to the development of new-onset diabetes mellitus following transplantation surgery. This occurs in part through β -cell apoptosis subsequently leading to a reduction in insulin secretion (47). Calcineurin is expressed in β -cells where it targets the NFAT family of transcription factors and the cAMP responsive element binding protein (CREB) transcriptional coactivator. CREB transcription factor mediates proliferative and survival effect of GLP-1 and the glucose signalling pathways (48). GLP-1 promotes islet-cell survival via cAMP through CREB-mediated induction of IRS2 (49). This promotes the activation of the survival kinase Akt as a response to insulin (50). Studies have shown that through reduced phosphorylation of Akt substrates calcineurin regulates survival and replication of β cells through the PI3K/Akt pathway (51). Cyclosporine is believed to alter mitochondrial permeability transition pore function (52). Cyclosporine is a potent inhibitor of the mitochondrial permeability transition

pore which disrupts β -cell function, as this stops the oscillatory activity that is necessary for insulin secretion (53). Studies have shown that tacrolimus and cyclosporine inhibit glucose uptake independent of insulin signalling as they remove GLUT4 from cell membranes through increased endocytosis (54).

1.2.3 Insulin signalling

Insulin receptors are present on every cell, but they are primarily expressed in skeletal muscle, liver, and fat tissue, which are the predominant insulin target sites. Insulin binds to target cell surface receptors; the insulin receptor is a heterotetramer consisting of β subunits and α subunits linked together through disulphide bonds (55). As illustrated in Figure 1.1, Insulin binds to cell surface protein receptors, resulting in tyrosine phosphorylation and subsequent phosphorylation of the insulin receptor substrate. This recruits and activates PI3K kinase and its downstream target Akt/PKB (56). The activation of Akt/PKB leads to phosphorylation of AS160, resulting in a reduction in Rab-GAP activity. This results in the translocation of GLUT4 and glucose uptake as depicted in Figure 1.1 (57). The serine/threonine kinase Akt signalling plays a major role in insulin-stimulated glucose uptake in adipose and skeletal muscle tissue. Akt signalling also inhibits glucose release from hepatocytes (58). The uptake of glucose via Akt signalling occurs through translocation of GLUTs to the cell membrane upon activation of insulin receptors in the presence of glucose (59). Protein tyrosine phosphatases (PTPs) can be classified into several sub-families based on their functional and structural characteristics. The three major sub-families include receptor-type PTPs (RPTPs), non-receptor-type PTPs (NRPTPs), and dual-specificity phosphatases (DSPs) (60). LAR is a member of the RPTPs family and PTP-1B, PTP-1C, and PTP-1D belong to the NRPTPs family (61). From the PTPs mentioned above, only PTP-1B is a target for obesity and diabetes treatment as PTP dephosphorylation activates both leptin and insulin signalling pathways (62). The negative regulatory effect of PTP-1B on the insulin signalling pathways is a therapeutic potential in type 2 DM (63). Patients with type 2 DM have disrupted GLUT4 expression and this contributes to insulin resistance (64). Disrupted intracellular signalling of GLUT4 translocation from intracellular vesicles to the cell membrane is responsible for resistance to insulin-stimulated glucose transport (65). Apart from insulin mediated glucose transport, basal glucose uptake in most cells is facilitated by the presence of GLUT1 (66). In this study the insulin signalling pathway was of interest. Understanding the effect small molecule tyrosine kinase inhibitors have on the insulin signalling pathway will help shed light on the mechanisms through which small molecule tyrosine kinase inhibitors improve glycaemic control in patients.

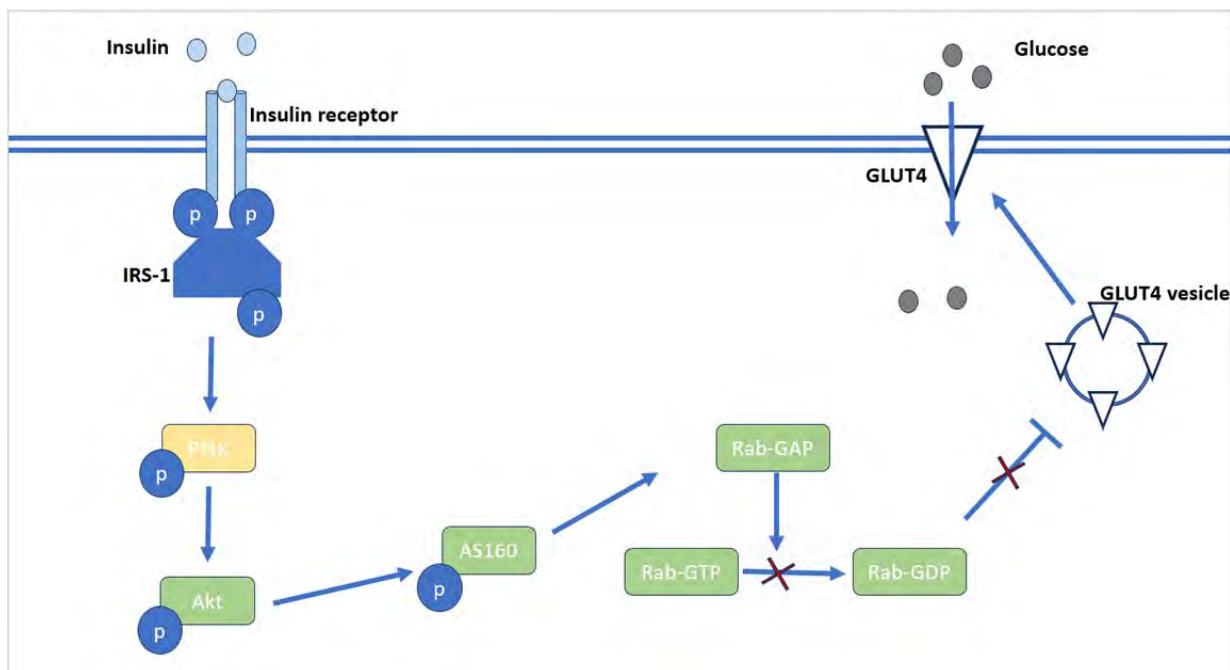


Figure 1. 1: An illustration of Akt's role in the insulin signalling pathway. Akt activation leads to phosphorylation of AS160 which reduces Rab-GAP activity resulting in reduced production of Rab-GDP which inhibits the translocation of GLUT4 vesicles.

1.3 Antihyperglycemic agents used for diabetes mellitus

1.3.1 Insulin

Insulin is the primary treatment for patients with type 2 diabetes mellitus when diet, physical activity, and other oral antidiabetic medications have not achieved glycaemic control (67). Efforts have been made over the last 2 decades to introduce new classes of antidiabetic medication, to address suboptimal blood glucose control, side effects, and limited access to treatment in low-income countries. Although various oral antidiabetic medications are available, insulin remains the most common treatment for patients with diabetes mellitus who are unable to achieve adequate glycaemic control with oral therapy or who have progressed to severe type 2 diabetes (68,69). Insulin therapy is classified based on onset and duration of action. Short acting insulin (regular insulin) has an onset of action of 30-60 mins following subcutaneous injection and a duration of action of 4-8 hours (70,71). Rapid-acting insulin was designed to optimally regulate postprandial hyperglycaemia, and have a pharmacokinetic profile similar to unmodified endogenous insulin following a meal (72). Long-acting (Basal) insulin has a long duration of action (24 hours). It was developed to mimic basal level insulin in healthy individuals during fasting states (73). Basal insulin like glargine have a delayed absorption from subcutaneous tissue resulting in a flat 24-hour insulin concentration profile (74). Insulin preparations also come as intermediate-acting, with an onset of action of 30-40 mins and duration of action of 6-8 hours (75). Despite being the mainstay in diabetes mellitus therapy insulin is associated with hypoglycaemia, hyperinsulinemia, and lipodystrophy dosing related challenges in patients. Hyperinsulinaemia is linked with weight gain (76,77).

Exogenous insulin to date is not able to replicate the physiological insulin profile resulting in poor overall glycaemic control (78).

1.3.2 Biguanides

Biguanides are a class of antidiabetic drugs derived from the *Galega officinalis* (goat's rue) plant (79). Oral biguanides include metformin, phenformin, and buformin. However, phenformin and buformin were withdrawn from clinical use in the late 1970s due to high incidence of lactic acidosis (80). Biguanides are indicated for treatment of non-insulin-dependent diabetes mellitus (type 2). Metformin is the first line treatment for type 2 diabetes mellitus, since its approval by the FDA metformin has become the most prescribed oral antidiabetic drug for type 2 diabetes mellitus (81,82). Though the exact mechanism biguanides exert their antidiabetic properties are yet to be fully understood. The widely accepted mechanism is that biguanides have an inhibitory effect on mitochondria complex I and inhibits the oxygen consumption rate, subsequently leading to energy stress, an increase in AMP/ATP ratio and activation of AMP kinase (83). Metformin further enhances the activated AMP kinase leading to suppression of hepatic gluconeogenesis and glycogenolysis and enhances hepatic glucose uptake (79,84). Metformin has also been shown to promote tyrosine kinase activity leading to enhanced peripheral and hepatic insulin sensitivity plus GLUT4 and GLUT1 translocation (85,86).

Adverse events associated with biguanides include gastrointestinal symptoms namely abdominal pain, nausea, vomiting, and diarrhoea. They have been shown to cause hepatotoxicity, coagulation, and acute pancreatitis (87). Metformin has been linked to malabsorption syndromes resulting in vitamin B₁₂ deficiency and electrolyte abnormalities (88). Metformin provides cardiovascular protection; studies have suggested that metformin increases the risk of cardiovascular diseases due to accumulation of homocysteine as a result of vitamin B₁₂ (89). It potentially causes vitamin B₁₂ deficiency through alterations in gut flora, competitive inhibition of absorption, motility or disrupting calcium dependent membrane activities on the terminal ileum (88).

1.3.3 Sulphonylureas

Sulphonylureas are a class of drugs utilized in the treatment of type 2 diabetes mellitus. They are the oldest class of oral antidiabetic medication, having been introduced in the 1950s (90,91). Sulphonylureas are classified into first and second generation. First generation sulphonylureas typically have a slower onset of action, long half-life, and increased risk of hypoglycaemia e.g. chlorpropamide, tolazamide, acetohexamide, and tolbutamide. Second generation sulphonylureas are more potent and are the preferred treatment for diabetes mellitus e.g. glimepiride, glipizide, glyburide, and gliclazide (92–94). Sulphonylureas primary action is through stimulation of insulin secretion (95). On the pancreatic β -cells sulphonylureas bind to and inhibit K_{ATP} channels, thus potassium efflux decreases and causes depolarisation of the β -cell membrane. Subsequently β -cell membrane depolarisation causes calcium channels to open leading to an influx of calcium which stimulates insulin secretion (96,97). Sulphonylureas exhibit glucose-independent stimulation of insulin secretion (98). Hence hypoglycaemia is the most prevalent adverse effect (99). They have been linked to weight gain leading to healthcare practitioners cautioning their use in obese patients (100). The American Diabetes Association (ADA) guidelines recommend taking the patient's weight into account when prescribing antidiabetic therapy (101). Due to their ability to bind to K_{ATP} channels, there

exists a potential concern of undesirable cardiac effects due to the presents of potassium channels in cardiac muscle (102). Open potassium channels counteract ischaemic effects that could impair cardiac function and induce arrhythmia (103).

1.3.4 Thiazolidinediones

Thiazolidinediones (TZDs) are a class of oral drugs utilized for the treatment of diabetes mellitus. Rosiglitazone and pioglitazone are the most commonly prescribed TZDs (104). They reduce insulin resistance through increased insulin mediated glucose uptake and inhibit hepatic glucose production (105). TZDs are agonist of peroxisome proliferation activated receptors- γ (PPAR- γ) (106). Upon binding to PPAR- γ a macromolecular complex is formed by PPAR- γ and the retinoic acid receptor. This macromolecular complex recruits an activator that promotes DNA transcription of peroxisome proliferator response elements involved in glucose metabolism (107). Activation of PPAR- γ results in increased GLUT4 expression (108). TZDs also have activity on renal PPAR- γ which increases sodium and fluid retention. This leads to adverse events namely oedema and heart failure (109). Another potentially limiting and common adverse effect is weight gain with an average increase in body weight ranging between 2-3kg (110).

1.3.5 Meglitinides

Meglitinides like sulphonylureas are oral insulin secretagogues. However, they are not structurally related. Commonly known meglitinides are mitiglinide, repaglinide, and neteglitinide (111). They inhibit K_{ATP} channels on β -cell membrane stimulating insulin secretion via binding to a different receptor from that of sulphonylureas (112). Meglitinides are characterized by rapid onset and short duration of action (113). In comparison, to sulphonylureas meglitinides have less incidence of hypoglycaemia (114). The common side effects associated with meglitinides treatment are hypoglycaemia and nausea (115).

1.3.6 Sodium-glucose co-transporter 2 (SGLT2) inhibitors

SGLT2 is selectively expressed in the kidney where it reabsorbs filtered glucose (116). SGLT2 inhibitors block the reabsorption of glucose from the proximal renal tubule into the blood stream, causing the excess glucose to be excreted in the urine (117). SGLT2 is responsible for approximately 90% of glucose reabsorption (118). Inhibiting SGLT2 lowers blood glucose concentration in type2 diabetes mellitus patients. Examples of SGLT2 inhibitors include dapagliflozin, canagliflozin, and empagliflozin (119). Due to glycosuria (elevated glucose levels in urine) a common side effect of SGLT2 inhibitors is urinary tract infection (120).

1.3.7 DPP-4 inhibitors

Ahrén B. (2007) described inhibition of dipeptidyl peptidase 4 (DPP-4) as a novel treatment for type 2 diabetes mellitus (121). DPP-4 is expressed in varies organs within the human body and it is also found in circulation. DPP-4 cleave oligopeptides after the second amino acid from the N-terminal end, leading to the deactivation of glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) (122). DPP-4 inhibitors such as linagliptin, sitagliptin, and vildagliptin, inhibit DPP-4 thus preventing the breakdown of incretins namely GLP-1 and GIP (123). This effect prolongs the half-life of GLP-1 and GIP subsequently promoting insulin secretion and reduces glucagon secretion thus lowering blood glucose concentration (124). DPP-4 are generally regarded as safe. Upper respiratory tract infection,

headache, and nasopharyngitis have are the common adverse effects associated with DPP-4 treatment (125).

1.3.8 Glucagon-like peptide-1 receptor agonist

In recent times the incretin system has become a point of focus as a target for the treatment of type 2 diabetes mellitus. GLP-1 has been of particular interest (126). The impact of incretin is reduced or limited in patients with type 2 diabetes mellitus (127). This reduces the patient's ability to secrete insulin. GLP-1 is a gastrointestinal hormone produced and released in response to nutrient ingestion (128). GLP-1 analogues e.g. exenatide and liraglutide have a high resistance to DPP-4 breakdown (124). These GLP-1 receptor agonist are delivered via the subcutaneous route (129). They enhance insulin secretion, inhibit glucagon secretion, suppress appetite, and slow the rate of gastric emptying (130). GLP-1 receptor agonists are linked to adverse effects such as local injection site reactions, nasopharyngitis, and GIT disturbances (131).

1.3.9 The need for new antidiabetic agents

As of 2014, 14 classes of antidiabetic drugs were available for the treatment of type 2 diabetes mellitus. However, only 36% of patients with diabetes mellitus obtain optimal glycaemic control with the currently available agents (132,133). Diabetes exerts a heavy burden on the healthcare system globally and with the prevalence of diabetes mellitus on the rise there is need for new, more effective, and cost-effective treatments (134). Between 1980 and 2011 in the U.S. the number of new diabetes mellitus diagnosis almost tripled from 493 000 to 1,5 million (135,136). The prevalence of diabetes mellitus in South Africa increased from 4,5% in 2010 to 12.7% in 2019 (137). In the next section, we are going to delve into small molecule tyrosine kinase inhibitors, highlighting their use as cancer treatments, and glycaemic perturbations reported to date.

1.4 Tyrosine kinases and cancer

Tyrosine kinases are enzymes that selectively phosphorylate tyrosine residues in specific proteins by transferring a phosphate group from ATP to the tyrosine residue. They alter the signalling cascade which modifies cell growth, differentiation, migration, death, and apoptosis, as illustrated in Figure 1.2 (138). Tyrosine kinase signalling pathways are typically responsible for preventing dysregulated cell proliferation and play a role in sensitising cells to apoptotic stimuli. In cancer cells, tyrosine kinase signalling pathways are often epigenetically or genetically altered, which gives cancer cells a selection advantage (139). Tyrosine kinases comprise the major group of all oncoproteins (140). Out of the 518 kinases found in the human kinome, approximately 90 are tyrosine kinases (141). Therapeutic agents that are utilized in diseases linked to unusual activation of tyrosine kinase resulting in abnormal downstream oncogenic signalling are at the forefront as targets for chemotherapy (142). Tyrosine kinases are primarily classified as receptor tyrosine kinases (RTKs), e.g., fibroblast growth factor receptors (FGFRs), epidermal growth factor receptors (EGFRs), platelet-derived growth factor receptors (PDGFRs), and insulin receptors (IRs) and non-receptor tyrosine kinases (NRTKs), e.g., Janus kinases (JAKs) and proto-oncogene tyrosine-protein kinase SRC (143). Receptor tyrosine kinases are more than cell surface transmembrane receptors, as they are also enzymes that have intracellular kinase activity (143). Non-receptor tyrosine kinases are cytoplasmic proteins varying in structure (144).

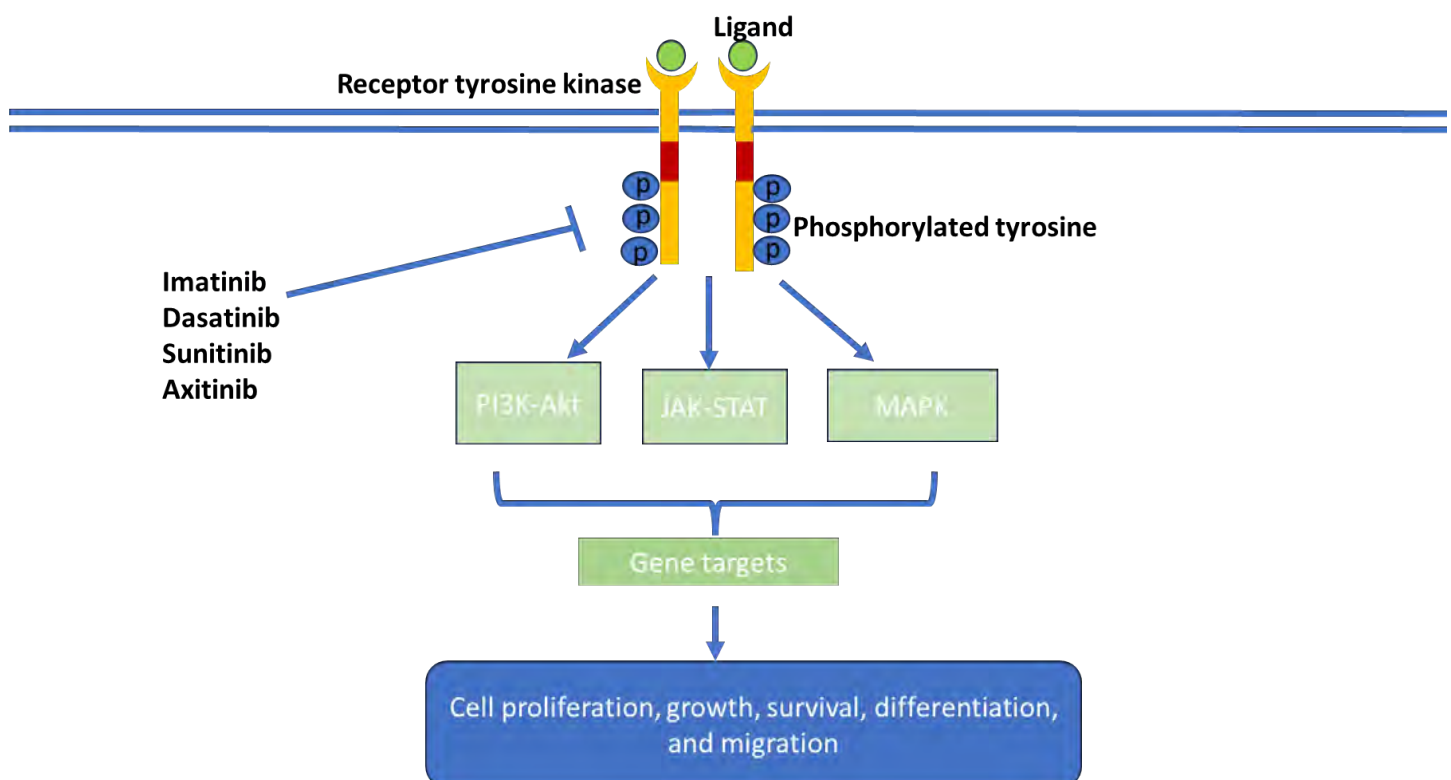


Figure 1. 2: Schematic model of tumorigenic signalling pathways and their inhibition by anti-cancer- tyrosine kinase inhibitors.

1.4.1 Tyrosine kinase mechanism of action

Similar to an insulin receptor activation, ligands bind to the receptor tyrosine kinase resulting in its conformational change, which enables the autophosphorylation of tyrosine residues (Figure 1.1). Activated receptors attract various signalling molecules which leads to the activation of kinases like the Serine/threonine kinase which leads to the phosphorylation and activation of mitogen-activated protein kinase thereby activating extracellular signal-regulated kinases 1 and 2 (145). Ligands such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are extracellular signalling molecules that induce receptor dimerization (139). Two types of ligand-receptor complexes can be formed, viz., one ligand may bind to two receptors resulting in a 1:2 ligand: receptor complex or two ligands can simultaneously bind to two receptor molecules resulting in a 2:2 receptor complex, for example, EGF: EGFR (epidermal growth factor: epidermal growth factor receptor) and VEGF: VEGFR (vascular endothelial growth factor: vascular endothelial growth factor receptor) (139,146). The activation loop has an open conformation after tyrosine phosphorylation and this gives access to ATP and substrates; ATP from Mg-ATP is transferred to the tyrosine residue (147). The ATP binding sites function as a docking site for numerous downstream signalling proteins containing SRC homology-2 (SH2) or protein tyrosine binding (PTB) domain (148). These docking proteins recruit other effector molecules which contain SH2, SH3, PTB, and Pleckstrin homology domain (139,148). As a result, signalling complexes are formed to an activated receptor and membrane which leads to activating a cascade of intracellular biochemical signals resulting in activation or regression of genes and this is what defines biological responses to signals (149). Receptor tyrosine kinases transfer complex information relating to cell growth from the cell's external environment to the cell nucleus thus activating transcriptional pathways which control cellular functions (148)..

1.4.2 Tyrosine kinase inhibitors

Cancer growth is usually promoted by the activation of mutations or gene amplifications of specific serine kinase or tyrosine kinases (150). The non-specificity of traditional chemotherapy drugs, impacting both cancerous and healthy cells, lead to a range of side effects and pose a narrow therapeutic index and potential drug resistance. Tyrosine kinase inhibitors in cancer therapy offer the advantage of selectively targeting and disrupting the upregulated activities of enzymes involved in cancer cell signalling, providing a more precise and less toxic treatment approach (151). The first tyrosine kinase inhibitor approved by the FDA was imatinib in 2001. By 2015, 28 small molecule tyrosine kinase inhibitors had been approved by the FDA, and by 2021 the FDA had approved 73 small molecule kinase inhibitors (152,153). Tyrosine kinase inhibitors have two classes, small molecules that target specific kinases and monoclonal antibodies that target receptor kinases or their ligands (150). Tyrosine kinases play an important role in modulating growth factor signalling (151). Tyrosine kinase inhibitors are orally active and work by competitive ATP inhibition at catalytic binding sites of oncogenic tyrosine kinases, as illustrated in Figure 1.2. Tyrosine kinase inhibitors differ from each other through targeting different kinases, differing pharmacokinetics, and adverse effects. Skin toxicity is an adverse effect observed in more than 50% of the patients on tyrosine kinase inhibitors (154). The use of tyrosine kinase inhibitors is limited by the development of resistance and lack of tumour response in the general population (151).

1.4.3 Tyrosine kinase inhibitors mechanism

Tyrosine kinase inhibitors are categorized by their binding modes into two classes: irreversible and reversible. Irreversible kinase inhibitors form covalent bonds with reactive nucleophilic cysteine residue proximal to the ATP binding site, blocking the ATP site resulting in irreversible inhibition (155). Reversible kinase inhibitors can be categorised into five types based on the conformation of the binding pockets and the Asp-Phe-Gly DFG motif which is a conserved amino acid sequence from the activation loop (156). Type I inhibitors competitively bind to ATP binding sites on active kinases that have aspartate residue of the DFG motif facing the catalytic site of the kinase. Type I½ inhibitors bind to a DFG-Asp in inactive conformation (157). Type II inhibitors bind to inactive kinase with the aspartate residue protruding outward from the ATP binding site (155,156). Type I, I½, and II inhibitors reside in a part of the adenine binding pocket (157). Type III inhibitors only bind to allosteric pockets next to the ATP binding site as they do not interact with or alter the ATP binding pocket (156). Type IV inhibitors bind to allosteric sites that are not on the ATP binding pocket. Type V includes kinase inhibitors like bisubstrate and bivalent inhibitors which display multiple binding modes (158). Irreversible kinase inhibitors are known as type VI inhibitors. Tyrosine inhibitors can inhibit either receptor or cytosolic tyrosine kinases. They compete with ATP for binding sites on tyrosine kinase (e.g., imatinib, gefitinib, PP1-AG1872) (Table 1). Other tyrosine kinase inhibitors such as lavendustin A exert their effect through allosteric inhibition on tyrosine kinase. Some tyrosine kinase inhibitors inhibit tyrosine kinase interaction with other proteins, including UCS15A. Tyrosine kinase inhibitors such as cetuximab inhibit ligands from binding to receptor tyrosine kinases (159).

1.5 Tyrosine kinase inhibitors and their glycaemic effects

Studies have shown that some small molecule tyrosine kinase inhibitors such as imatinib, erlotinib, and sunitinib exhibit antihyperglycaemic effects, the mechanisms of which are not yet fully understood (160). However, certain tyrosine kinase inhibitors like nilotinib have been observed to cause hyperglycaemia (161). Understanding how these drugs cause antihyperglycemic effects could provide further insight on glucose handling of pharmacological targets and shed light on whether tyrosine inhibitors could be useful as pharmacophores to develop novel agents towards the management of DM. Small molecule tyrosine kinase inhibitors have been shown to reverse or even prevent type 1 and type 2 DM by reducing insulin resistance and improving β cell dysfunction, thus reversing hyperglycaemia (162). Literature indicates that tyrosine kinase inhibitors can cause hypoglycaemia in both type 1 and type 2 DM (163). Improvement in HbA1c and glycaemia has been noted in several cases, leading to either termination or reduction of insulin therapy when patients are being treated with imatinib and sunitinib (164). A retrospective study investigated the blood glucose concentration in 17 diabetic and 61 non-diabetic patients being treated with imatinib, sunitinib, dasatinib, and sorafenib. A mean decline in blood glucose concentration was observed with the use of all four drugs; imatinib had a mean decline of blood glucose concentration of 9 mg/dL, sunitinib 14 mg/dL, dasatinib 53 mg/dL, and sorafenib 12 mg/dL. The decrease in blood glucose concentration observed in this retrospective study was statistically significant and it was noted that 47% of patients with diabetes were able to successfully discontinue their diabetic treatment including insulin therapy (165).

This literature is envisaged to provide and consolidate evidence of glycaemic aberrations associated with the small molecule tyrosine kinase inhibitors. We envisage that corroborating retrospective evidence together *in vivo* and *in vitro* observation may assist in shedding deeper mechanistic insight on how small molecule tyrosine kinase inhibitors modulate glucose handling. As mentioned in the introduction, some small kinase inhibitors exhibit hypoglycaemic effects, hence understanding and differentiating the mechanisms of actions of small molecule tyrosine kinase inhibitors is crucial. This may pave the way for the repurposing of these drugs in diabetes management. We envisage discussion in this study will provide a motive for further research and development to explore the worthiness of repurposing small molecule tyrosine kinase inhibitors for the management of DM. In the next section, we present a discussion of the small molecule tyrosine kinase inhibitors outlining the indications and highlighting the known glycaemic effects to date, of each one. Table 1 provides a summary of some small molecule tyrosine kinase inhibitors investigated for their antidiabetic activity.

1.5.1 Imatinib

Imatinib was approved in 2001 by the FDA after successful clinical trials for the treatment of all phases of Philadelphia chromosome-positive (Ph⁺) chronic myeloid leukaemia. It was then approved for the treatment of gastrointestinal stromal tumours (GIST) in 2002 (166,167). Imatinib is a selective and potent inhibitor of the protein tyrosine kinase BCR-ABL, KIT and platelet-derived growth factor receptors (PDGFR- α and PDGFR- β) (168). The protein tyrosine kinase BCR-ABL is a ligand-independent (constitutively) activated tyrosine kinase that causes myeloid leukaemia (CML) (166). Imatinib is a type II tyrosine kinase inhibitor and a phenylamino-pyrimidine derivative (169). After binding to the ATP binding site of the ABL domain of the BCR-ABL tyrosine kinase protein, imatinib traps the chimeric protein in an inactive conformation thus inhibiting it with nanomolar potency (170). The BCR-ABL is constitutively activated and is only present in the leukemic clone, hence imatinib's selectivity. Imatinib inhibition of KIT and tyrosine kinase PDGFR is the basis of its selective treatment in GIST. This selectivity results in reduced side effects. Inhibition of KIT may lead to myelosuppression, and dermatologic toxicity, while ABL inhibition may lead to rare cardiac events and inhibition of PDGFR may lead to altered bone metabolism and fluid retentions (171).

BCR-ABL positive cells express high affinity for the GLUT-1 glucose transporter and increased glucose uptake. This supports the theory that the control of glucose substrate flux is a vital mechanism of the antiproliferative action demonstrated by imatinib (172). The potential therapeutic benefit of imatinib in GIST cells is via decreased glucose uptake (173,174). Treatment of BCR-ABL positive cells with imatinib resulted in approximately 90% of the cell surface transporter GLUT-1 being internalized (175). Furthermore, imatinib treatment has been found to significantly reduce glucose uptake by reduced plasma membrane-bound GLUT-4 in GIST cells (176). Boren et al. have shown that imatinib treatment might reduce *de novo* fatty acid and nucleic acid synthesis through restriction of glucose-6phosphate 1- dehydrogenase activities and hexokinase in myeloid tumour cells (177). In a study by Gottschalk et al., human BCR-ABL positive cell lines treated with imatinib 0.1–1.0 $\mu\text{mol/L}$ demonstrated a decrease in glucose uptake from the media via the suppression of glycolytic cell activity and increased mitochondrial Krebs cycle activity. However, we should note that these observations are reported in cancerous cells, which could suggest that a poor-glucose uptake is a cytotoxic tool to kill cancerous cells (178).

In a clinical observation of seven diabetic chronic myeloid leukaemia patients being treated with imatinib, six patients showed improvement in fasting blood glucose concentration which led to a reduction of their insulin dosage or oral antidiabetic drugs (179). Imatinib has been shown to increase insulin sensitivity, as studies have shown that imatinib treatment led to stable insulin plasma levels and progressive reduction of HbA1c fraction (180). The exact molecular mechanisms in which imatinib confers its antidiabetic properties is not clearly understood and this is a strong motive of this study. So far, the proposed hypothesis of how imatinib improves glycaemia include the preservation of beta cell viability and mass. Imatinib is thought to inhibit beta cell apoptosis through increased activation of NF-KB, an antiapoptotic factor and via decreased activation of proapoptotic MAPK JNK. Insulin sensitivity is improved via inhibition of tumour necrosis factor- α production (181). This is believed to occur via imatinib's inhibition of the non-receptor tyrosine kinase c-Abl (179). Studies show that a moderate dose of imatinib efficiently neutralize high-fat induced peripheral insulin resistance (182). Increased insulin sensitivity may also be due to imatinib's slimming effect (180,182). Imatinib inhibits the phosphorylation of several kinases such as extracellular regulated kinase 1 and 2 (ERK1 and ERK2), AKT and PDGFR. This inhibition of phosphorylation leads to better signalling and functioning of effectors with improvements in insulin sensitivity (183).

1.5.1.1 c-Abl

Xia et al. (2014) observed that inhibition of c-Abl enhanced NKx2.2 a positive regulator for insulin gene expression. These findings compliment studies that reported an increase in serum levels of c-peptide in patients on imatinib treatment (184,185). β -cell c-Abl is a promising potential therapeutic target for the treatment of DM. Studies have observed that c-Abl activation enhances insulin sensitivity in key insulin target tissue namely liver, muscle and, fat cells (186). These finding offer an alternative to the hypothesis proposed by some authors, that imatinib improves insulin sensitivity (182). However recent studies suggest that imatinib enhances insulin production (184). From the above c-Abl tyrosine kinase negatively regulates insulin expression in β -cells, hence the inhibition of c-Abl by imatinib enhances insulin production.

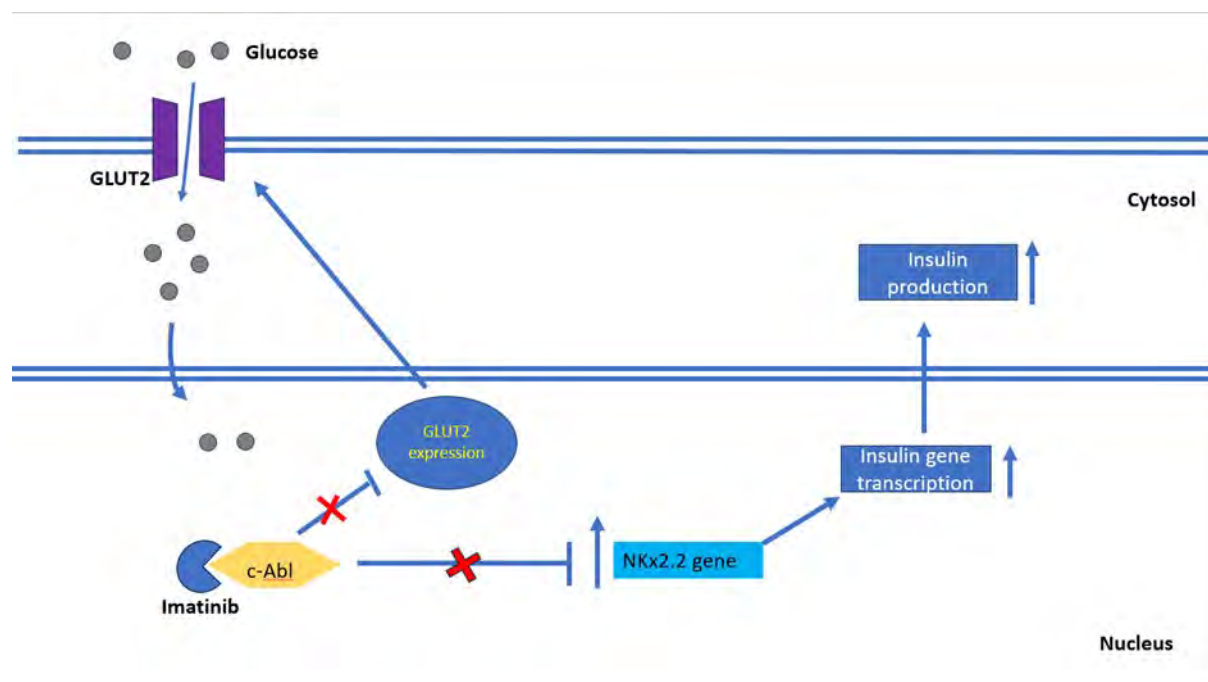


Figure 1. 3: An illustration of enhanced insulin gene transcription through inhibition of c-Abl. Imatinib bound to c-Abl leading to increase NKx2.2 activity and ultimately induced insulin production.

1.5.1.2 ERK 1/2

ERK1/2 have been shown to be stimulated by glucose in MIN6, INS-1, and β TC3 pancreatic β -cell lines (187). Notably ERK1/2 are not required for glucose stimulated insulin secretion, however it has been observed that glucose increases ERK1/2 levels and activity in the nucleus of β -cells. This implies ERK1/2 are potentially associated in regulating gene transcription in these cells (188). Upon activation via phosphorylation in response to glucose, the mitogen-activated protein kinase ERK1/2 promotes nuclear translocation and/or binding of β /NeuroD1, MaFA, and PDX to the insulin promoter. These transcription factors then synergistically activate insulin gene transcription (189). A study analysing mRNA levels, inhibition of ERK1/2 using kinase-defective ERK2 resulted in reduced prepro-insulin mRNA after 24 hours, potentially linking ERK1/2 to insulin gene transcription (190). Another study demonstrated that dominant negative ERK2 inhibitors suppress glucose stimulation of the rat insulin promoter (191).

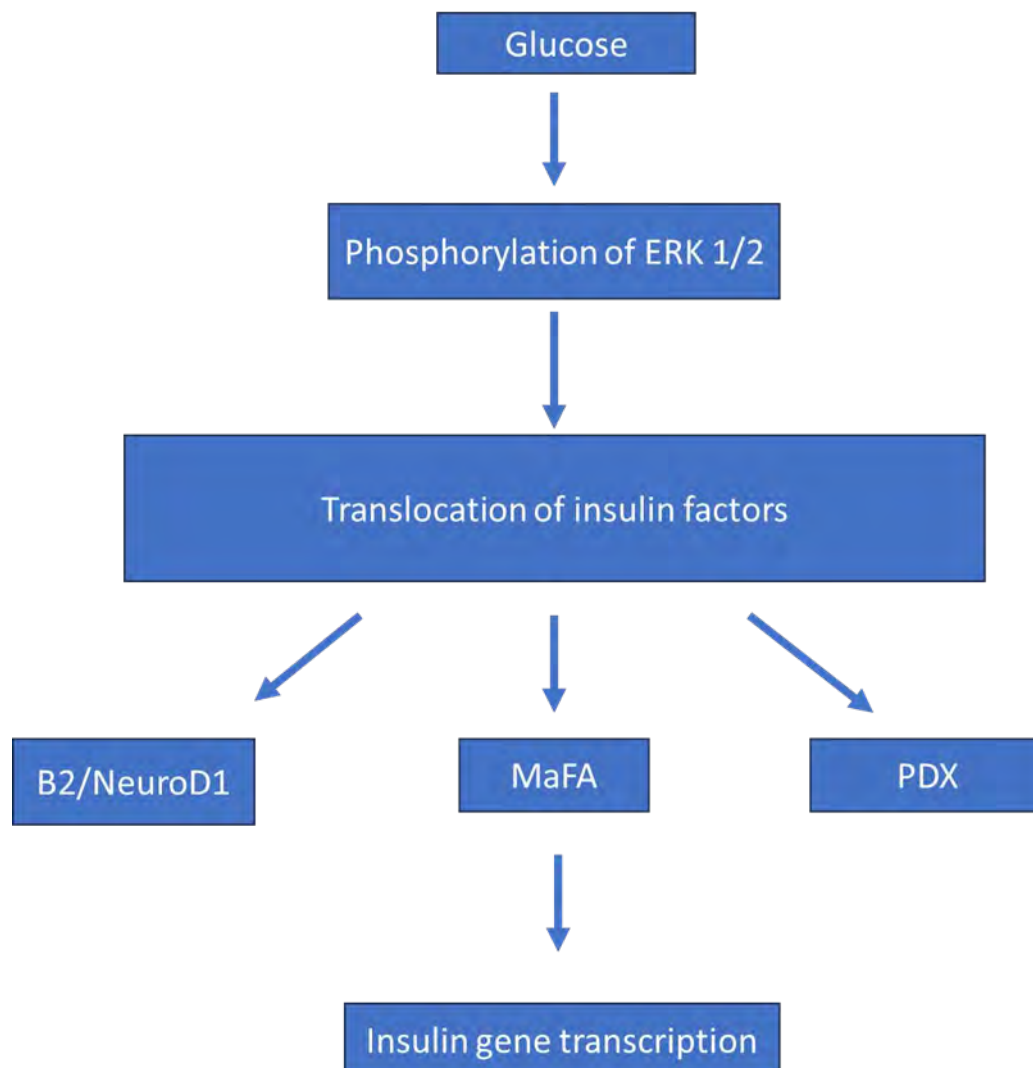


Figure 1. 4: A diagrammatic illustration of glucose stimulated insulin gene transcription through phosphorylation of ERK1/2.

1.5.1.3 PDGFR

PI3K activation is associated with the translocation of GLUT4. PDGFR promotes PI3K activity in 3T3-L1 adipocytes, however PDGFR is not capable of stimulating GLUT4 translocation and subsequently glucose uptake (192). Despite the similarities between insulin and PDGFR pathways, PDGFR activation does not stimulate GLUT4 translocation, due to insufficient PDGF receptors as demonstrated by studies in 3T3-L1 adipocytes (193). A study in which PDGFR were overexpressed in CHO-GLUT4myc cells, PDGFR was shown to activate PI3K induced GLUT4 translocation and glucose uptake (194). Whiteman et al. (2003) simultaneous administration of PDGR and insulin did not increase the translocation of GLUT4 above the levels of each hormone alone. This suggests that PDGR and insulin both maximally stimulate transport by receptor tyrosine kinase activated PI3K-dependent signalling pathway in fat cells (193).

1.5.2 Axitinib

Axitinib is a small molecule tyrosine kinase inhibitor derived from indazole. It is an oral multitargeted potent tyrosine kinase receptor inhibitor. Axitinib selectively inhibits VEGFR-1, -2, and -3 at sub-nanomolar concentrations *in vitro* (195). Axitinib was approved by the FDA for the treatment of advanced renal cell carcinoma in 2012 (167). It is a second-generation type IIA receptor inhibitor of VEGFR with sub-nanomolar potency (169). The VEGFR is a component of the VEGF/VEGFR signalling pathways which plays a role in vascular development during embryogenesis and angiogenesis during adulthood. These pathways are also implicated in the growth, metastasis, and survival of tumours (196). Axitinib binds to the intracellular tyrosine kinase domain of VEGFR and inhibits autophosphorylation of VEGFRs thus blocking downstream signalling pathways, cell survival, and tube formation at a picomolar concentration (197). Axitinib is more selective against VEGFR tyrosine kinase than the tyrosine kinase inhibitors such as soraferinib, sunitinib, and pazopib, as they are multitargeting (198). Tyrrell and Pwint observed a decrease in blood glucose concentrations and HbA1c levels in a patient receiving axitinib, 5 mg twice daily (199). *In vitro* studies by Hudson et al. (2014) showed that axitinib increases [C-14] deoxy glucose uptake and translocation of GLUT-1 transporters from cytosolic pools to the cell surface membrane in pancreatic adenocarcinoma (PDAC) cells (200).

1.5.3 Sunitinib

Sunitinib is a potent orally bioavailable ATP-competitive type I1/2B inhibitor. It is a multitargeted receptor tyrosine kinase inhibitor of PDGFR- α and β , FMS-like tyrosine kinase 3, VEGFR-1, -2 and 3, colony-stimulating factor 1 receptor and glial cell-line derived neurotrophic factor receptor (201). These receptors are associated with tumour growth and angiogenesis. Sunitinib is used in the treatment of renal cell carcinoma and gastrointestinal stromal tumours (202). The development of sunitinib was brought by the development of resistance to imatinib in gastrointestinal stromal tumour potent caused by mutation of KIT (203). Sunitinib forms polar bonds with Asp86, Glu81, and Lue83 in the CDK2 hinge region (169).

In a study that was done at Seoul National University Bundang Hospital in the Republic of Korea, 10 out of 48 diabetic patients with metastatic renal cell carcinoma, were reported to have a significant decrease in blood glucose levels after treatment with sunitinib dose for 4 weeks. However, their blood glucose concentrations reverted to normal 2 weeks after treatment

cessation. Sunitinib doses were not disclosed by the authors, however since no special note was highlighted, we propose that the doses were within the global recommendations of 50 mg daily for an adult patient. One of the 10 patients was able to discontinue their diabetic medication, and 3 patients had dose reductions of their oral antidiabetics during the 4-week sunitinib treatment. Conversely, 38 non-diabetic patients showed no significant changes in blood glucose levels during the 4 weeks of sunitinib treatment (204). In a retrospective review, all 19 type 2 diabetes mellitus patients showed a reduction in blood glucose concentrations after 4 weeks of sunitinib treatment. Some studies reported discontinuation of diabetic medications as a result of sunitinib treatments, while others reported patients having normalized blood glucose (205).

The exact blood glucose lowering mechanism of tyrosine kinase inhibitors is unknown. It has been suggested that tyrosine kinase inhibitors' effect on c-KIT tyrosine kinase is in part responsible for the alteration in blood glucose levels. C-KIT is associated with β -cell survival (206). Sunitinib is believed to have a direct effect on β -cells resulting in glucose-induced insulin secretion which leads to lowered blood glucose levels in patients. Sunitinib stimulates insulin secretion in a concentration and dose-dependent manner (207). Billemont et al. (2008) suggested that sunitinib affects insulin resistance by interfering with insulin-like growth factor-1 pathway. Louvet et al. (2008) using data from mouse models suggested that inhibition of PDGF downstream-mediated inflammatory response improves pancreatic β -cell survival and insulin resistance (208).

1.5.4 Nilotinib

Nilotinib is an orally administered type IIA BCR-ABL inhibitor, second-generation tyrosine kinase inhibitor developed by Novartis for treatment against imatinib-resistant mutants of BCR-ABL. Nilotinib binds to the inactive DFG-out conformation of the BCR-ABL (209). Nilotinib is a high-affinity aminopyrimidine-based ATP competitive inhibitor that reduces cell proliferation and viability through selective inhibition of BCR-ABL autophosphorylation (210). To maximize efficacy, nilotinib was designed to bind more tightly to the BCR-ABL than imatinib, by incorporating a N-methylpiperamine moiety while keeping the amide pharmacophore to preserve the H-bond interaction to Glu286 and Asp381. Hence nilotinib is able to maintain binding to the inactive conformation of the ABL kinase domain (211). Out of the 33 imatinib-resistant BCR-ABL mutants, nilotinib is active against 32 except for the T315I mutant. Nilotinib has more lipophilic interactions than imatinib; it has 4 hydrogen-bond interactions with the ABL kinase domain, which results in nilotinib expressing a higher affinity for binding than imatinib (212).

Nilotinib and imatinib are similar in structure but reports have shown them to have opposite effects on glucose metabolism (161). Iurlo et al. (2015) observed that patients on nilotinib had significantly higher levels of insulin, C-peptide, fasting plasma glucose, insulin resistance, and LDL cholesterol compared to patients who were being treated with imatinib and dasatinib (213). It was however concluded that nilotinib treatment does not induce diabetes mellitus or impair fasting glucose to a significantly higher extent than imatinib and dasatinib, but nilotinib causes a worse glucometabolic profile. Hence it has been advised that patients on nilotinib require dose monitoring of glucose and lipid metabolism. A large phase III trial comparing the efficacy of nilotinib and imatinib showed that 53% of the patients being treated with nilotinib 400 mg b.i.d were observed to have hyperglycaemia, 50% of patients treated with 300 mg b.i.d and only 30% of patients treated with imatinib 400 mg/day were observed to have hyperglycaemia (214). Nine out of thirteen chronic myeloid leukaemia patients being treated

with nilotinib showed increased fasting glucose levels as compared to baseline pre-nilotinib therapy (161). In a study by Philipp le Courte an increase in grade ≥ 3 hyperglycaemia was observed during nilotinib therapy (214). Fasting 1-h and 2-h plasma glucose concentrations increased after 3 months of nilotinib treatment (215). Based on fasting glucose (8.1 and 7.3 mmol/L), 2 patients fulfilled the criteria of diabetes mellitus while on nilotinib therapy and 2 patients had impaired glucose tolerance. It is worth noting that most studies have concluded that the hyperglycaemia caused by nilotinib is clinically insignificant as it is usually mild, transient, and manageable (216). However, understanding the mechanism by which hyperglycaemia is brought about is crucial, especially for patients with both chronic myeloid leukaemia and diabetes mellitus.

The mechanism by which nilotinib impairs glucose metabolism is not clearly known. In one case of pre-existing severe type 2 diabetes mellitus, nilotinib was shown to directly affect glucose metabolism via impairment of endogenous insulin secretion. When nilotinib was discontinued the impairment of endogenous insulin secretion was reversed (217). Insulin resistance which was calculated by the mathematical model HOMA2-IR increased significantly during nilotinib therapy (215). Insulin sensitivity index (HOMA2-%S) significantly decreased as well as ISI_{0,120}. HOMA2-%S and HOMA2IR represent hepatic insulin resistance while ISI_{0,120} represents peripheral insulin resistance. The cABL domain is associated with the insulin signalling pathway. It has been hypothesized that by blocking the c-ABL, tyrosine kinase therapy affects the insulin receptor pathway (218). Plasma concentration of adiponectin has been observed to have decreased during nilotinib therapy. Adiponectin exerts a potent insulin-sensitizing effect (215,219).

1.5.5 Gefitinib

Gefitinib is a 4-anilinoquinazoline derivative, a potent and selective ATP competitive inhibitor of EGFR and HER-2 kinases (220). Gefitinib binds to the ATP binding pockets of EGFR's active form, hence it is classified as a Type-I kinase inhibitor. Gefitinib forms H-bonds with Met793 in the hinge region (153). Mutations in the extracellular segment of the EGFR such as deletion have been shown to result in constitutive growth factor-independent EGFR activation leading to the overexpression of the EGFR, which causes the progression of numerous malignancies (221). A common mutation of the EGFR is the L858R which results from exon 19 substitution of leucine with arginine, and this accounts for 40% of mutations in non-small cell lung cancer. The other mutation is exon 19 deletion. These mutations cluster around the active site cleft of the tyrosine kinase domain of the EGFR (222). Studies of direct binding measurements have shown that gefitinib binds 20-fold more tightly to the L858R mutant form of the EGFR as compared to the wild-type receptors. Cells with mutant EGFR tend to be more sensitive to tyrosine kinase inhibitors than cells that express the wild-type kinase (223). Gefitinib is considered to be relatively selective, however, it demonstrates moderate activity in other receptor tyrosine kinase resulting in common adverse events such as diarrhoea, rash, nausea, and dry skin (224). In 2004 gefitinib became the first epidermal growth factor receptor tyrosine kinase inhibitor for molecular targeted chemotherapy of unresectable non-small cell lung cancer in Japan. The antineoplastic effect of gefitinib comes from inhibiting the activation of ErbB-1 (EGFR) (225). Clinically gefitinib is used for the treatment of chemo-resistant non-small cell lung cancer (220).

Gefitinib has been shown to have a dramatic effect on a limited number of patients. Studies have suggested that it is ineffective in 70%–80% of patients with non-small cell lung cancer

(226). EGFR inhibitors, erlotinib, and gefitinib seem to have secondary pharmacological mechanisms of action, as they appear to be inhibitors of Tumour Necrosis Factor Alpha (TNF- α). They may prevent progression of type 1 diabetes mellitus as they appear to be effective in the treatment of related TNF- α mediated inflammatory diseases. This is achieved through the inhibition of the TNF- α (227). Inflammation associated with TNF- α has been linked to the development of insulin resistance (228). Gefitinib may be of value in the treatment of type I diabetes mellitus by inhibition of TNF- α , leading to a reduction in inflammation, decreased insulin resistance, and slowed progression of β -cell destruction (227).

In a rat model study, gefitinib improved insulin sensitivity in rats with type 2 diabetes. This is hypothesised to occur through the increase in the PI3K/AKT pathway of insulin signalling (229). Gefitinib has an inhibitory action on receptor-interacting protein kinase 2 (RIPK2). RIPK2 propagates immune responses that are associated with dysglycemia during obesity and insulin resistance (230,231). In a mice model study, gefitinib improved glucose control independently of RIPK2. In the absence of RIPK2, gefitinib improved insulin sensitivity and lowered insulin secretion (232). One study showed that there was a significant decrease in fluorodeoxyglucose (FDG) uptake in gefitinib-sensitive cell lines (233). FDG uptake is a marker for tissue uptake of glucose (234). In the same study immunoblots showed the translocation of GLUT3 from the plasma to the cytosol (233).

1.5.6 Dasatinib

Dasatinib is a type 1 inhibitor of ABL tyrosine kinase and a type I1/2A inhibitor of LYN. It is an orally available ABL inhibitor which is different from imatinib such that it binds to both the active and inactive conformations of the ABL kinase domain (235). In 2006, the FDA granted accelerated approval for the treatment of chronic myeloid leukaemia and Ph⁺ acute lymphoblastic leukaemia (Ph⁺ ALL) with dasatinib in patients that had demonstrated resistance or intolerance to prior therapy with imatinib (236,237). Resistance in a large proportion of patients on imatinib therapy results from point mutations of the BCR-ABL gene and activation of alternative signalling pathways the likes of SRC family kinase (236). Dasatinib is a potent ATP competitive inhibitor, it has less stringent binding requirements as compared to imatinib; hence it shows inhibition activity against many imatinib-resistant BCR-ABL mutations. Dasatinib is active against 14 out of 15 imatinib-resistant BCR-ABL mutants (238). The one mutant not inhibited by dasatinib is the T315I mutant, which is a single mutation deep within the ATP-binding pockets of the ABL tyrosine kinase (210). Dasatinib is a multikinase inhibitor that shows activity against PDGFR- β , KIT, and SRC family kinase, and because of this range of inhibition, dasatinib has many side effects including haemorrhage, teratogenicity, fluid retention, and myelosuppression (239).

In a retrospective study on blood glucose concentrations in eight diabetic and non-diabetic patients treated with dasatinib, a mean decrease in blood glucose of 53 mg/dL was observed. Some patients on dasatinib treatment were able to discontinue their antidiabetic medication (240). A case report of a 57-year-old diabetic chronic myeloid leukaemia patient had their insulin requirements declined upon the introduction of dasatinib. Within 2 weeks of dasatinib treatment, insulin treatment was discontinued. The patient's fasting C-peptide immunoreactivity increased two-fold, suggesting that dasatinib allowed for the recovery of insulin production (241). In another case report, a 65-year-old woman with type 2 diabetes mellitus, had her glycaemic index improved to <6.0% upon dasatinib treatment initiation. The quantitative insulin sensitivity check index rapidly improved followed by an improvement in

insulin sensitivity. This case report demonstrated that dasatinib might have negative as well as positive effects on glucose metabolism (242). In a retrospective study, patients that were treated with dasatinib had a mean decrease in HbA1c of 0.80 absolute point and these patients required 8.2 less total daily insulin units on average. This led to the conclusion that dasatinib demonstrates antidiabetic effects and may be considered for use as a novel diabetic therapy. The mechanism by which dasatinib instigates these antidiabetic effects has not been stated and remains unknown (243).

Evidence suggests that tyrosine kinase play a role in regulating insulin secretion. Various mechanisms underpin the involvement of SRC kinase, including their potential regulation through myristoylation, dynamic palmitoylation, and acylation. All of which contribute to the inhibition of insulin secretion (244). SRC kinase pathways are integral in the extracellular calcium-dependent secretion of pancreatic enzymes and insulin (245). Dasatinib's inhibition of SRC kinase pathways leads to the reduction of fasting glucose observed in the studies mentioned.

SRC-family kinase are proto-oncogenes that play a role in cell processes, including cell differentiation, cell growth, cell morphology, mortality, and survival (246). Insulin secretion and neurotransmitter release both occur via exocytosis (247,248). Exocytosis is a ubiquitously occurring mechanism responsible for cell-to-cell communication (249). It has been suggested that SRC-family tyrosine kinase inhibit neurotransmitter release, and it is believed that insulin secretion and neurotransmitter release have similar regulatory mechanisms. Cheng et al. (2007) observed that SU-6656 and PP2, structurally different SRC-family tyrosine kinase inhibitors, enhanced Ca^{2+} -induced insulin secretion in INS-1 cells and rat pancreatic islets in both a time and dose-dependent manner (245). This study illustrates that one or more SRC-family tyrosine kinase exert a tonic inhibitory role on Ca^{2+} -dependent insulin secretion. The role of SRC-family tyrosine kinase in Ca^{2+} -induced insulin secretion is illustrated in Figure 3.

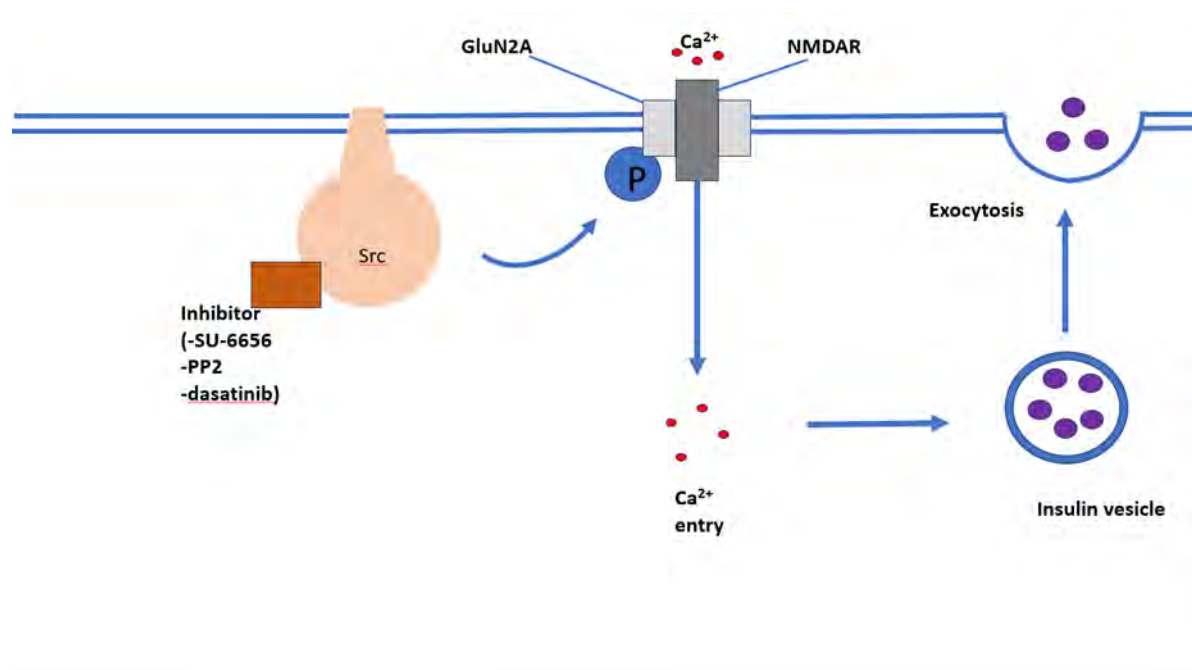


Figure 1. 5: An illustration of the mechanism through which the SRC-family kinase pathway enhances insulin secretion. The binding of inhibitors such as SU-6656, PP2, and dasatinib enhances Ca^{2+} induced insulin secretion.

SRC-family tyrosine kinase are non-receptor tyrosine kinase. They are expressed in high levels on secondary vesicles and plasma membranes of cells that are specialised for exocytosis. Cells that fall under this category include neuronal and endocrine cells (250). Evidence from studies suggests that protein tyrosine kinase are associated with the regulation of insulin secretion (251). Small molecule tyrosine kinase inhibitors have been observed to induce increases in insulin secretion in INS-1 cells (252). On the other hand, some studies have reported decreased insulin secretion due to small molecule tyrosine kinase inhibitors in adult rat islets (253). These varying observations highlight that different protein tyrosine kinases have different or opposing outcomes on insulin secretion. The exact function of protein tyrosine kinase in insulin secretion is unknown.

1.5.7 Erlotinib

Erlotinib is an orally available 4-anilinoquinazoline derivative and a reversible tyrosine kinase inhibitor of the EGFR, it is classified as a Type I and Type I1/2B kinase inhibitor (167,254). When erlotinib binds to the ATP binding pocket of active EGFR it functions as a Type I inhibitor but when it binds to EGFR with its inactive DFG-in and C-helix out conformation erlotinib functions as a Type I1/2B inhibitor (167,255). Through inhibiting the EGFR erlotinib blocks tumour cell division, initiates programmed cell death and produces cell cycle arrest in EGFR-overexpressing human tumour cells (256). Erlotinib has been approved for the treatment of refractory non-small cell lung cancer and advanced pancreatic cancer in combination with gemcitabine chemotherapy (257). The most prevalent adverse events reported in non-small cell lung cancer and pancreatic cancer patients undergoing erlotinib therapy are diarrhoea and rash (258,259).

Studies have shown that several small molecule tyrosine kinase inhibitors including erlotinib, imatinib, and sunitinib have antihyperglycemic activity (160). Onder and Heybeli et al. (2024) In a case report showcased a 73-year-old man with type 2 diabetes mellitus who was diagnosed with non-small cell lung cancer, experienced hypoglycaemia episodes, and his blood glucose level progressively declined after approximately one month of erlotinib 150mg/day therapy (260). Costa and Huberman et al. (2006) reported a 72-year-old woman with type 2 diabetes mellitus also diagnosed with non-small cell lung cancer experienced episodes of hypoglycaemia and a decline in fasting blood glucose to <90 mg/dl within the first 4 weeks of erlotinib 150mg/day (259). The precise mechanism through which erlotinib improves glycaemic control is unknown (261).

EGFR modulates pancreatic β -cell proliferation and insulin secretion (262). Studies have reported that inhibition of EGFR promotes insulin generation due to greater islet autophagy and reduced islet macrophage infiltration. Erlotinib treatment leads to decreased proinflammatory cytokines production, decreased renal oxidative stress, and preservation of pancreatic function (263). There is no consensus on how inhibition of EGFR by erlotinib improves hyperglycaemia. Mice without EGFR were reported to have physiologically abnormal islets, demonstrating that a functional EGFR pathway is required for normal pancreatic development (264). Inhibition of EGFR should result in hyperglycaemia episodes. Our study sought to investigate this claim by looking closely at erlotinib's effects on the insulin signalling pathway. In the next section below, we will briefly explore the cell lines used in this study and highlight their relevance

Table 1.1: Summary of small kinase inhibitors investigated for their antidiabetic activity

Small Kinase inhibitor	Mechanism of action	Pharmacological uses	Glycaemic effect
Imatinib	Binds to ATP binding site of the ABL domain of the BCR-ABL(170) Binds to ATP binding site of the ABL domain of the BCR-ABL(170)	Philadelphia chromosome positive (Ph⁺) chronic myeloid leukaemia and gastrointestinal stromal tumours (166) Philadelphia chromosome positive (Ph⁺) chronic myeloid leukaemia and gastrointestinal stromal tumours (166)	Hypoglycaemic (179) Hypoglycaemic (179)
Axitinib	Selectively inhibits VEGFR-1, -2, and -3, and also inhibits PDGFRβ and KIT. Its benzamide group forms H-bonds with αC-Glu885 and the DFG-Asp1046 on the juxta-membrane of the VEGFR (265) Selectively inhibits VEGFR-1, -2, and -3, and also inhibits PDGFRβ and KIT. Its benzamide group forms H-bonds with αC-Glu885 and the DFG-Asp1046 on the juxta-membrane of the VEGFR (265)	Treats advanced renal cell carcinoma (153) Treats advanced renal cell carcinoma (153)	Hypoglycaemic (199) Hypoglycaemic (199)
Nilotinib	Selectively inhibits autophosphorylation of BCR-ABL (210) Selectively inhibits autophosphorylation of BCR-ABL (210)	Treatment against imatinib-resistant mutants of BCR-ABL (209) Treatment against imatinib-resistant mutants of BCR-ABL (209)	Hyperglycaemic (215) Hyperglycaemic (215)
Gefitinib	Inhibit the activation of ErbB-1 (EGFR) (225) Inhibit the activation of ErbB-1 (EGFR) (225)	Locally advanced or metastatic non-small cell lung cancer (266) Locally advanced or metastatic non-small cell lung cancer (266)	Hypoglycaemic (227) Hypoglycaemic (227)

Sunitinib	Inhibits the VEGFR, platelet-derived growth factor receptor, and c-Kit (267) Inhibits the VEGFR, platelet-derived growth factor receptor, and c-Kit (267)	Renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumour (GIST) (268) Renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumour (GIST) (268)	Hypoglycaemic (204) Hypoglycaemic (204)
Dasatinib	Inhibits BCR-ABL, SRC family, and PDGFR β (269) Inhibits BCR-ABL, SRC family, and PDGFR β (269)	Philadelphia chromosome-positive acute lymphoblastic leukaemia or chronic myeloid leukaemia (236) Philadelphia chromosome-positive acute lymphoblastic leukaemia or chronic myeloid leukaemia (236)	Hypoglycaemic (165) and Hyperglycaemic (270) Hypoglycaemic (165) and Hyperglycaemic (270)
Erlotinib	Inhibits the epidermal growth factor receptor (271) Inhibits the epidermal growth factor receptor (271)	Non-small cell lung cancer and pancreatic cancer (272) Non-small cell lung cancer and pancreatic cancer (272)	Hypoglycaemic (273) Hypoglycaemic (273)

1.6 Skeletal muscle cell line (C2C12)

The C2C12 cell line is an immortalized mouse myoblast cell line. C2C12 cells are commonly utilized in pharmaceutical science and biomedical research (274,275). Skeletal muscle play a crucial role in post-prandial glucose regulation as they facilitate insulin-stimulated glucose uptake through their expression and translocation of GLUT4 (276,277). Skeletal muscle account for approximately 80% of glucose uptake via insulin activity (278). The desensitization of skeletal muscle to insulin through various mechanism leads to elevated blood glucose levels, which is a key characteristic of type 2 diabetes mellitus (279). The C2C12 cell model contains biochemical features, cross striations, and characteristic muscle proteins, which makes them ideal for skeletal muscle metabolism examination (276). The C2C12 myoblast cell line has been widely used *in vitro* to examine the progression of metabolic diseases. Due to C2C12 well-documented evidence that has been conducted on their facilitation of glucose metabolism, oxidative stress, insulin resistance, insulin signalling pathway, and glucose transport at the molecular and cellular levels hence they were utilized in this study (280,281).

1.7 Liver cell line (HepG2)

Hepatocytes are responsible for the initial handling of carbohydrates from intestinal absorption (282,283). During the post-prandial period, the liver is a major site of glucose utilization accounting for 1/3 to 60 % of the glucose (284). The liver is central in regulating glycaemia in humans through its role in regulating *de novo* glucose synthesis (gluconeogenesis) and glycogen breakdown (glycogenolysis) (283,285,286). The liver utilizes excess glucose to produce fatty acids (287). Elevated blood glucose concentrations during post-prandial periods stimulate pancreatic β -cells to secrete insulin, which acts on the liver (279,283,288). Glucose predominantly enters hepatocytes through GLUT2 (facilitative glucose transporter). The

transport of glucose occurs through passive transport (energy-independent) (289). HepG2 is a human derived hepatoma cell line that is widely used as an *in vitro* alternative to human hepatocytes (290). HepG2 cells are frequently used in hepatotoxicity studies, glycogen storage, lipid accumulation, insulin resistance, and drug metabolism (291). Characteristics of cell lines include easy handling, unlimited life span, and stable phenotype. HepG2 cells has a epithelial-like morphology which enables them to perform various hepatic functions (286,291,292). HepG2 cells are capable of performing limited metabolic functions compared to primary hepatocytes (287,292). HepG2 cells were utilized in this study to examine glucose utilization alterations and their effect on insulin signalling pathway. Due to their shared physiological function in lipid and glucose metabolism with primary normal human hepatocytes, HepG2 cells were selected in this study.

1.8 JUSTIFICATION OF THE STUDY

Studies indicate that small molecule tyrosine kinase inhibitors alter glucose metabolism in patients diagnosed with a range of cancers as alluded to in the preceding sections. Small molecule tyrosine kinase inhibitors have varied impacts on blood glucose concentrations, with some lowering blood glucose concentrations while others elevate blood glucose concentrations. The exact mechanism through which these compounds alter glycaemic control remains less understood. This study seeks to gain a deeper insight of small molecule tyrosine kinase inhibitors on glucose handling *in vitro*. Given that the incidence of both diabetes and cancer is projected to rise, there is need to effectively utilise the available pharmacotherapies. This may pave the way for the repurposing of these drugs in diabetes. Findings from this study is envisaged to provide a motive for further research to explore worthiness of repurposing small molecule tyrosine kinase inhibitors for the management of diabetes mellitus. By examining the impact of small molecule tyrosine kinase inhibitors on glucose uptake, metabolism, and signalling pathways in target tissues such as muscle and liver, this research will contribute to a better understanding of the complex interplay between kinases and glucose homeostasis. Ultimately, the findings may have significant clinical implications for the development of novel therapies targeting kinases to improve glucose regulation and prevent diabetes-related complications. Understanding the role that small molecule tyrosine kinase inhibitors play in glucose metabolism will be of great aid to healthcare professionals when it comes to which small molecule tyrosine kinase inhibitor to administer to a patient. Patients at risk of hypoglycaemic complications are being considered.

1.9 Aim of the study

This study aimed to investigate the effects of small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) on glucose metabolism and the insulin signalling pathway in skeletal muscle and liver cell lines *in-vitro*.

1.10 Objectives of the study

1 *In vitro* screening of small molecule tyrosine kinase inhibitors on cell viability in:

- a) Skeletal muscle cell line (C2C12)
- b) Hepatic cell line (HepG2)

2 *In vitro* screening of small molecule tyrosine kinase inhibitors on glucose utilisation in:

- a) Skeletal muscle cell line (C2C12)
- b) Hepatic cell line (HepG2)

3 *In vitro* screening of small molecule tyrosine kinase inhibitors to determine the expression of GLUT4 and translocation in:

- a) Skeletal muscle cell line (C2C12)

4 *In vitro* screening of small molecule tyrosine kinase inhibitors to determine the expression of Akt in:

- a) Skeletal muscle cell line (C2C12)
- b) Hepatic cell line (HepG2)

6 *In vitro* screening of small molecule tyrosine kinase inhibitors to determine the expression of IL6 in:

- a) Skeletal muscle cell line (C2C12)
- b) Hepatic cell line (HepG2)

7 Investigating the effect of dasatinib on glucose metabolism in wortmannin and palmitic acid treated cells:

- a) Skeletal muscle cell line (C2C12)
- b) Hepatic cell line (HepG2)

8 To investigate enzyme inhibitory activity of small molecule tyrosine kinase inhibitors against α - amylase and α -glucosidase.

CHAPTER 2: METHODOLOGY

2.1 Chemicals and drugs

The following chemicals and drugs were purchased from Sigma-Aldrich, St Louis, USA: Acarbose, porcine pancreatic α -amylase, dinitro-salicylic acid, starch, sodium potassium tartrate, α -glucosidase from *saccharomyces cerevisiae*, p-nitrophenyl- α -glucopyranoside, sodium phosphate monobasic, sodium phosphate dibasic, para-nitrophenyl phosphate, Sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), Potassium chloride (KCl), Dulbecco's modified eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), Fetal bovine serum (FBS), and penicillin-streptomycin, Bovine Serum albumin, Triton X100, Rabbit polyclonal anti-phospho-Akt antibody, Anti-Akt2 antibody produced in rabbit, GLUT4 antibody, IL-6 antibody, Tween 20, Paraformaldehyde, Tetramethylbenzidine(TMB), Horseradish peroxidase (HRP), Hydrogen peroxide (H₂O₂), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Axitinib, Dasatinib, Erlotinib, Imatinib.

2.2 Equipment

The following equipment and materials were used in the study: Hotplate stirrer (H3760-HSE)(230V, 60Hz; (Lasec Laboratories) Benchmark Scientific Inc. PO BOX 709, Edison, NJ 08818, USA), Incubator (FSOH4)(220/240V 50Hz, 0.5kW; Labcon (Pty) Ltd 15 Aschenberg street Chamdor Krugersdorp Transvaal), BV1000 vortex mixer (230VAC 50Hz, 0.75amps;

Benchmark Scientific Inc. PO BOX 709, Edison, NJ 08818, USA; made in Taiwan), SpectraMax M3 multi-mode microplate reader (100 – 240V~3.5A 50 – 60Hz; manufactured in China, designed in California, USA), UV-VIS Spectrophotometer (UVmini-1240)(AC 220 – 240~ 50/60Hz 160VA; SHIMADZU CORPORATION, made in China), Water bath, Microplate shaker Intl plug (888610242, 150-1000rpm, AC100- 240V.50/60Hz,71VA, L9CF61024008, Fisher brand, assembled in China), BioTek, EPOCH|2 microplate reader (24V ,120W), Inverted microscope (BS-2092,200-240V 50/60HZ,0.8A Lasec), BIO-RAD TC20 Automated cell counter ,(508BR09575, 30W, made in Singapore), Centrifuge (166145,1-14, made in Germany, SIGMA), Bayer Accu-Check Performa glucometer and contour plus test strips.

2.3 Biological assays

2.3.1 Preparation of compounds for *in vitro* testing

A stock solution (5000µg/mL) for imatinib, dasatinib, axitinib, and erlotinib was prepared by weighing 0.05g of each compound and dissolved in DMSO (100 µL). The final stock solution was made up to 1 ml by the required medium (DMEM or EMEM). The $C_1V_1 = C_2V_2$ formula was utilized to further dilute the compounds to desired concentrations (5, 10, 20, 40, and 80 µg/mL). Before each assay, the desired concentrations were freshly prepared using distilled water, media, or an appropriate buffer. In each assay, a reference drug was employed.

2.3.2 Cell culture

The assays were conducted using C2C12 skeletal muscle cells and HepG2 liver cells. The C2C12 and HepG2 cells were cultured in a humidified incubator with 5% CO₂ at 37°C, in tissue culture flasks (T25 and T75). The cells were maintained in Dulbecco's modified eagle's medium (DMEM) and minimum essential medium eagle (MEM) for C2C12 and HepG2, respectively. The medium was supplemented with 10% FBS (10%) and penicillin-streptomycin (1%). After the cells had grown to confluence (approximately 80%), they were trypsinized and then transferred to new flasks until ready for seeding into either 24 or 96 plates.

2.3.3 Skeletal muscle differentiation

After seeding, the skeletal muscle cells (C2C12) were further differentiated to form myotubes. The C2C12 myoblasts differentiation into myotubes was attained by switching the medium from DMEM supplemented with FBS (10%) to DMEM supplemented with FBS (0.2%) and penicillin-streptomycin (1%) for four days. The media was changed daily, and the extent of differentiation was observed and confirmed under an inverted microscope.

2.3.4 Cell viability

Principle

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is conducted to ascertain the cell viability of the cultured cells. The MTT assay is a calorimetric assay that relies on the intracellular reduction of the yellow MTT to form purple formazan granules as a measure of cellular activity. MTT reagent is a mono-tetrazolium salt that contains a positively charged quaternary tetrazole ring core with four nitrogen atoms surrounded by three aromatic rings including one thiazolyl ring and two phenyl moieties. The reduction of MTT occurs at the core tetrazole ring. MTT is reduced in the mitochondria of metabolically active cells by succinate dehydrogenase to produce a water-insoluble purple formazan. Due to

MTT's lipophilic structure, and positive charge, it is able to permeate through the cell membrane and the mitochondrial inner membrane of viable cells. When cells die, they lose their ability to convert MTT into formazan, hence the formation of colour presumably serves as an indicator of the viable cells. The total formazan produced is directly proportional to the number of viable cells, thus colour intensity is directly proportional to the number of viable cells, and a spectrophotometer is used to measure absorbance at 570 nm (293,294). The total formazan produced is directly proportional to the number of viable cells, thus colour intensity is directly proportional to the number of viable cells, and a spectrophotometer is used to measure absorbance at 570 nm (293,294).

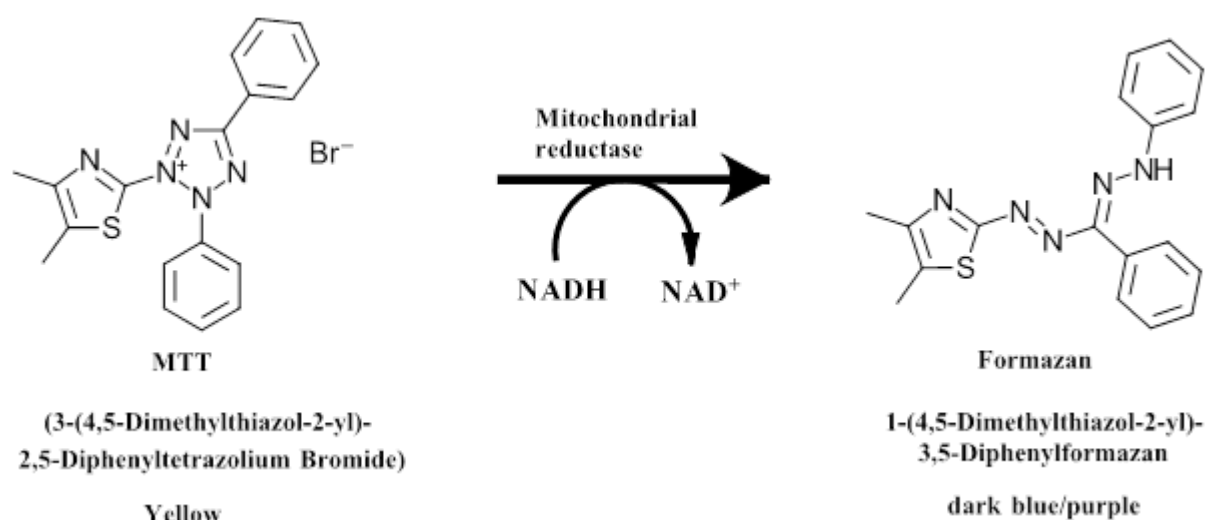


Figure 2. 1: Enzymatic reduction of MTT to formazan

Protocol

To assess cell viability, the 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was conducted in the presence of selected drugs (axitinib, dasatinib, erlotinib, and imatinib). The C2C12 and HepG2 cells were seeded into 96-well clear bottom tissue culture plates at densities of 4.65×10^4 cells/mL and 4.3×10^4 cells/mL, respectively, and cultured until they reached approximately 80% confluence. The cells preparations were separately exposed to different concentrations (5, 10, 20, 40, and 80 $\mu\text{g/mL}$) of the small molecule kinase inhibitors. After 24 hours, MTT solution (200 μL , 5 mg/ml) dissolved in PBS (10%), and FBS free media (90%) was added to each well. The plate was then incubated for three hours in the dark at 37°C. Thereafter, the media was removed and replaced with DMSO (200 μL) followed by incubation for five minutes. The absorbance was measured at 570 nm using a UV-VIS Spectrophotometer. The assay was performed in triplicates. The cell viability was calculated as follows:

$$\text{Equation 1: Percentage viability (\%)} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

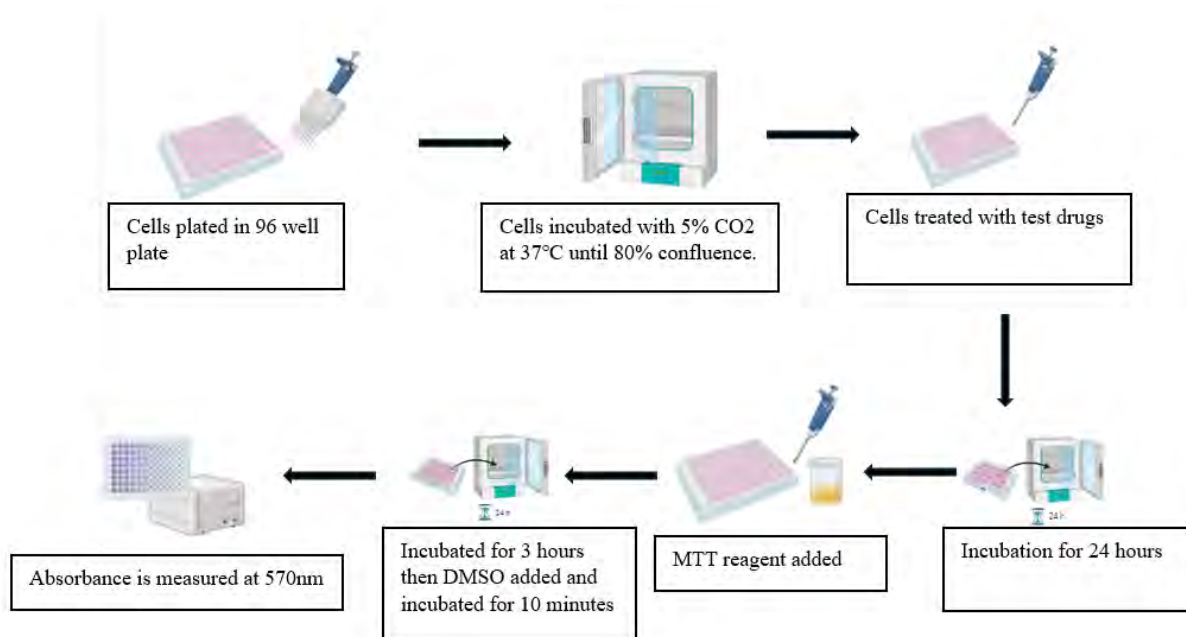


Figure 2. 2: Workflow of MTT assay protocol

2.3.5 Accu-Check Performa glucometer appraisal and validation for glucose uptake measurement

Bayer Accu-Check Performa glucometers, and contour plus test stripes are commercially utilized to measure blood glucose concentrations. To validate the use of these glucometers and glucose stripes for measuring cell culture medium glucose, the following experimental procedure was performed. The glucose concentration of the cell culture medium was initially calculated based on its composition. The values were then converted to mmol/L, the unit used by the glucometer for glucose measurements. Thereafter, 4 mL of cell culture medium was aspirated from the stock solution, then serially diluted to make the following theoretical concentrations; 24.97, 12.48, 6.24, 3.12, and 1.56 mmol/L. Then the glucometer was used to record the actual glucose concentration of the dilutions as depicted in Figure 2.3 below. Each sample was tested in triplicate.

Figure 2.3 shows the glucometer validation for media glucose concentration measurement Experiment; theoretical glucose concentration vs glucometer glucose reading. The R^2 value observed from the plot was 0.9972. The R^2 is very close to 1, indicating that the regression line approximates the actual data very well. This means that the glucometer readings are statistically similar to the theoretical glucose concentrations, hence the glucometer was used to estimate cellular glucose uptake. These findings validate the use of glucometers and test strips for relative comparisons of glucose concentrations.

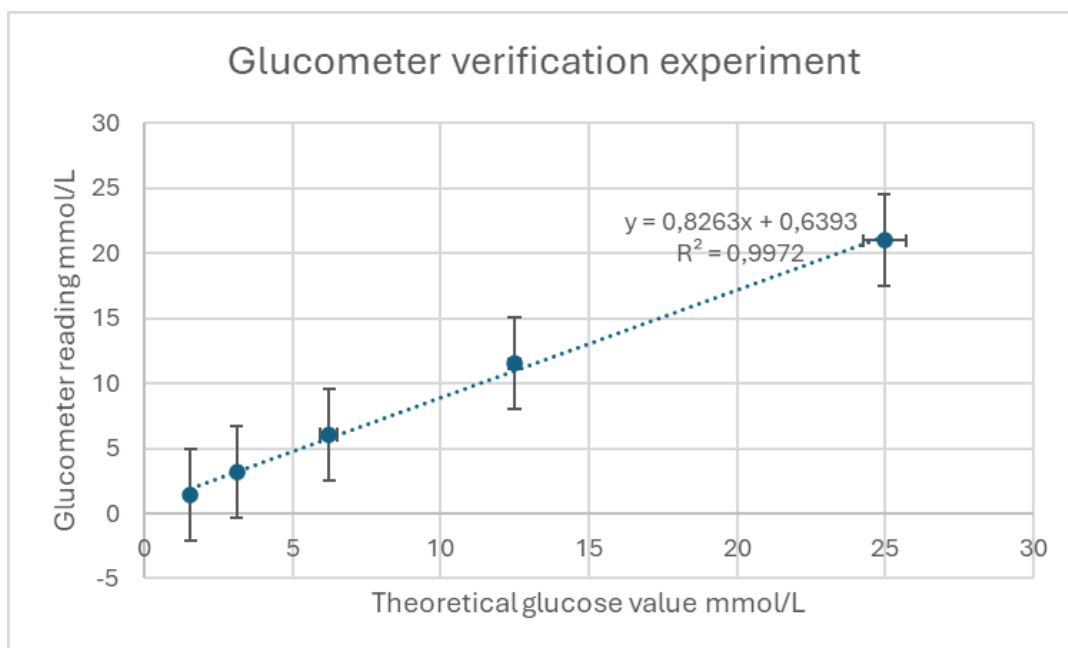


Figure 2. 3: Theoretical Glucose Values vs. Glucometer Readings. Theoretical glucose concentrations were set at 24.97, 12.48, 6.24, 3.12, and 1.56 mmol/L. Glucometer readings were taken in triplicate for each theoretical value, allowing for a comparison between the expected and observed glucose levels.

2.3.6 Estimation of cellular glucose uptake

The glucose uptake was estimated by measuring the concentration of glucose present in the medium after 24 hours according to the method described by Cruz-Bermúdez et al. (295). To achieve this, HepG2 (3.7×10^4) cells/mL and C2C12 (3.96×10^4) cells/mL were plated in 24-well plates and left to adhere and reach confluence. The C2C12 were further differentiated as described above. Thereafter, the culture medium was aspirated from the wells and washed with Phosphate-Buffered Saline (PBS) (200 μ L) three times. Media Glucose concentration was measured before incubation to serve as Time 0 (T0). Thereafter, the C2C12 and HepG2 were treated with the selected drugs at (5, 10, 20, 40, and 80 μ g/mL) in medium supplemented with 10% FBS and 1% penicillin-streptomycin and preincubated at 37°C for 24 hours (T24). The cells receiving only medium (200 μ L) served as a control while insulin (0.05 units) was utilized as a standard drug. After the 24-hour incubation period, the media glucose concentration was measured using an Accu-Check Performa glucometer (295). The assay was performed in triplicates and repeated twice.

The estimation of glucose uptake was determined using the following formula:

$$\text{Equation 2: Glucose uptake (\%)} = \frac{\text{Medium glucose (T0)} - \text{Medium glucose (T24)}}{\text{Medium (T0)}} \times 100$$

2.3.7 Glucose uptake studies in Wortmannin and treated cells

Dasatinib due to its pronounced effect on promoting glucose uptake was further investigated in its ability to affect glucose uptake in the presence of a PI3K inhibitor, wortmannin. The goal was to assess whether dasatinib utilizes the insulin signalling pathway or an alternative pathway. The C2C12 and HepG2 cells plated in 24-well plates where glucose concentration measurements were performed following co-administration of dasatinib (5, 10, 20, 40, and 80 $\mu\text{g/mL}$) and wortmannin 25 nM. Cells exposed exclusively to 25 nM wortmannin and those maintained in medium alone served as control groups.

2.3.8 Glucose uptake studies in palmitic acid-induced insulin resistance

The effects of dasatinib on insulin-resistant C2C12 and HepG2 cell lines regarding glucose uptake were investigated as outlined below. C2C12 and HepG2 cell lines plated in 24-well plates were exposed to palmitic acid (250 μM) for 24 hours to induce insulin resistance (296,297). Cells not exposed to palmitic acid served as a control groups. Following palmitic acid-induced insulin resistance the cells were treated with dasatinib at 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. Cells not treated with dasatinib served as the second control group.

2.3.9 In-Cell Enzyme-Linked Immunosorbent Assay

Principle

Enzyme-linked immunosorbent assay (ELISA) is a highly versatile and sensitive technique that can be used for qualitative or quantitative determinations of practically any antigen or antibody. ELISA is a heterogeneous enzyme immunoassay technique used in clinical analysis. The introduction of substrates that produce coloured, fluorescent, or luminescent reaction products allows for the detection of reactants at extremely low concentrations. Cell-based ELISA is also known as In-cell ELISA. In-cell ELISA is an immunocytochemistry method used to detect and quantify target proteins or post-translational modifications of the target protein, in cultured cells. Highly specific and well-characterized primary antibodies derived from mice or rabbits are utilized. The primary antibody immobilizes the antigen of interest. The immobilized antigen is then recognized and bound by a specific secondary antibody tagged by a reporter enzyme. Absorbance is measured at 450 nm using spectrophotometry.

Protocol

As illustrated by Figure 2.4 HepG2 (4.25×10^4) cells/mL and C2C12 (4.09×10^4) cells/mL were seeded into 96-well plates. They were preincubated at 37°C with 5% CO₂ until they reached approximately 80% confluence. Assays were conducted in C2C12 cells to investigate the expression of Akt and GLUT4, as well as the translocation of GLUT4. Two separate 96-well plates were used for each assay condition. In the first 96-well plate, cells were exposed to insulin for 1 hour, followed by treatment with small molecule tyrosine kinase inhibitors. In the second 96-well plate, cells were treated with small molecule tyrosine kinase inhibitors without prior insulin exposure. This dual setup allowed for a comparative analysis of the effects of insulin and tyrosine kinase inhibitors on the relevant cellular pathways. The cells were treated with different small molecule tyrosine kinase inhibitors concentrations (5, 10, 20, 40, and 80 $\mu\text{g/mL}$) in EMEM (HepG2) and DMEM (C2C12) and preincubated at 37°C for 24 hours. After that, the medium was aspirated, and paraformaldehyde (100 μL of 8%) was added to each well for cell fixation. The plate was then incubated for 15 minutes at room temperature on a microplate shaker set at 300 rpm. Afterwards, the paraformaldehyde was aspirated, and each

well was washed four times with PBS (200 μ L). Following the washes, 200 μ L of 2X permeabilization buffer containing Triton X-100 (250 μ L in 24.75 ml 1X PBS) was added to each well. Thereafter, the plate was incubated for 30 minutes at room temperature while shaking at 300 rpm. After incubation, the permeabilization buffer was aspirated, and 200 μ L of blocking buffer (1X BSA dissolved in PBS) was added to each well. The plate was then incubated at room temperature for 2 hours while shaking. Afterwards, the blocking buffer was aspirated, and 100 μ L of primary antibody (GLUT 4, Akt, or IL-6) 2 μ L in 10 mL incubation buffer was added to each well separately. The plate was then incubated overnight at 4°C. After incubation, the primary antibody was aspirated, and the plate was washed three times with 250 μ L of wash buffer (625 μ L 400X Tween-20 in 250 mL of 1X PBS). Next, 100 μ L of a secondary antibody (anti-rabbit IgG) specific to the primary antibody was added to each well. The plate was then incubated at room temperature for 2 hours while shaking. After incubation, the plate was washed four times using wash buffer (250 μ L) after which 100 μ L of horseradish peroxidase substrate (HRP) (0.05 mg/mL) was transferred into each well and incubated for 30 minutes whilst shaking at 300rpm. The main colorimetric substrate for HRP is TMB (3, 3', 5, 5'-tetramethylbenzidine). TMB produces a deep blue colour during the enzymatic degradation of hydrogen peroxide by HRP. To stop the reaction, HCL (100 μ L, 0.1M) was added into each well and absorbance was read at 450nm using a spectrophotometer (UVmini-1240). The assays were performed in triplicates and repeated twice. To determine GLUT 4 translocation, the same procedure was followed but without the cell permeabilization step in order to only capture GLUT 4 on the cell surface. The expression and translocation of GLUT 4 were examined exclusively in C2C12 cells due to their ability to express GLUT 4. The relative percentage expression or translocation was calculated as follows:

Equation 3: Relative percentage expression = $\frac{\text{Average absorbance of treated wells}}{\text{Average absorbance of control}} \times 100$

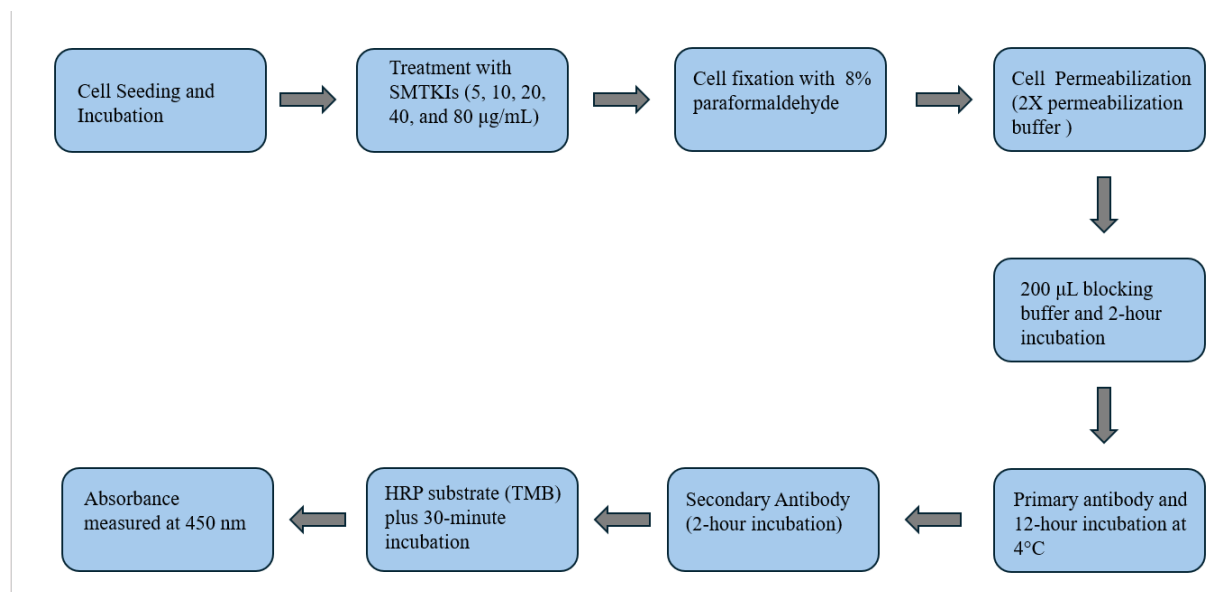


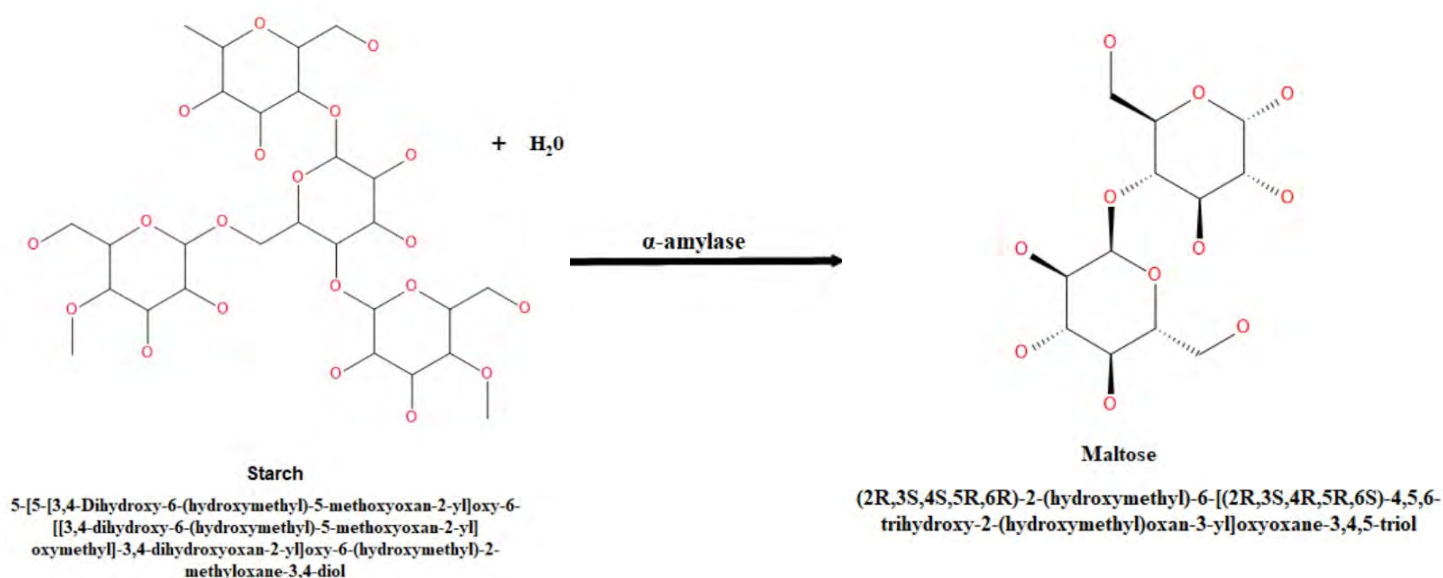
Figure 2. 4: ELISA workflow

2.4 Enzymatic assays

2.4.1 Alpha-amylase inhibition assay

Principle

This assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method. The α -amylase activity is measured using a colorimetric method with DNSA reagent. In this approach, alpha-amylase converts the substrate (starch) into maltose as illustrated by Figure 2.5, and the amount of maltose released is measured by the reduction of DNSA. The maltose produced then reduces the pale-yellow alkaline DNSA reagent to an orange-red colour as shown in Figure 2.5. The intensity of this colour change is directly proportional to the maltose concentration in the sample, and the absorbance is measured using a spectrophotometer at a wavelength of 540 nm. The presence of an alpha-amylase inhibitor, such as acarbose, reduces maltose production, consequently decreasing the reduction of DNSA and the absorbance detected.



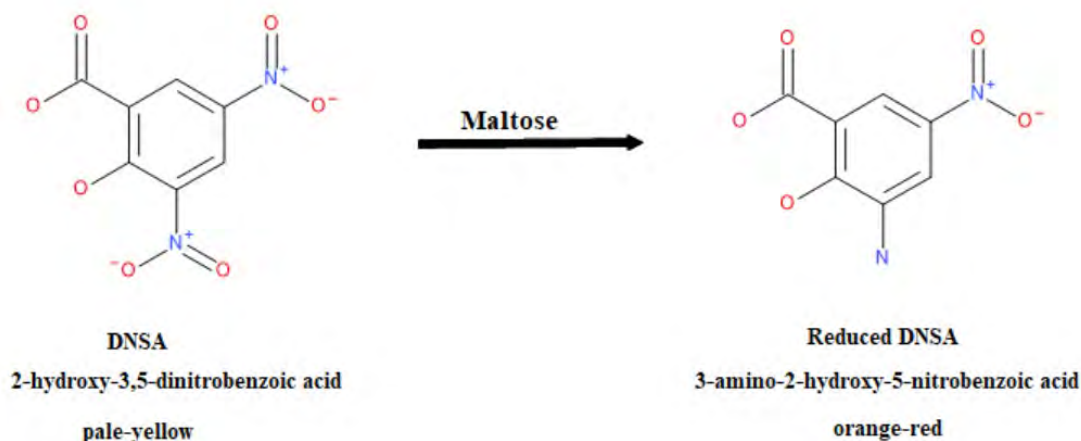


Figure 2. 5: Reduction of DNSA reagent by maltose (from starch) to an orange-red colour
Protocol

The alpha-amylase inhibition assay was performed using the DNSA method with slight modifications (4). In a microplate, 20 μ L of Phosphate buffer saline (0.01M, pH 7.4), 20 μ L of starch (5% wv), 20 μ L of amylase (4 units/ml), and 20 μ L of small molecule kinase inhibitors at different concentrations (5, 10, 20, 40 and 80 μ g/mL) were incubated for 30 minutes at 37 degrees. Thereafter, 20 μ L of DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) was added. And. The plate was sealed and secured in a Ziploc plastic, after which it was carefully mounted into a water bath to boil for 15 minutes. Thereafter, the mixture was cooled to ambient temperature and was diluted with distilled water (170 μ L) after which the absorbance was measured at 540 nm using a spectramax M3-multimode microplate reader (Molecular devices, China). Acarbose was used as the standard drug at concentrations of 5, 10, 20, 40, and 80 μ g/mL. The absolute control with 100% enzyme activity was prepared by replacing small molecule kinase inhibitors with buffer (20 μ L). The assay was performed in triplicates. The α -amylase inhibitory activity was expressed as percent inhibition and was calculated using the equation given below:

Equation 4:

$$\text{Inhibition of } \alpha\text{-amylase activity (\%)} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.4.2 Alpha-glucosidase inhibition assay

Principle

Alpha-glucosidase is an exoenzyme hydrolysing terminal glycosidic bonds and releasing α -glucose from the nonreducing end of the substrate chain. Alpha-glucosidase acts on the substrate *p*-nitrophenyl- α -D-glucopyranoside (pNPG) to form *p*-nitrophenol and α -D-glucopyranoside as illustrated by Figure 2.6. The absorbance of *p*-nitrophenol liberated is measured with a spectrophotometer at 405 nm. Potent inhibitors such as acarbose inhibit α -

glucosidase activity on the substrate, resulting in reduced absorbance, which is a measure of the *p*-nitrophenol formed.

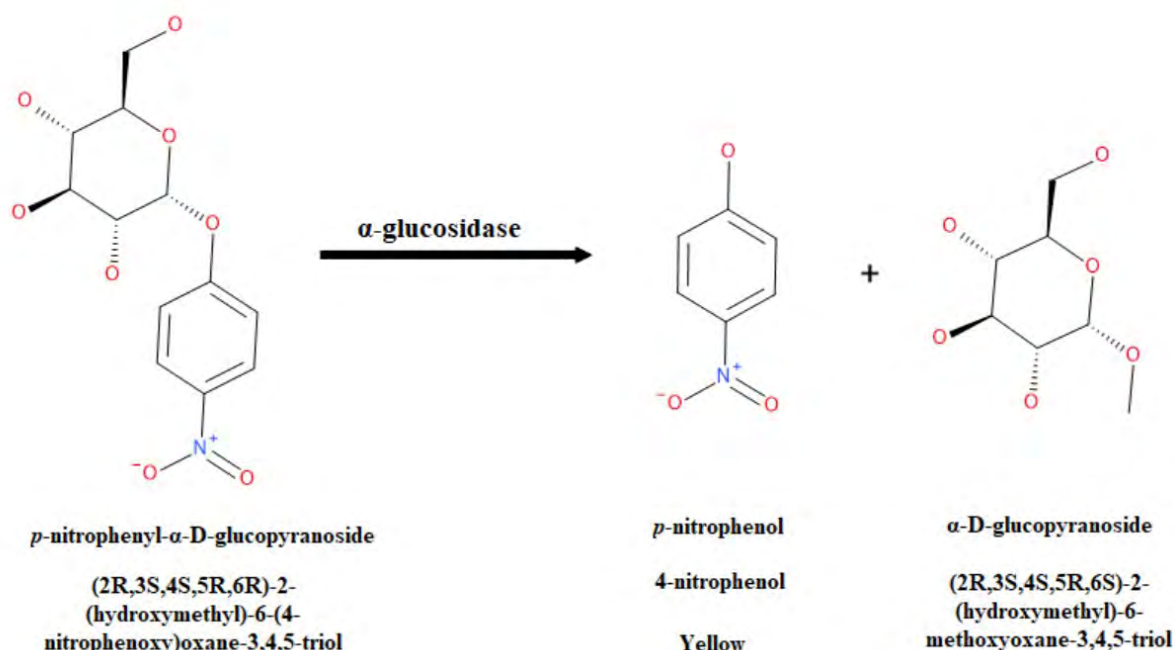


Figure 2. 6: Formation of *p*-nitrophenol and α -D-glucopyranoside from *p*-nitrophenyl- α -D-glucopyranoside through the activity of α -glucosidase

Protocol

The effect of small molecule tyrosine kinase inhibitors on alpha-glucosidase activity was assessed according to the standard method with minor modification (5). In a 96-well plate, a reaction mixture containing 100 μ L of phosphate buffer (0.1 M, pH = 6.8), 40 μ L of alpha-glucosidase (0.1 U/mL), and 80 μ L small molecule tyrosine kinase inhibitors at different concentrations (5, 10, 20, 40, and 80 μ g/mL) were preincubated at 37°C for 10 min. The reaction was initiated by the addition of 20 μ L of pNPG (2 mM), the mixture was then incubated at 37°C for 10 min. The reaction was stopped by adding 100 μ L of Na₂CO₃ (0.1 M). The degree of inhibition of α -glucosidase was assessed by measuring the absorbance caused by the production of the yellow-coloured *p*-nitrophenol at 405 nm utilizing a spectrophotometer. Acarbose was used as the standard drug at concentrations of 15, 30, 60, 120, and 240 μ g/. Wells with phosphate buffer instead of small molecule tyrosine kinase inhibitors served as absolute controls with 100% enzyme activity. The assay was performed in triplicates, and the inhibitory activity of alpha-glucosidase was determined as percentage inhibition using the following formula:

Equation 5:

$$\text{Inhibition of } \alpha\text{-glucosidase (\%)} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.5 Data and Statistical Analysis:

Each experiment was performed in triplicate and repeated twice to ensure reproducibility, and the data was presented as mean $\pm$ SD on separate column graphs for clarity and represented by an error bar ($n = 3$). Statistical analysis was performed using Excel Office 365. One-way analysis of variance (ANOVA) trailed by Tukey post-test was used to test for statistical significance difference at p -values ≤ 0.05 . The results of these analyses are presented on the corresponding graphs, with asterisks (*) denoting statistically significant differences ($p \leq 0.05$) between the control and treatment groups.

CHAPTER 3: RESULTS

3.1 Cell viability assay

A cytotoxicity study was performed in C2C12 and HepG2 cell lines on the small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at concentrations of 5, 10, 20, 40, and 80 $\mu\text{g/mL}$ as presented in Figures 3.1 and 3.2, respectively. Cell viability represents the proportion of live, healthy cells within a sample. The control group was assigned a baseline of 100% cell viability. Consequently, a viability percentage below 80% is deemed cytotoxic. In C2C12 cells exposed to small molecule tyrosine kinase inhibitors, it was observed that exposure to axitinib resulted in a percentage viability greater than 80, and a percentage viability greater than 100 was observed at 10 $\mu\text{g/mL}$. Statistical significance was noted at 5 and 80 $\mu\text{g/mL}$. Exposure to dasatinib resulted in a percentage viability greater than 80 across all concentrations, with the exception of 80 $\mu\text{g/mL}$, which showed a statistically significant lower percentage viability compared to the control. Furthermore, at 10 $\mu\text{g/mL}$, the percentage viability was greater than 100, demonstrating statistical significance compared to the control. Exposure to imatinib and erlotinib demonstrated percentage viability greater than 100 between 5 and 40 $\mu\text{g/mL}$. It was noted that at a concentration of 80 $\mu\text{g/mL}$, imatinib demonstrated a statistically significant reduction in percentage cell viability to 55. The percentage cell viability observed in HepG2 cells treated with dasatinib and erlotinib across all concentrations was closely comparable to the control percentage viability of 100. HepG2 cells exposed to axitinib were observed to show percentage viability similar to the control between 5 and 40 $\mu\text{g/mL}$, while at 80 $\mu\text{g/mL}$ a decrease in percentage viability was noted. HepG2 cells exposed to imatinib demonstrated percentage viability that was greater than 80, however a slight decrease in percentage viability that was statistically significant in comparison to the control was noted at 20, 40, and 80 $\mu\text{g/mL}$.

C2C12 cell line

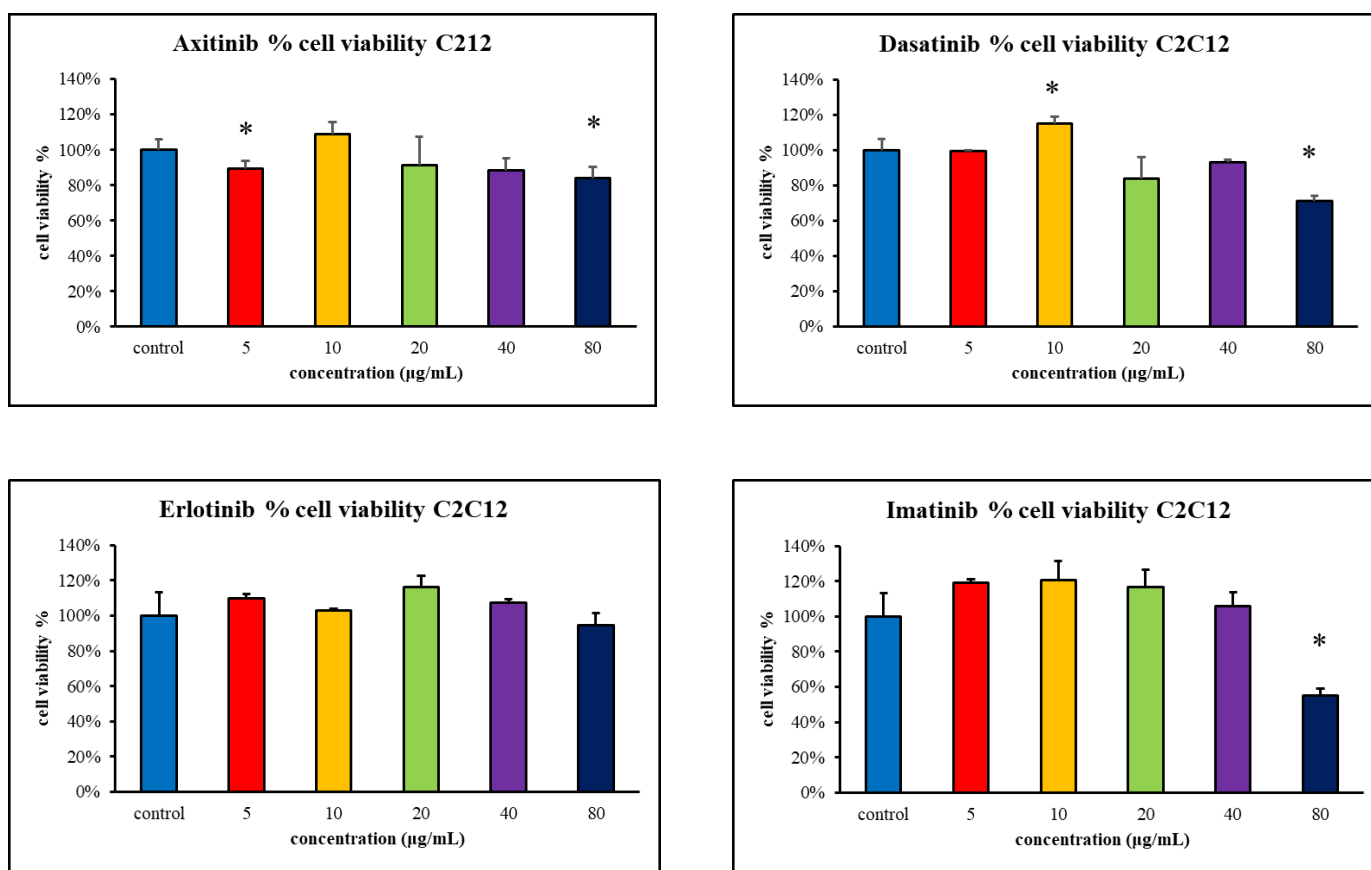


Figure 3. 1: Percentage viability in C2C12 cells treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 µg/mL. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

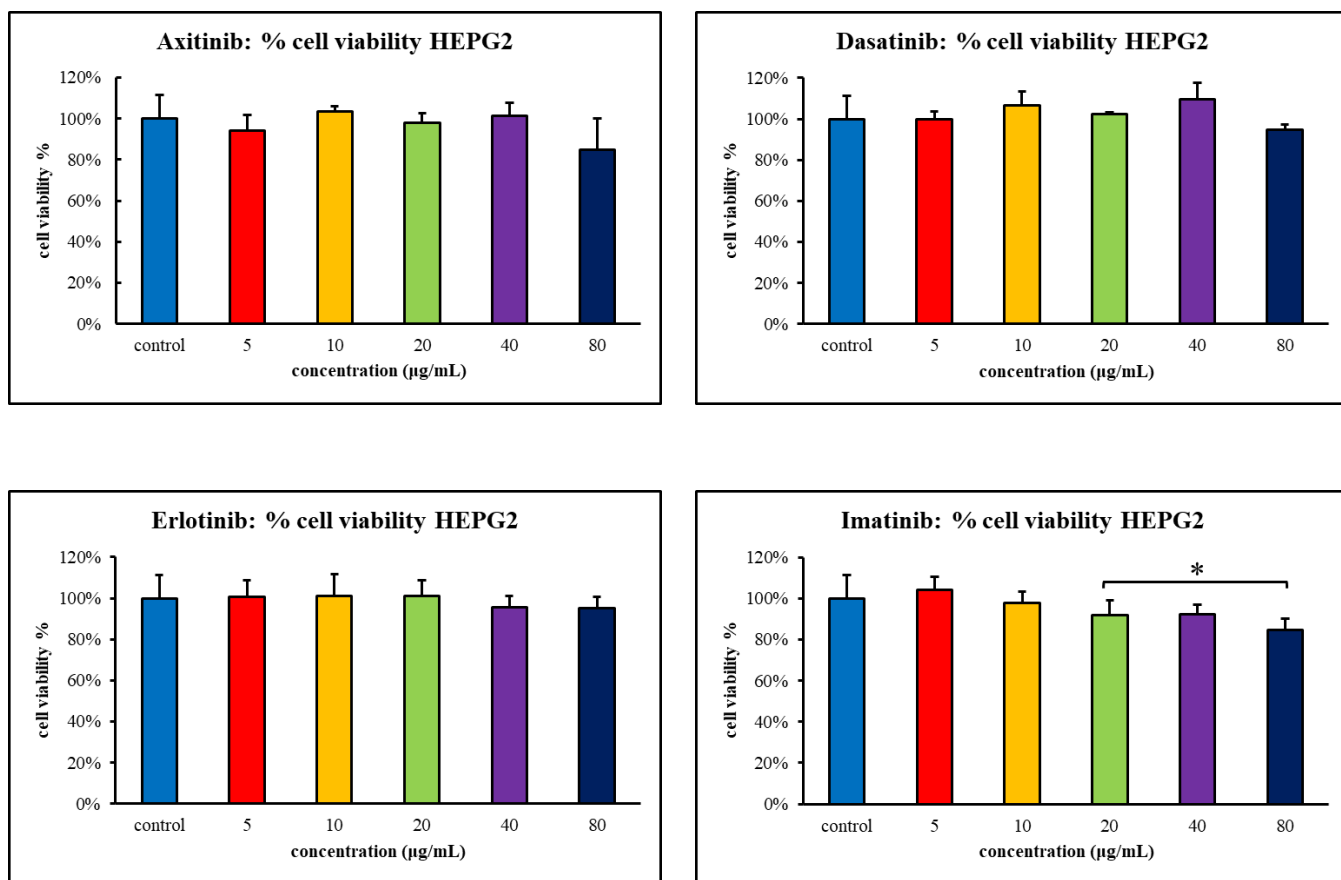


Figure 3. 2: Percentage viability in HepG2 cells treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 µg/mL. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.2 Estimated glucose uptake

An estimated glucose uptake study was performed in C2C12 and HepG2 cell lines. The percentage glucose uptake was estimated after treating C2C12 and HepG2 cell lines with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at concentrations of 5, 10, 20, 40, and 80 µg/mL. Percentage glucose uptake is presented in Figures 3.3 and 3.4, respectively. In the C2C12 cell line exposure to axitinib was observed to have a lower percentage glucose uptake at 5 and 10 µg/mL which was statistically significant in comparison to the control. An increase in percentage glucose uptake was observed from 5 to 10 µg/mL. C2C12 cells treated with axitinib at concentrations of 20, 40, and 80 µg/mL percentage glucose uptake was observed to be similar to the control. Exposure to dasatinib led to a slight increase in percentage glucose uptake that was statically significant at 5 and 10 µg/mL in comparison, to the control, with the rest of the concentrations demonstrating percentage glucose uptake similar to the control. Exposure to erlotinib at 5, 10, and 20 µg/mL was observed to demonstrate percentage glucose uptake similar to the control, at 40 µg/mL a slight increase in percentage glucose uptake that was statistically significance was observed.

C2C12 cells treated with imatinib demonstrated a statistically significant increase in glucose uptake compared to the control group. Insulin was observed to elicit a higher percentage glucose uptake that was statistically significant in comparison to the control.

HepG2 cells treated with axitinib were observed to have a lower glucose uptake percentage than the control, with an increase in the concentration of axitinib an increase in glucose uptake was observed. Treatment with dasatinib resulted in lower glucose uptake compared to the control and HepG2 cells treated with insulin. HepG2 cells treated with erlotinib and imatinib demonstrated elevated glucose uptake compared to the control and cells treated with insulin. This increase in glucose uptake appeared to be independent of concentration.

C2C12 cell line

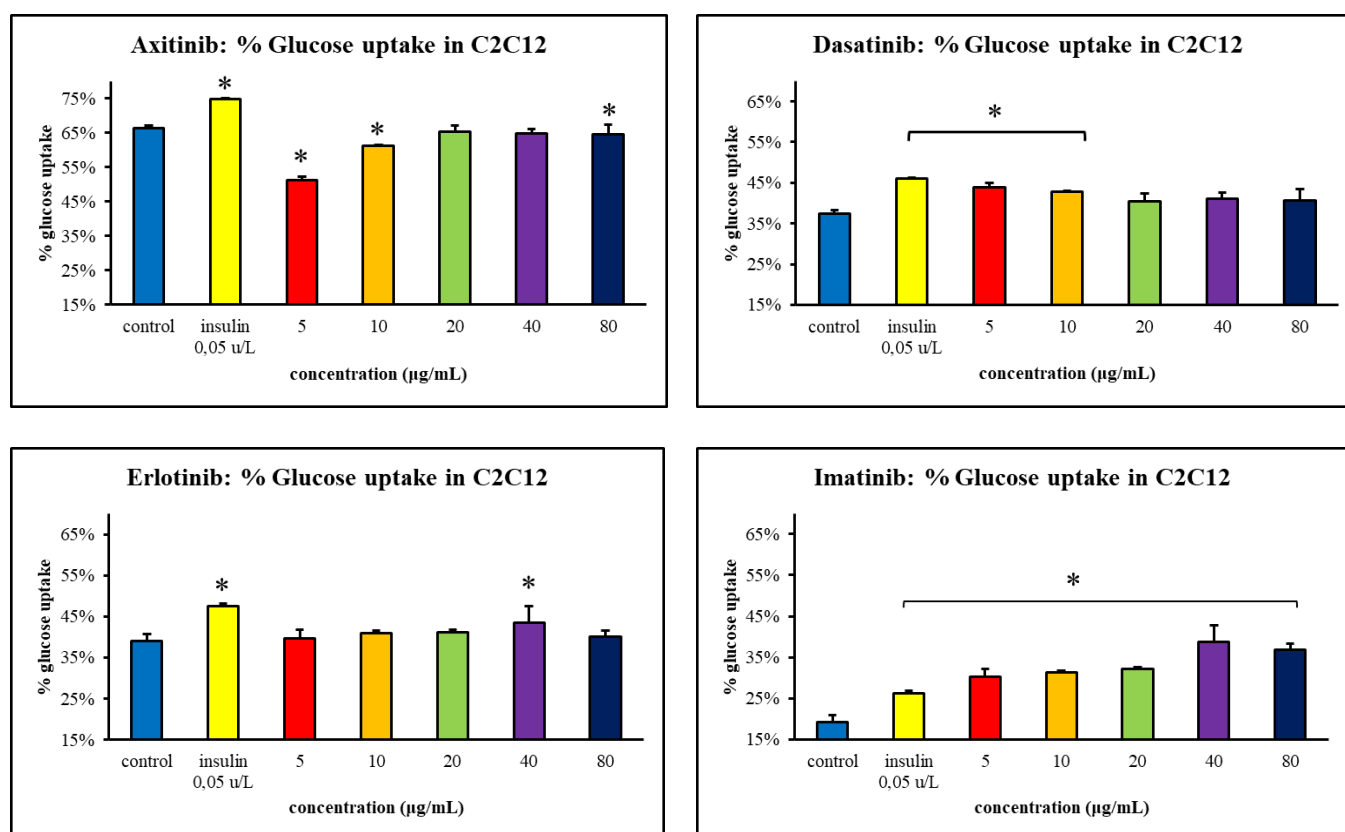


Figure 3. 3: Percentage glucose uptake in C2C12 cells treated with small molecule tyrosine kinase inhibitors at different concentrations (axitinib, dasatinib, erlotinib, and imatinib) 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

HepG2 cell line

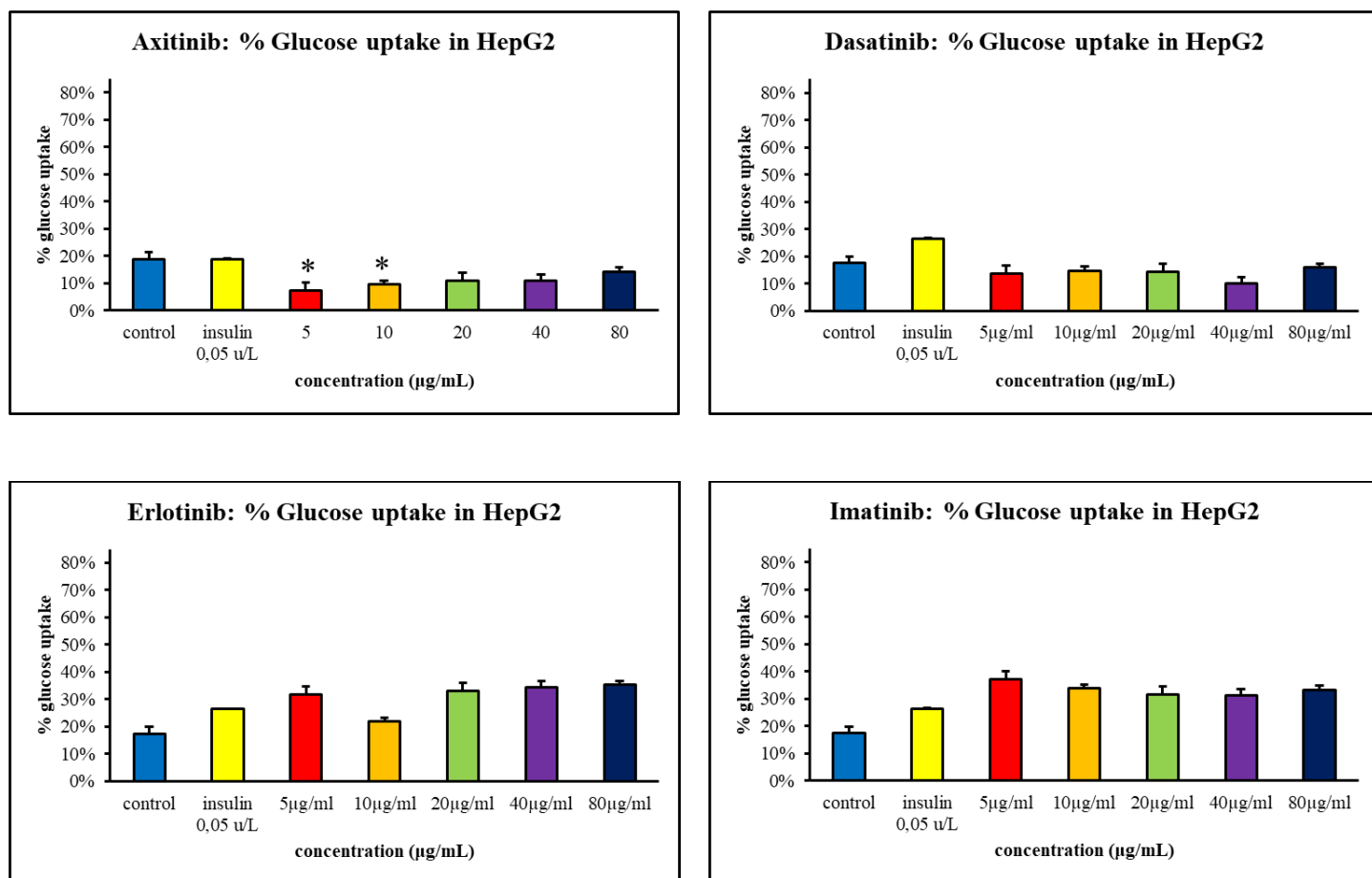


Figure 3. 4: Percentage glucose uptake in HepG2 cells treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

3.3 In-Cell ELISA

3.3.1 GLUT4 translocation

The percentage translocation of the GLUT4 protein in C2C12 cells treated with small-molecule tyrosine kinase inhibitors is depicted in figure 3.5. C2C12 cells not exposed to small molecule tyrosine kinase inhibitors or insulin served as the control. It was observed that GLUT4 translocation in C2C12 cells gradually increased in the presence of axitinib, dasatinib, and erlotinib, which was statistically significant compared to the control as their concentrations increased. In C2C12 cells exposed to imatinib, a statistically significant decrease in GLUT4 translocation was observed. At the initial concentration (5 µg/mL) imatinib demonstrated GLUT4 translocation that was statistically higher in comparison to the control. As the concentration increased it was noted that at 80 µg/mL, the GLUT4 translocation in C2C12 cells exposed to imatinib was equivalent to the control.

C2C12 cell line

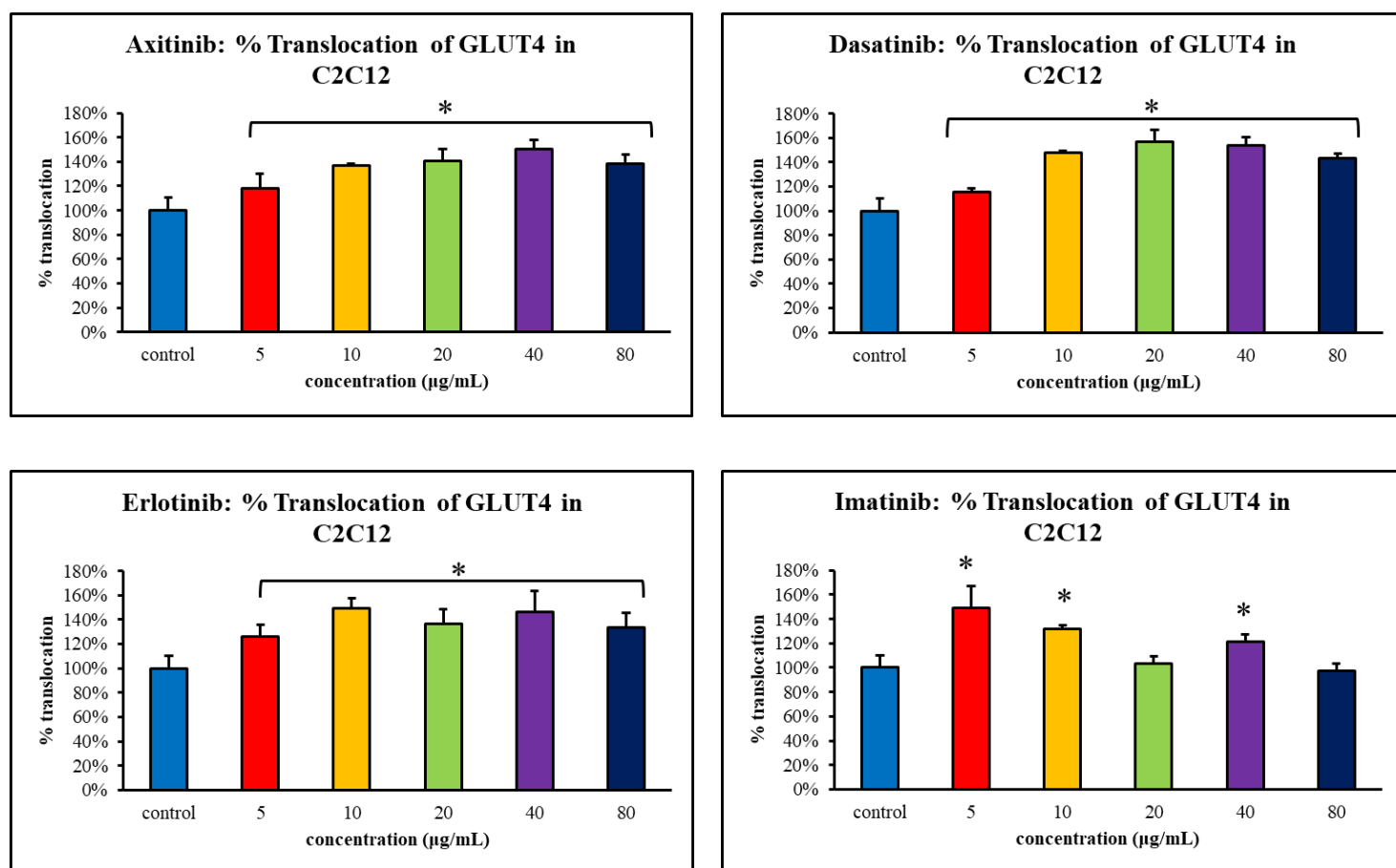


Figure 3. 5: Percentage translocation of GLUT4 in C2C12 cells treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean ± SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

3.3.2 GLUT4 translocation (in C2C12 cells stimulated by insulin for 1 hour before treatment with small molecule tyrosine kinase inhibitors)

The percentage translocation of the GLUT4 protein in C2C12 cells that were exposed to insulin and then treated with small molecule tyrosine kinase inhibitors is illustrated in Figure 3.6. The control consisted of insulin-treated cells in the absence of small-molecule tyrosine kinase inhibitors. The intention was to investigate the effect small-molecule tyrosine kinase inhibitors had on the GLUT4 translocation in C2C12 cells after exposure to insulin. The control was considered as 100% GLUT4 translocation. As the concentration of axitinib increased the percentage GLUT4 translocation was observed to increase from below the control at 5µg/mL to a percentage comparable to the control at 80µg/mL. In C2C12 treated with dasatinib it was observed that at the lowest concentration (5µg/mL) the percentage GLUT4 translocation was lower in comparison to the control. As the concentration of dasatinib increased a rise in percentage GLUT4 translocation that was statistically significant compared to the control at 20, 40, and 80µg/mL was observed. At these concentrations the percentage GLUT4 translocation was statistically higher in comparison to the control. As the concentration of

imatinib increased the percentage GLUT4 translocation was observed to increase from below the control at 5 μ g/mL to a percentage comparable to the control at 80 μ g/mL, it was however observed that at 5 μ g/mL the percentage GLUT4 translocation was lower than the control with statistical significance. In comparison to the control, C2C12 cells exposed to erlotinib were observed to demonstrate a lower GLUT4 translocation percentage, with the difference at 5 and 10 μ g/mL being statistically significant. It was noted that as the concentration of erlotinib increased the percentage of GLUT4 translocation increased.

C2C12 cell line

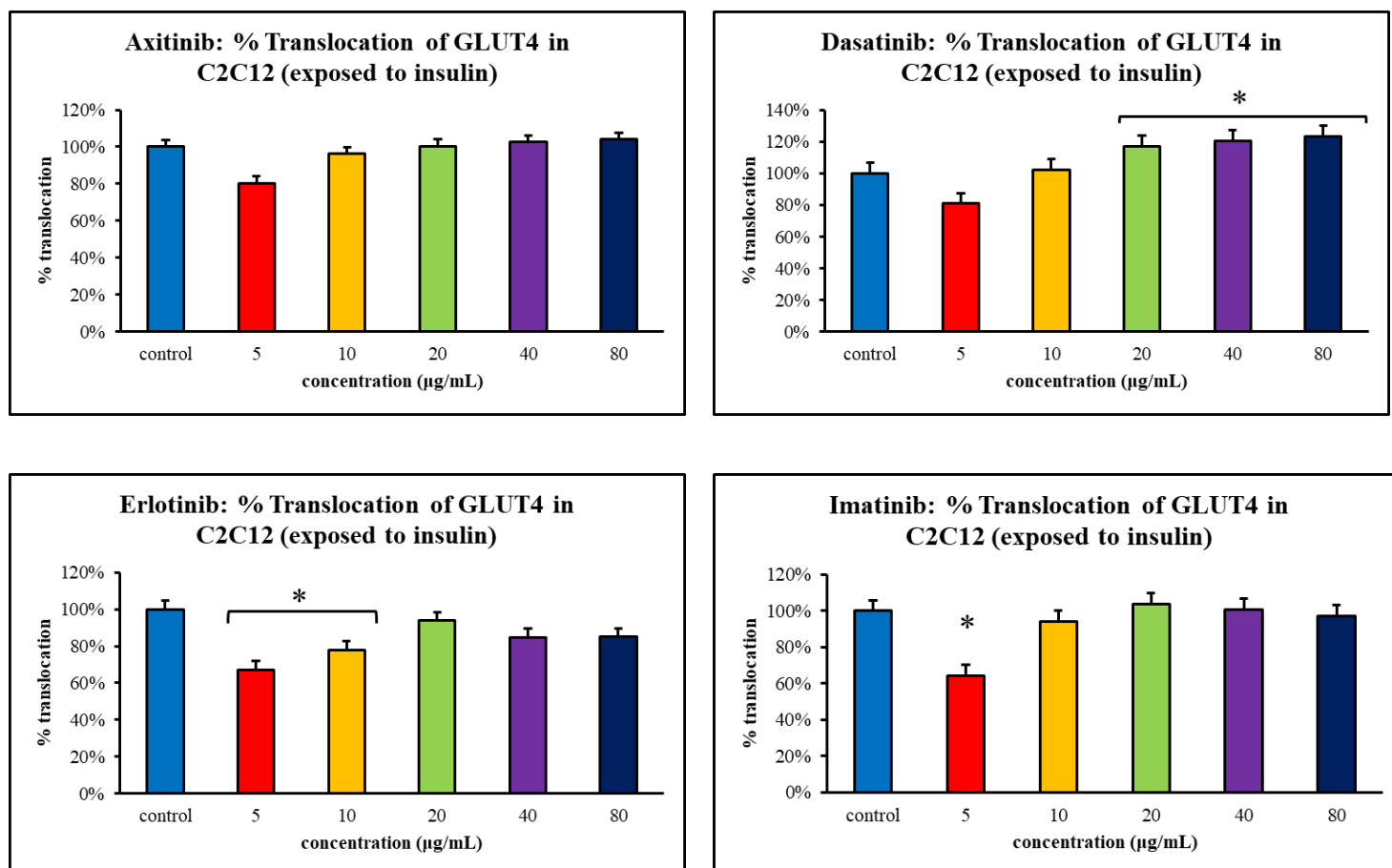


Figure 3. 6: Percentage translocation of GLUT4 in C2C12 cells exposed to insulin followed by small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 μ g/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

3.3.3 GLUT4 expression

The percentage expression of the GLUT4 protein in C2C12 cells treated with small-molecule tyrosine kinase inhibitors is depicted in Figure 3.7. C2C12 cells not exposed to small molecule tyrosine kinase inhibitors or insulin served as the control. C2C12 cells treated with axitinib at a concentration of 5 μ g/mL demonstrated a statistically significant reduction in percentage expression of GLUT4 compared to the control group. However, at higher concentrations ranging from 10 to 80 μ g/mL, the percentage expression of GLUT4 in axitinib-treated cells

were comparable to those observed in the control group. Similarly, C2C12 cells exposed to dasatinib showed a statistically significant decrease in percentage expression of GLUT4 at 5 $\mu\text{g/mL}$ when compared to the control. Notably, at a concentration of 40 $\mu\text{g/mL}$, dasatinib-treated cells exhibited a statistically significant increase in GLUT4 expression compared to the control. At concentrations of 10, 20, and 80 $\mu\text{g/mL}$, the GLUT4 expression in dasatinib-treated cells remained comparable to that of the control group. In C2C12 cells treated with erlotinib, a progressive reduction in percentage GLUT4 expression was observed as the concentration increased from 5 to 20 $\mu\text{g/mL}$. This reduction became statistically significant at 20 $\mu\text{g/mL}$ compared to the control group. C2C12 cells exposed to imatinib exhibited a higher percentage GLUT4 expression compared to the control group. Notably, as the concentration of imatinib increased from 10 to 40 $\mu\text{g/mL}$, a corresponding increase in GLUT4 expression was observed.

C2C12 cell line

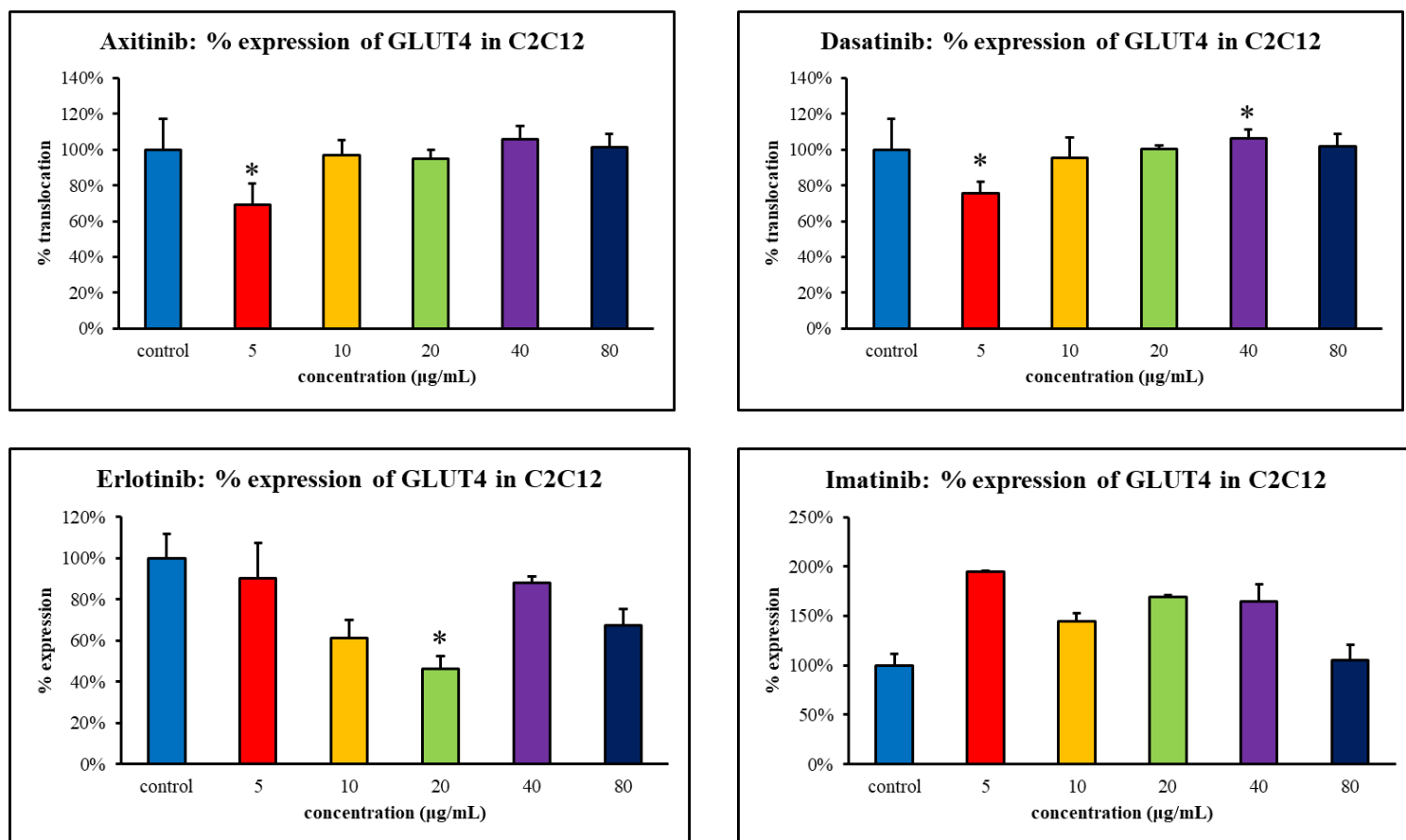


Figure 3. 7: Percentage expression of GLUT4 in C2C12 cells treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.3.4 GLUT4 expression (in C2C12 stimulated by insulin for 1 hour before treatment with small molecule kinase inhibitors)

GLUT4 percentage expression was assessed in C2C12 exposed to insulin and then treated with small-molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 µg/mL. All the C2C12 cells utilized in this assay were all exposed to insulin, the control served as 100% GLUT4 expression. As shown in Figure 3.8, C2C12 cells treated with axitinib displayed elevated GLUT4 expression compared to the control group except for a concentration of 5 µg/mL where GLUT4 expression was comparable to the control. The statistical significance was only observed at 10 and 40 µg/mL compared to the control. C2C12 cells treated with dasatinib showed a statistically significant increase in GLUT4 expression across all tested concentrations compared to the control with 5 to 10 µg/mL showing a greater increase compared to higher concentrations (20, 40, and 80 µg/mL). C2C12 cells treated with erlotinib showed a statistically significant increase in GLUT4 expression across all tested concentrations compared to the control. However, it was noted that as the concentration of erlotinib increased the expression of GLUT4 decreased. As shown in figure 3.8, C2C12 cells treated with imatinib exhibited GLUT4 expression percentage that were comparable to the control. However, a slight increase in GLUT4 expression was observed as the concentration of imatinib increased.

C2C12 cell line

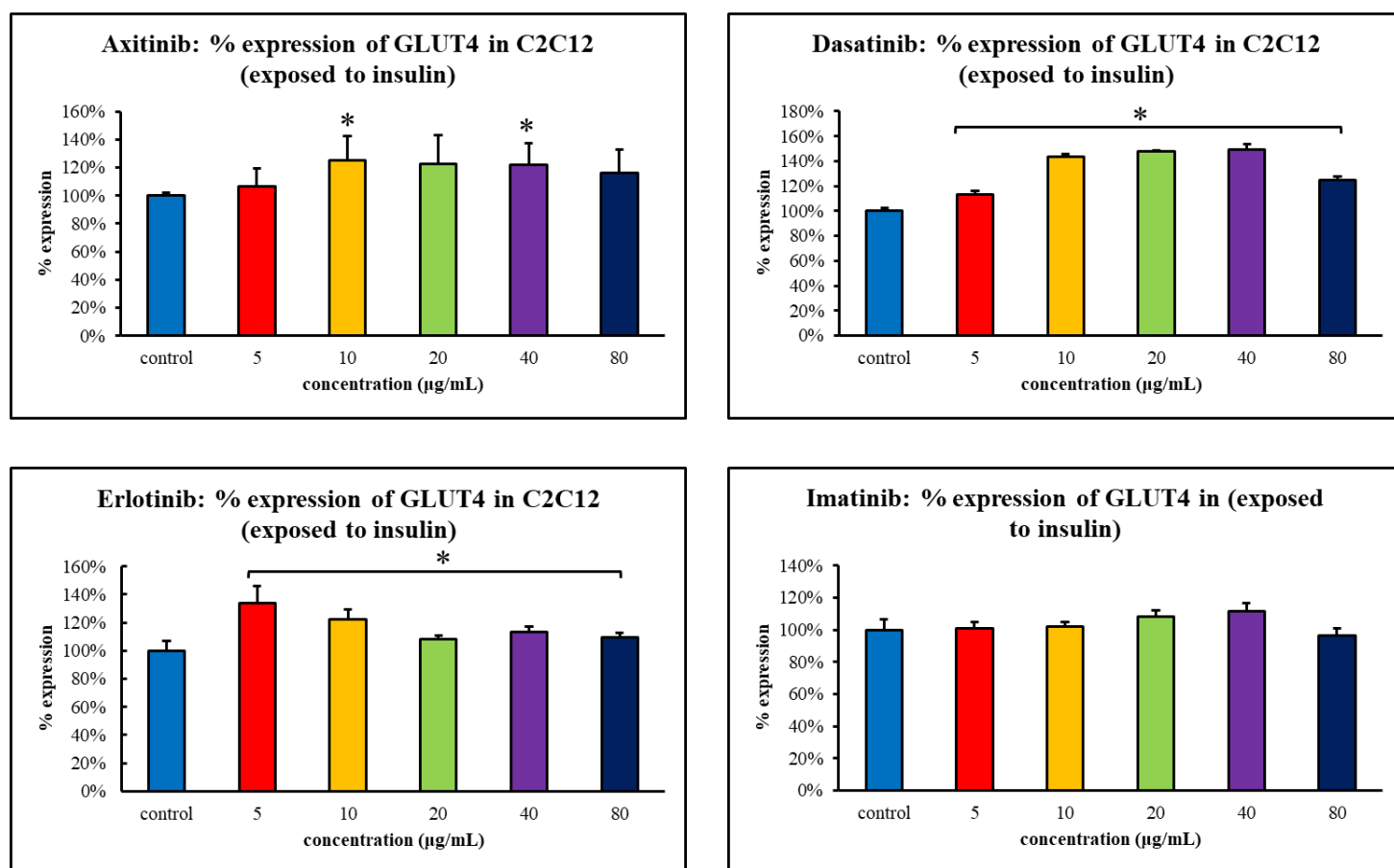


Figure 3. 8: Percentage expression of GLUT4 in C2C12 cells exposed to insulin followed by small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.3.5 Akt expression

Percentage expression of the Akt protein in C2C12 and HepG2 cells exposed to small-molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80µg/mL is displayed in Figure 3.9 and 4.0 respectively. It was observed that as the concentration of axitinib increased the percentage expression of Akt was reduced as visualized in Figure 3.9. Across all concentrations analysed, the percentage expression of Akt remained comparable/similar to the control. In C2C12 cells treated with dasatinib, an increase in percentage expression of Akt was observed as the concentration of dasatinib increased. From 10 µg/mL to 80µg/mL, the percentage expression of Akt was higher compared to the control group. In C2C12 cells treated with imatinib, an increase in concentration resulted in a corresponding increase in the percentage expression of Akt. With the exception of the 10 and 80µg/mL concentrations, imatinib exposure led to elevated Akt expression compared to the control group. Exposure to erlotinib resulted in a statistically significant reduction in Akt expression in C2C12 cells compared to the control, particularly at

concentrations between 10 and 80 $\mu\text{g/mL}$. However, within the 10 to 40 $\mu\text{g/mL}$ range, a gradual increase in Akt expression was observed.

HepG2 cells treated with axitinib exhibited a percentage Akt expression comparable to the control. However, as the concentration of axitinib increased, a slight decrease in the percentage expression of Akt was observed. In HepG2 cells treated with dasatinib, the percentage expression of Akt decreased as the concentration of dasatinib increased. At a concentration of 40 $\mu\text{g/mL}$, the expression of Akt was significantly lower compared to the control. It was observed that in HepG2 cells treated with erlotinib, the percentage expression of Akt decreased as the concentration of erlotinib increased. At a concentration of 20 and 40 $\mu\text{g/mL}$, the expression of Akt was significantly lower compared to the control. In HepG2 cells treated with imatinib from 5 to 20 $\mu\text{g/mL}$ a decrease in percentage expression of Akt was observed, while between 20 and 80 $\mu\text{g/mL}$, an increase in percentage expression of Akt was observed with 80 $\mu\text{g/mL}$ showing percentage expression of Akt greater than the control with statistical significance.

C2C12 cell line

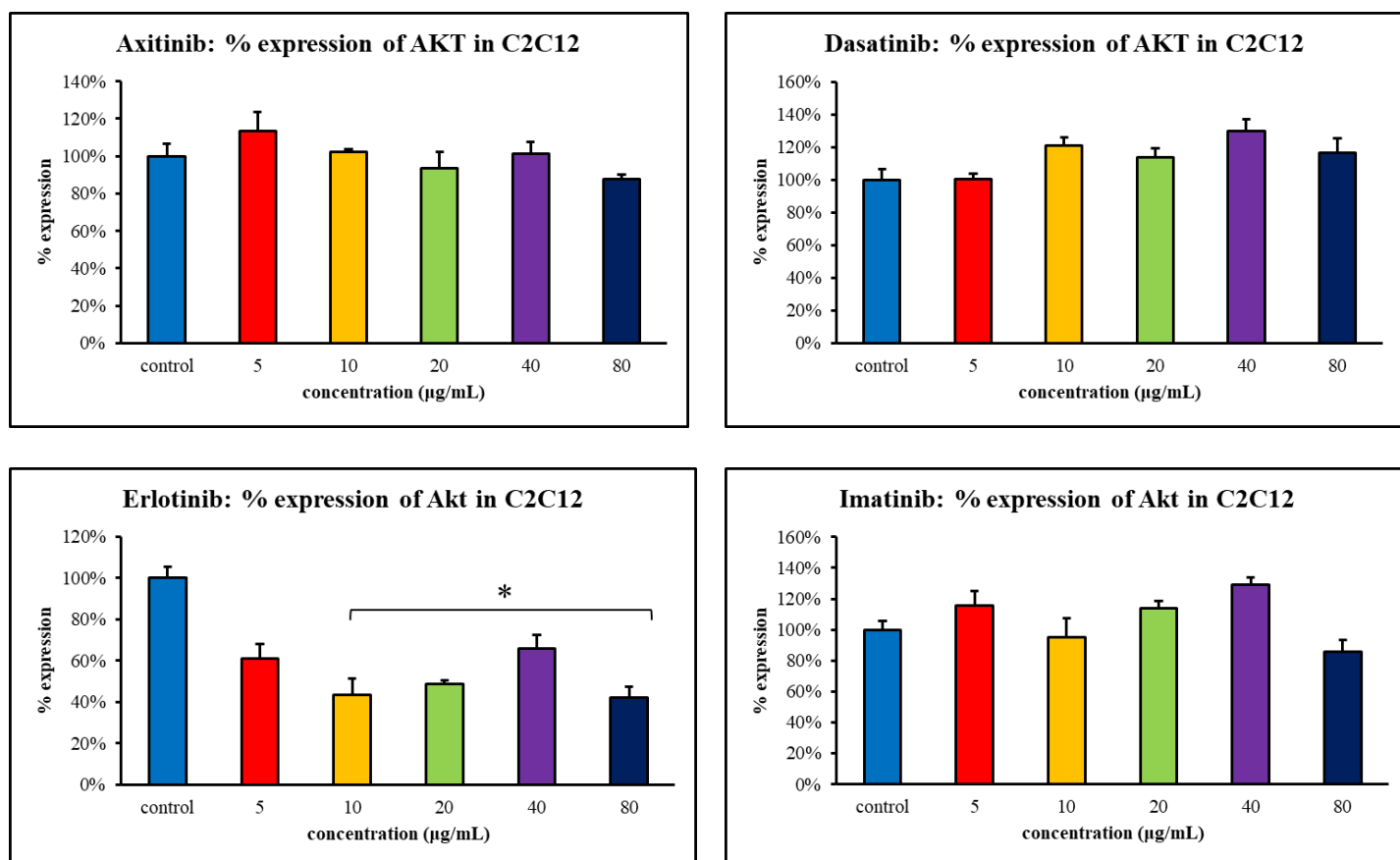


Figure 3. 9: Percentage expression of Akt in C2C12 cells treated with small molecule tyrosine kinase inhibitors treatment at different concentrations (axitinib, dasatinib, erlotinib, and imatinib) 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

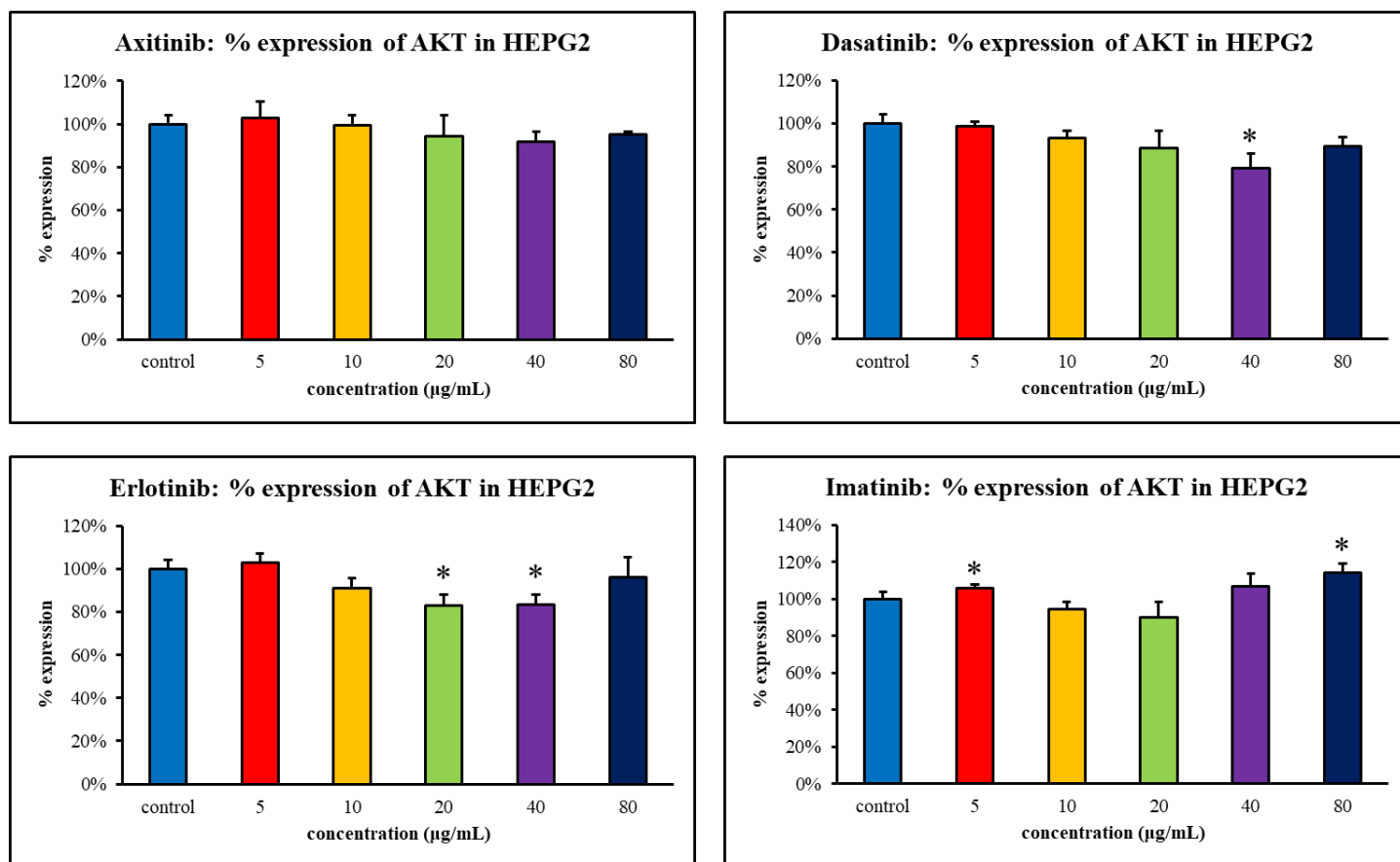


Figure 3. 10: Percentage expression of Akt in HepG2 cells treated with small molecule tyrosine (axitinib, dasatinib, erlotinib, and imatinib) kinase inhibitors treatment at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.3.6 Akt expression (in C2C12 cells that were exposed to insulin for 1 hour before treatment with small molecule kinase inhibitors)

Figures 3.11 and 3.12 depict the percentage expression of Akt in C2C12 and HepG2 cells exposed to insulin and then treated with small-molecule tyrosine kinase inhibitors. Cells that were only exposed to insulin served as the control. C2C12 cells treated with axitinib exhibited a statistically significant increase in percentage expression of Akt compared to the control. Additionally, as the concentration of axitinib increased, a corresponding increase in percentage expression Akt was observed. C2C12 cells treated with dasatinib exhibited an increase in percentage expression of Akt compared to the control. Furthermore, as the concentration of dasatinib increased, a corresponding increase in percentage expression Akt was observed. C2C12 cells treated with erlotinib demonstrated a slightly higher percentage expression of Akt compared to the control. However, this difference was statistically significant at concentrations of 10, 40, and 80 µg/mL. Imatinib exposure resulted in a higher percentage expression of Akt compared to the control, with the exception of the 5 µg/mL concentration.

HepG2 cells treated with axitinib were observed to have lower levels of Akt expression and demonstrated a decreasing trend between 5 and 40 $\mu\text{g/mL}$. However, at 5 $\mu\text{g/mL}$ the level of Akt expression in cells treated with axitinib was greater compared to the control. Exposure to dasatinib was observed to result in elevated Akt expression in HepG2 cells compared to the control between 5 and 20 $\mu\text{g/mL}$. At 5 $\mu\text{g/mL}$ Akt expression was elevated compared to the control with statistical significance. Between 10 and 40 $\mu\text{g/mL}$ HepG2 cells treated with dasatinib showed a decreasing expression of Akt. HepG2 cells treated with erlotinib demonstrated elevated Akt levels between 5 and 20 $\mu\text{g/mL}$ compared to the control. However, at 40 and 80 $\mu\text{g/mL}$ the cells were observed to have lower levels of Akt expression in comparison to the control. Treatment of HepG2 cells with imatinib was observed to result in lower Akt levels compared to the control with statistical significance at 5 $\mu\text{g/mL}$.

C2C12 cell line

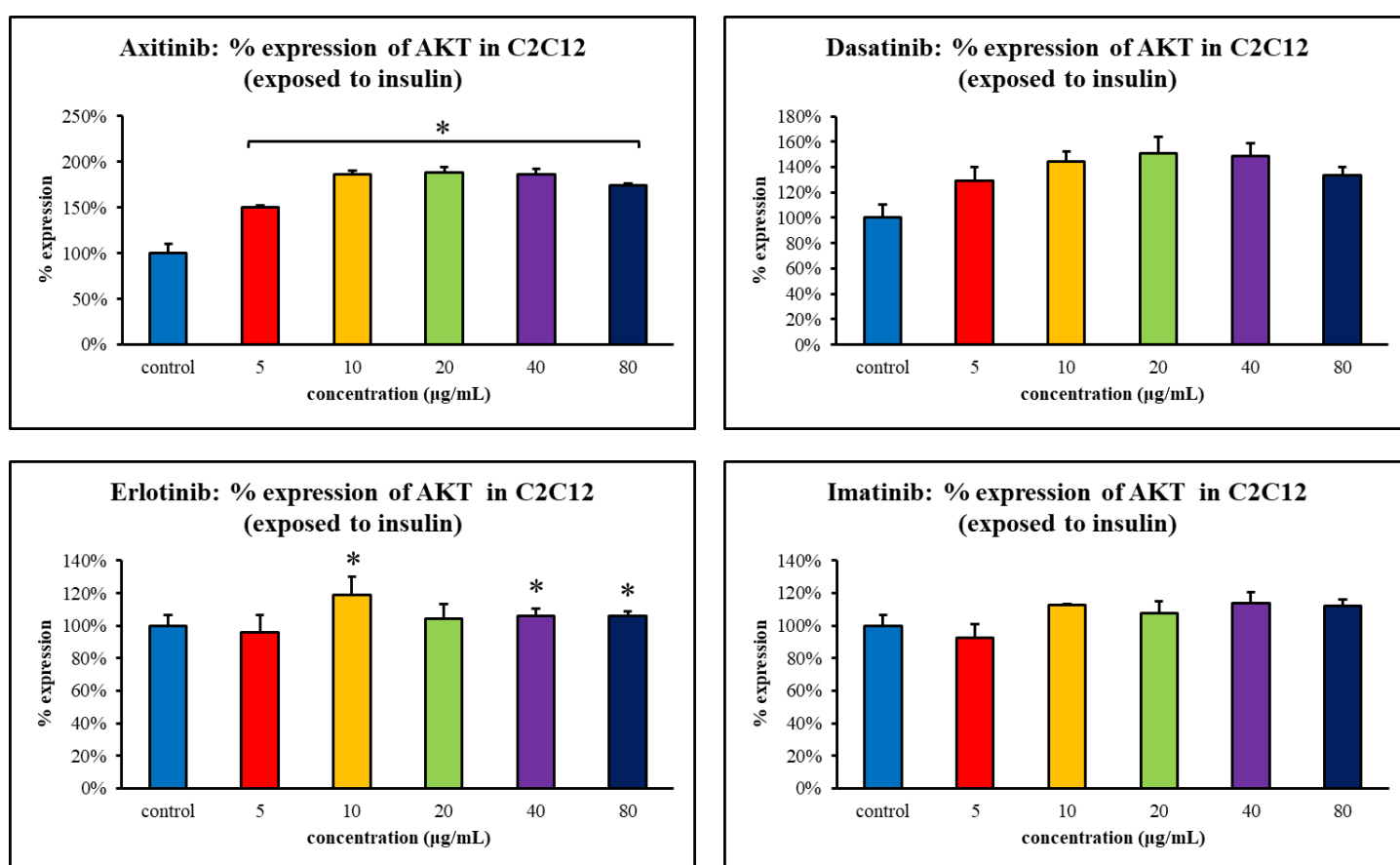


Figure 3. 11: Percentage expression of Akt in C2C12 cells exposed to insulin then treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) treatment at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

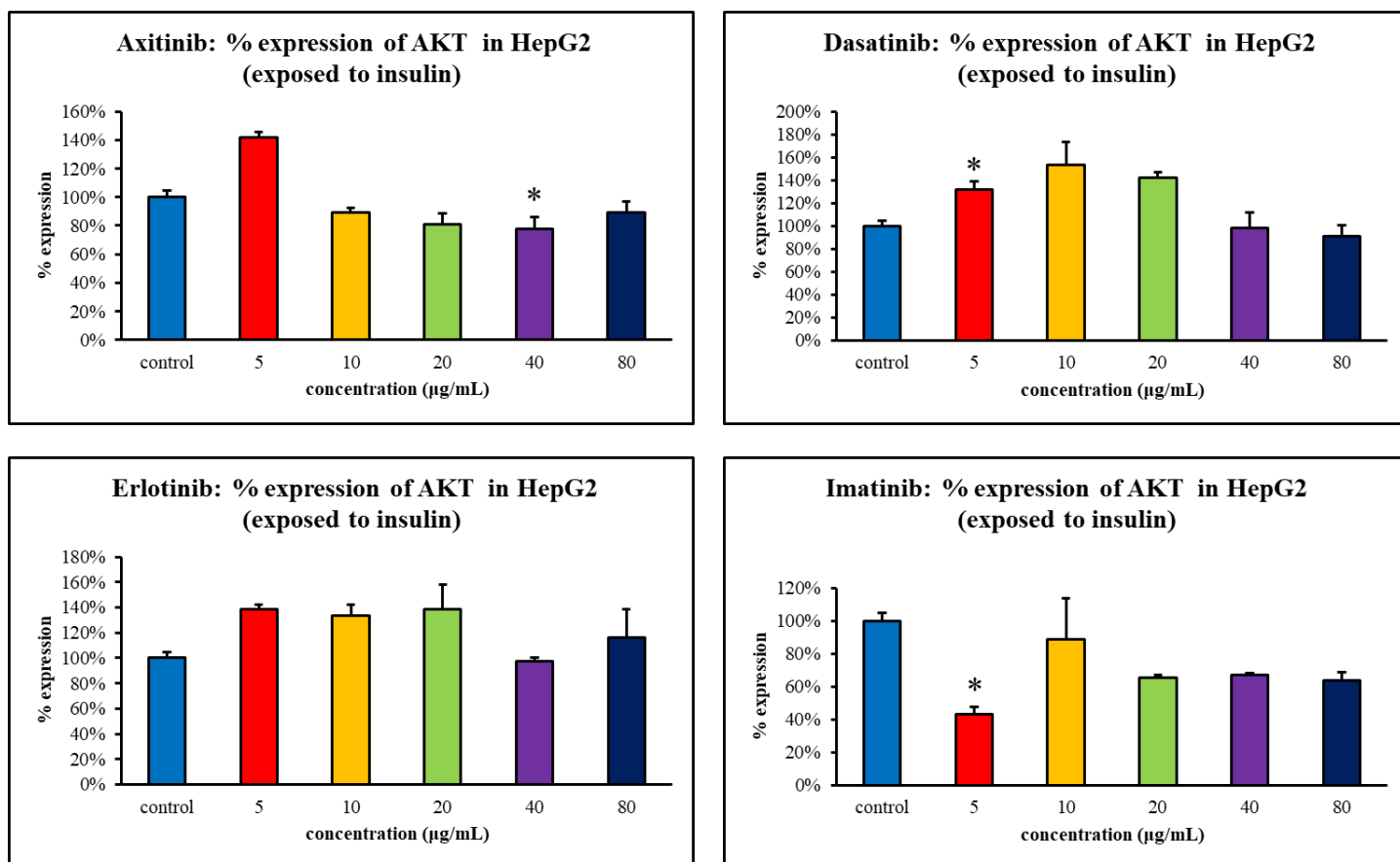


Figure 3. 12: Percentage expression of Akt in HepG2 cells exposed to insulin then treated with small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) treatment at different concentrations 5, 10, 20, 40, and 80 µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

3.3.7 Interleukin 6 (IL-6) cellular expression

Interleukin-6 percentage expression was assessed in C2C12 and HepG2 cells treated with axitinib, dasatinib, erlotinib, and imatinib at 5, 10, 20, 40, and 80 µg/mL is depicted in Figures 3.12 and 3.13 respectively. C2C12 and HepG2 cells that were not exposed to small molecule tyrosine kinase inhibitors served as the control. In C2C12 cells treated with axitinib, IL-6 percentage expression at 5 µg/mL was comparable to the control. However, between 10 and 80 µg/mL, a statistically significant elevation in IL-6 expression was observed compared to the control. Similarly, in C2C12 cells treated with dasatinib, IL-6 percentage expression at 5 µg/mL was comparable to the control. However, between 10 and 80 µg/mL, a statistically significant elevation in IL-6 expression was observed compared to the control. C2C12 cells exposed to erlotinib demonstrated an increasing percentage of IL-6 expression as the concentration of erlotinib increased. A statistically significant increase in IL-6 expression was observed at 80 µg/mL compared to the control. In C2C12 cells treated with imatinib, an increase in IL-6 expression was observed. A dose-dependent increase was noted between 5 and 20 µg/mL, with the 5 µg/mL concentration showing a statistically significant lower IL-6 expression compared

to the control. Between 20 and 80 $\mu\text{g/mL}$, imatinib-treated cells demonstrated a statistically significant elevated IL-6 expression compared to the control.

In HepG2 cells treated with axitinib, IL-6 expression remained comparable to the control across all investigated concentrations. In contrast, imatinib treatment resulted in IL-6 expression comparable to the control at 5 $\mu\text{g/mL}$, followed by a significant increase at 10 and 20 $\mu\text{g/mL}$ before decreasing to control levels at 80 $\mu\text{g/mL}$. Similarly, dasatinib treatment showed no significant change at 5, 10, and 20 $\mu\text{g/mL}$, but at higher concentrations of 40 and 80 $\mu\text{g/mL}$, a statistically significant increase in IL-6 expression was observed.

C2C12 cell line

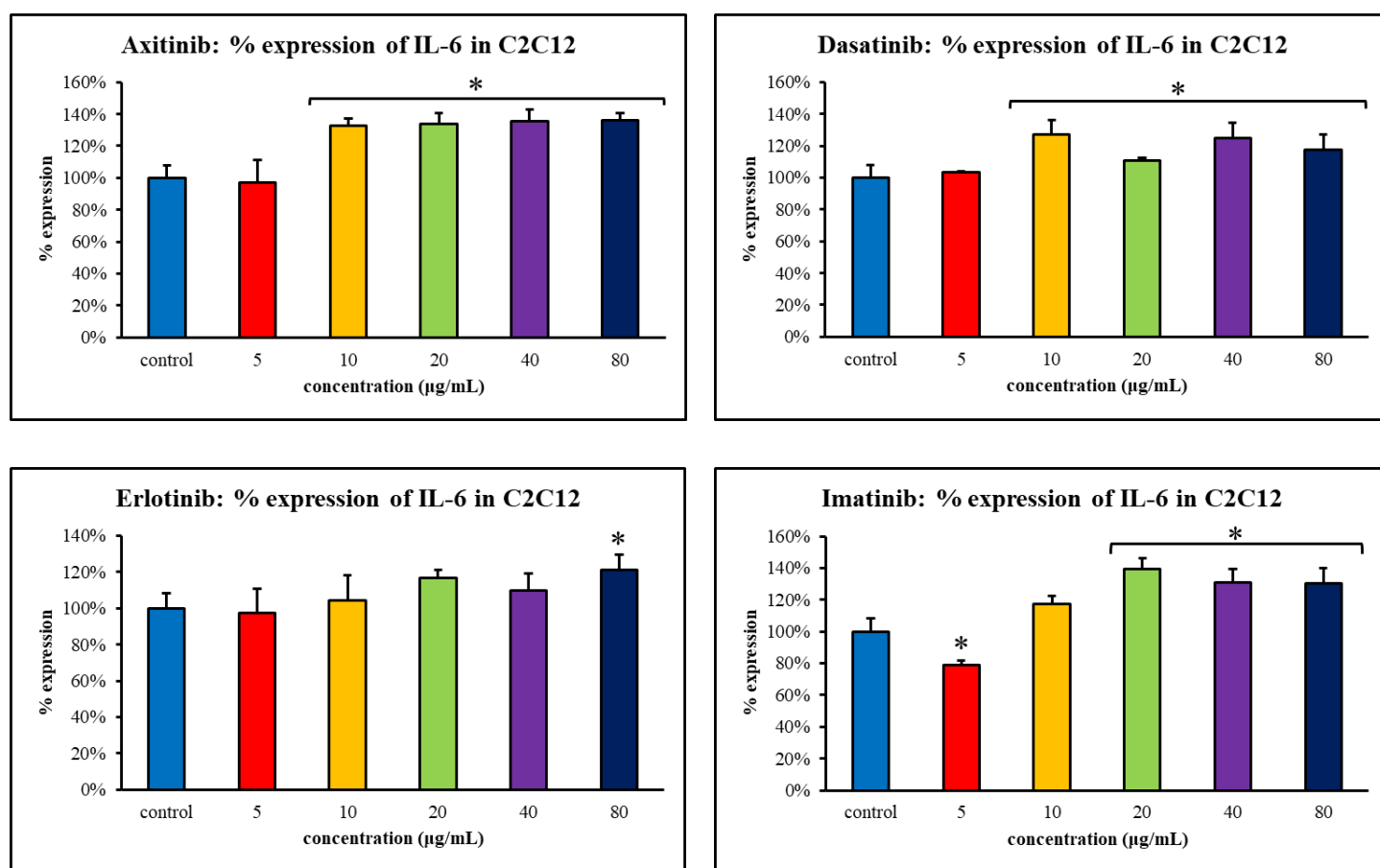


Figure 3. 13: Percentage expression of IL-6 in C2C12 cells treated with small molecule tyrosine kinase inhibitors treatment (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

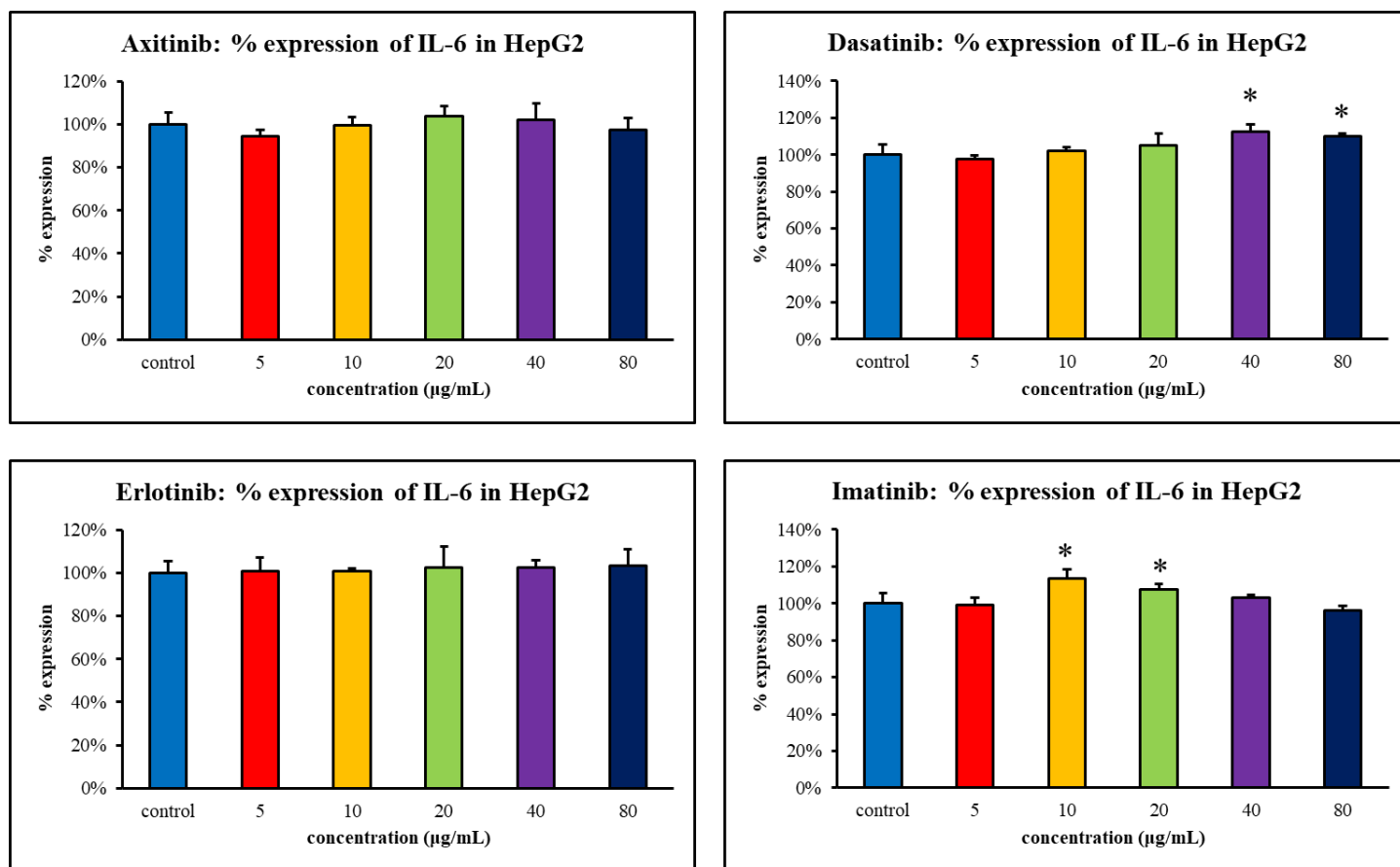


Figure 3. 14: Percentage expression of IL-6 in HepG2 cells treated with small molecule tyrosine kinase inhibitors treatment at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at (p < 0.05).

3.3.8 Media Interleukin 6 (IL-6)

Figure 3.14 and Figure 3.15 depict the percentage of IL-6 in medium that was harvested from C2C12 and HepG2 cells treated with small molecule tyrosine kinase inhibitors treatment at different concentrations (axitinib, dasatinib, erlotinib, and imatinib) 5, 10, 20, 40, and 80µg/mL, respectively. It was observed that the concentration of IL-6 in the medium from C2C12 cells treated with axitinib, had a concentration of IL-6 that was lower than the control with statistical significance. A slight increase in IL-6 concentration was observed as the concentration of axitinib increased, though the overall levels remained below the control. In the medium harvested from C2C12 cells treated with dasatinib, an increase in IL-6 concentration was observed as the dasatinib concentration increased. However, at all concentrations of dasatinib, the IL-6 levels remained lower than those of the control. This reduction was statistically significant at 5 µg/mL. It was observed that as the concentration of erlotinib increased the concentration of IL-6 in medium increased. Initially, the IL-6 concentration was comparable to the control, but at higher concentrations of erlotinib, IL-6 levels exceeded those of the control. In the medium harvested from C2C12 cells treated with imatinib, IL-6 concentration increased with rising dasatinib concentrations. At 5 and 10 µg/mL, IL-6 concentrations were lower with statistical significance than those of the control. However,

as the concentration increased to 80 $\mu\text{g/mL}$, IL-6 concentrations became comparable to the control. In HepG2 cells treated with axitinib, the concentration of IL-6 in the medium was significantly lower than that of the control across all concentrations, showing statistical significance. It was observed that the concentration of IL-6 in the medium from HepG2 cells treated with dasatinib was comparable to the control at 5 $\mu\text{g/mL}$ however between 10 and 80 $\mu\text{g/mL}$ IL-6 concentrations were lower with statistical significance than those of the control. In HepG2 cells treated with erlotinib, the concentration of IL-6 in the medium was observed to decrease as the concentration of erlotinib increased from 5 $\mu\text{g/mL}$ which had IL-6 levels higher than the control to 80 $\mu\text{g/mL}$ where the IL-6 concentrations were lower than the control with statistical significance. It was observed that as the concentration of imatinib increased the concentration of IL-6 in the medium decreased to a level comparable to the control.

Media utilized in C2C12 cell line

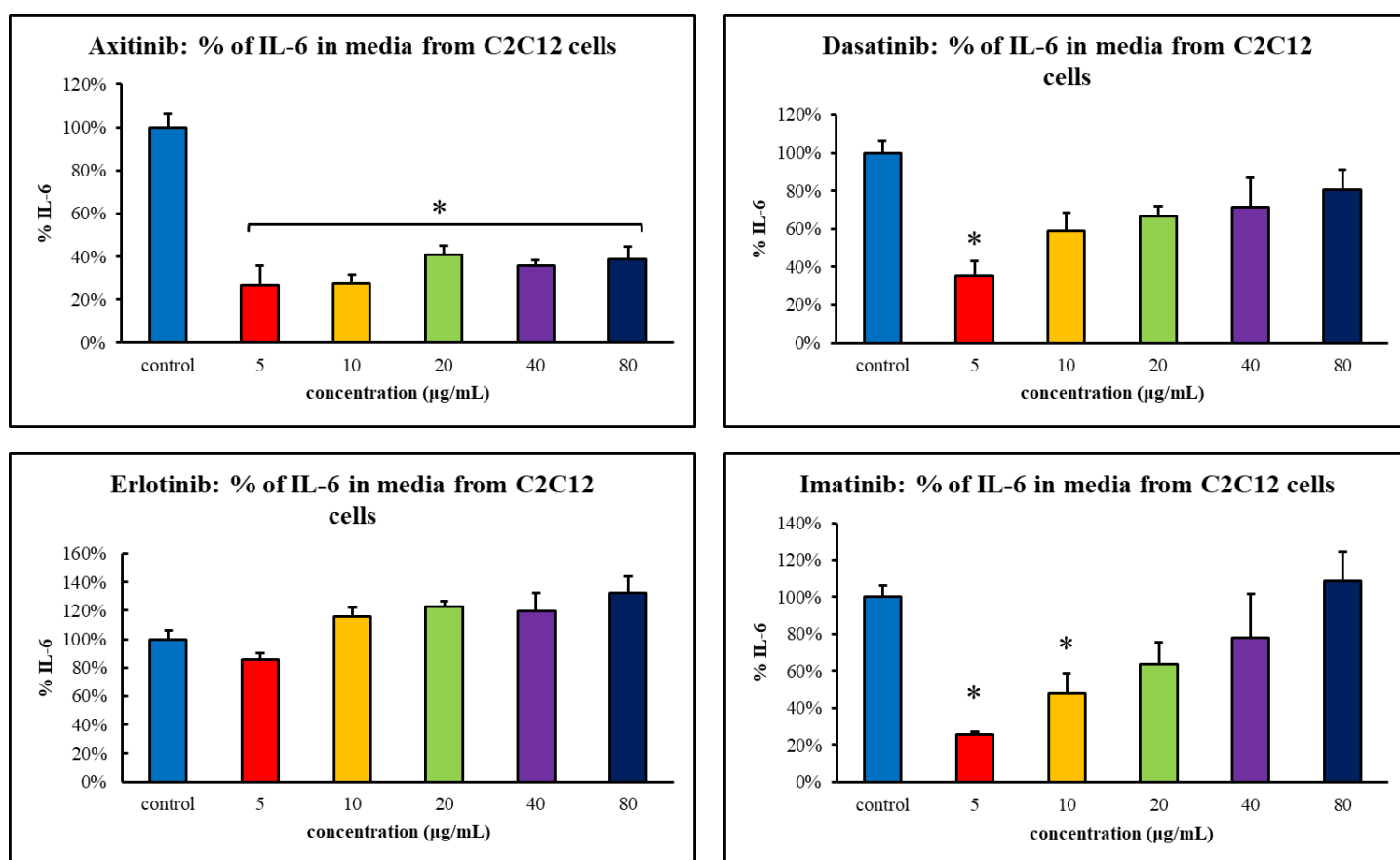


Figure 3. 15: Percentage of IL-6 in medium used in C2C12 cells treated with small molecule tyrosine kinase inhibitors treatment at different concentrations (axitinib, dasatinib, erlotinib, and imatinib) 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

Media utilized in HepG2 cell line

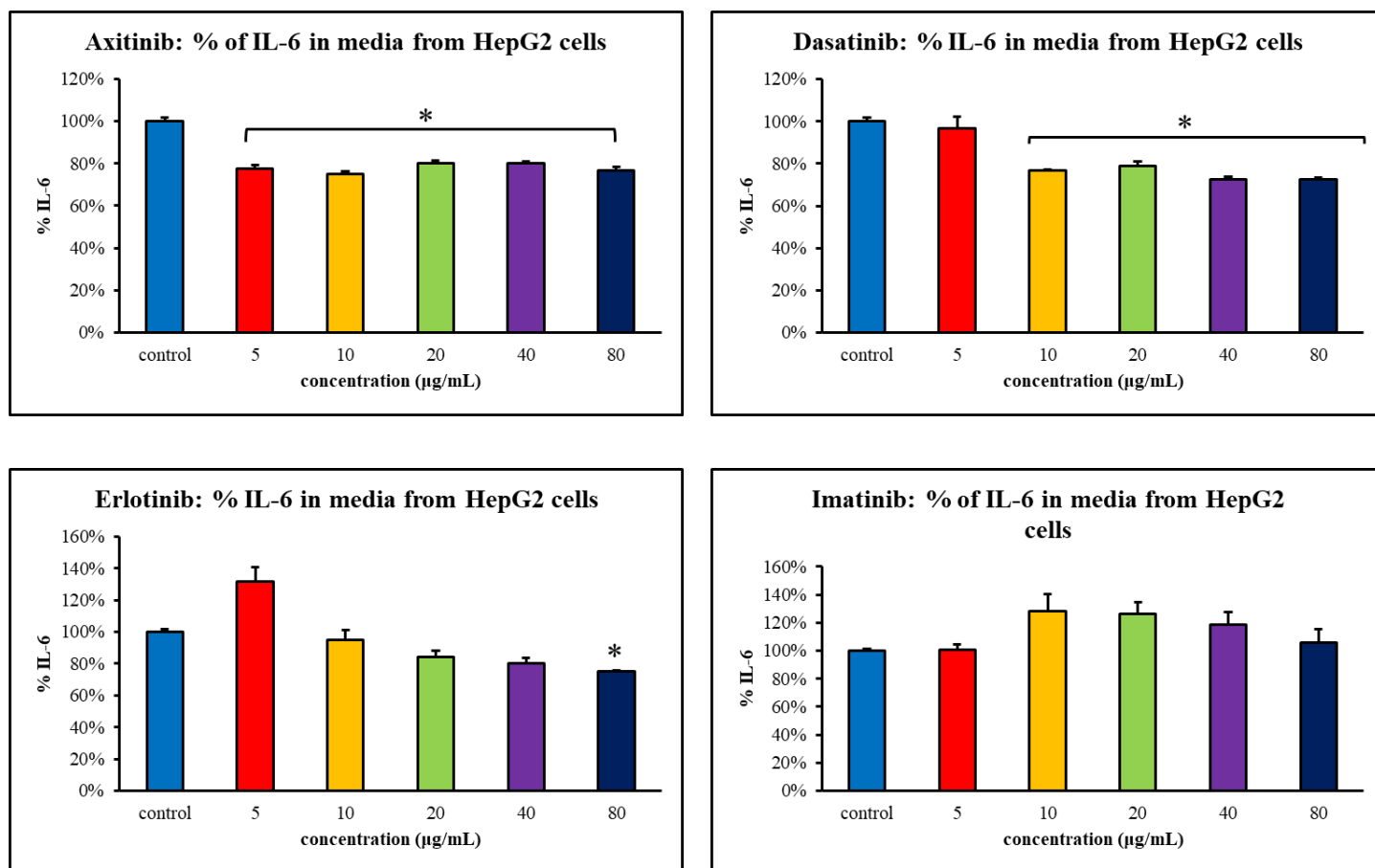


Figure 3. 16: Percentage of IL-6 in medium used in HepG2 cells treated with small molecule tyrosine kinase inhibitors treatment (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.3.9 Justification for the investigation of dasatinib

Dasatinib was identified to be the only drug that showed percentage translocation of GLUT4 that was increasing and above the control in C2C12 that were exposed to insulin. It was the only compound that showed an increase in percentage translocation of GLUT4 that was also above the control in both C2C12 that were exposed to insulin and those that were not. Axitinib and erlotinib only demonstrated an increase in percentage translocation of GLUT4 that was also above the control in C2C12 that were not exposed to insulin. Akt percentage expression was noted to increase as the concentration of dasatinib increased in C2C12 cells exposed to insulin and those that were not.

3.4 Further assays performed with Dasatinib

3.4.1 Effect of Dasatinib on glucose uptake in the presence of PI3K-inhibitor (Wortmannin)

Figures 3.16 and 3.17 depict the estimated glucose uptake in C2C12 and HepG2 cells in which the co-administration of dasatinib and wortmannin for 24 hours was performed. Cells that were not exposed to dasatinib or wortmannin served as the absolute control. In Figure 3.16 and 3.17 25mM wortmannin represented cells that were only exposed to wortmannin. C2C12 cells only exposed to wortmannin were observed to show glucose uptake that was lower with statistical difference in comparison to the absolute control. As depicted in Figure 3.16, C2C12 cells exposed to dasatinib and wortmannin concurrently displayed lowered glucose uptake that was lower with statistical significance compared to the absolute control. With increasing concentrations of dasatinib, an increase in glucose uptake was observed indicating a reversal of the PI3K-inhibitory effect of wortmannin. It was observed that as the concentration of dasatinib increased an increase in glucose uptake was observed. Similarly, as depicted in Figure 3.17, HepG2 cells only exposed to wortmannin were observed to show glucose uptake that was lower with statistical difference in comparison to the absolute control. HepG2 cells exposed to dasatinib and wortmannin concurrently displayed lowered glucose uptake that was lower than the absolute control with statistical significance. However, as the concentration of dasatinib increased a slight increase in glucose uptake was noted. In both C2C12 and HepG2 cells, exposure to dasatinib resulted in increased glucose uptake in comparison to the wortmannin control. A reversal of wortmannin's inhibitory effect on PI3K was observed in C2C12 and HepG2 cells treated with dasatinib.

C2C12 cell line

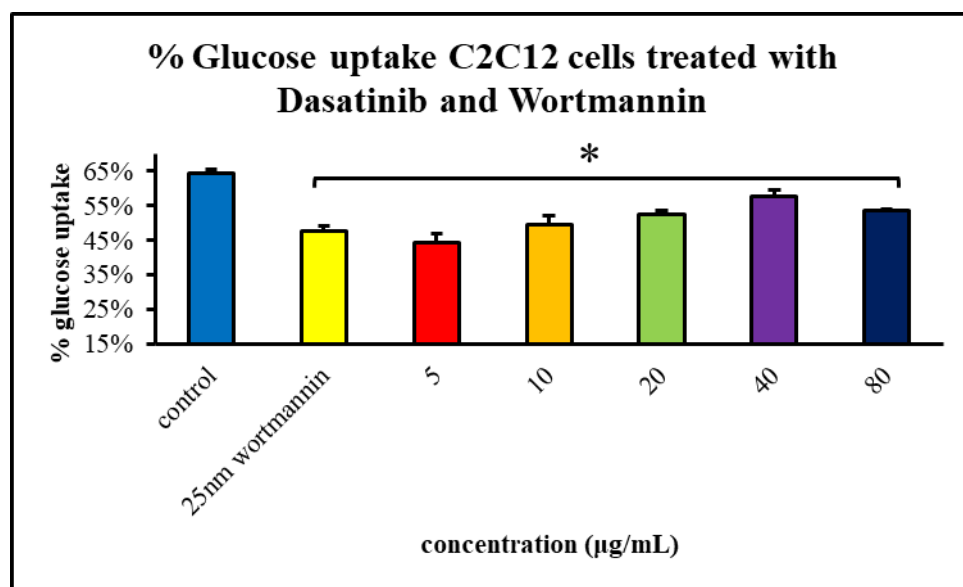


Figure 3. 17: Percentage glucose uptake in C2C12 cells concurrently treated with wortmannin and dasatinib at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

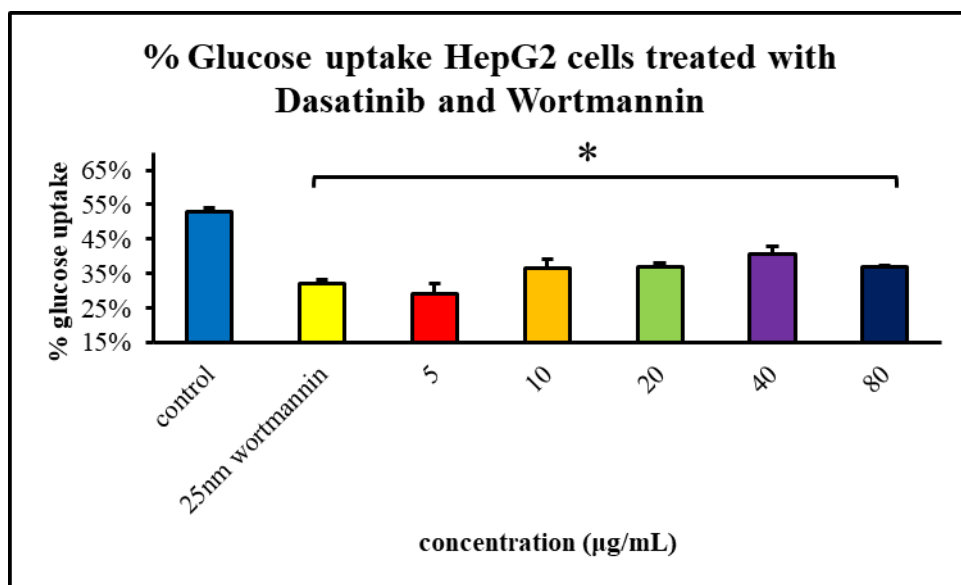


Figure 3. 18: Percentage glucose uptake in HepG2 cells concurrently treated with wortmannin and dasatinib at different concentrations 5, 10, 20, 40, and 80µg/mL. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.4.2 Effect of dasatinib on glucose uptake in palmitic-induced insulin resistance

Palmitic acid was used to induce insulin resistance in C2C12 and HepG2 cell lines. As displayed in Figures 3.18 and 3.19, the C2C12 and HepG2 cells that were not exposed to palmitic acid, served as the absolute control (yellow bar on the graph). Cells that were exclusively exposed to palmitic acid were the control. In C2C12 cells not exposed to palmitic acid a higher glucose uptake that was statistically significant compared to the absolute control was observed. Meanwhile, cells exposed to dasatinib showed glucose uptake that was comparable to the control. The C2C12 cells treated with palmitic acid alone exhibited glucose uptake levels comparable to those observed in C2C12 cells that were subsequently treated with dasatinib following palmitic acid exposure.

In HepG2 cells not exposed to palmitic acid, a statistically significant increase in glucose uptake was observed compared to the absolute control. In HepG2 cells treated with dasatinib, glucose uptake was generally comparable to the control, except at 10 µg/mL, where a significantly higher glucose uptake than the absolute control was observed. The HepG2 cells treated with palmitic acid alone exhibited glucose uptake levels comparable to those observed in HepG2 cells that were subsequently treated with dasatinib following palmitic acid exposure.

C2C12 cell line

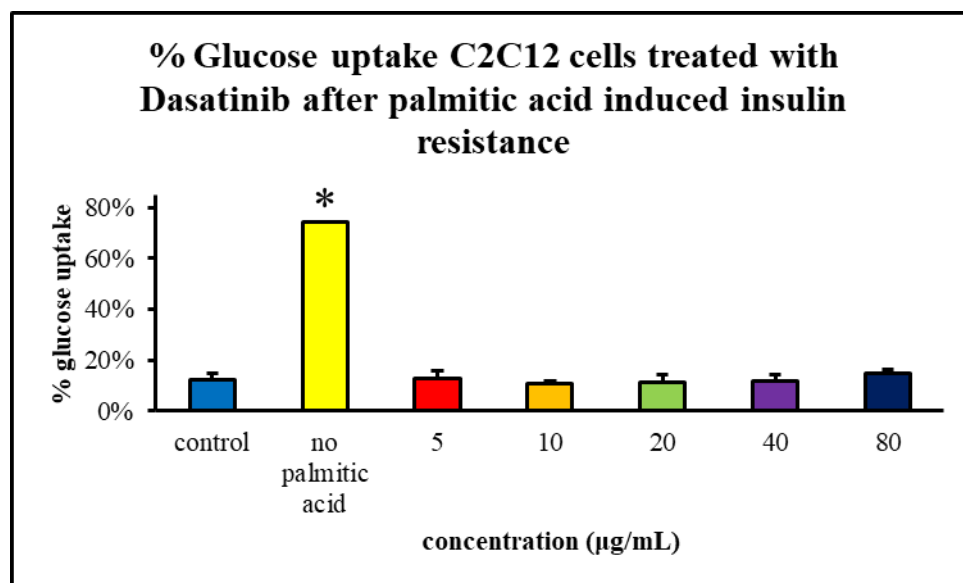


Figure 3. 19: Percentage glucose uptake in C2C12 cells treated with dasatinib at different concentrations 5, 10, 20, 40, and 80µg/mL after palmitic acid-induced insulin resistance. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

HepG2 cell line

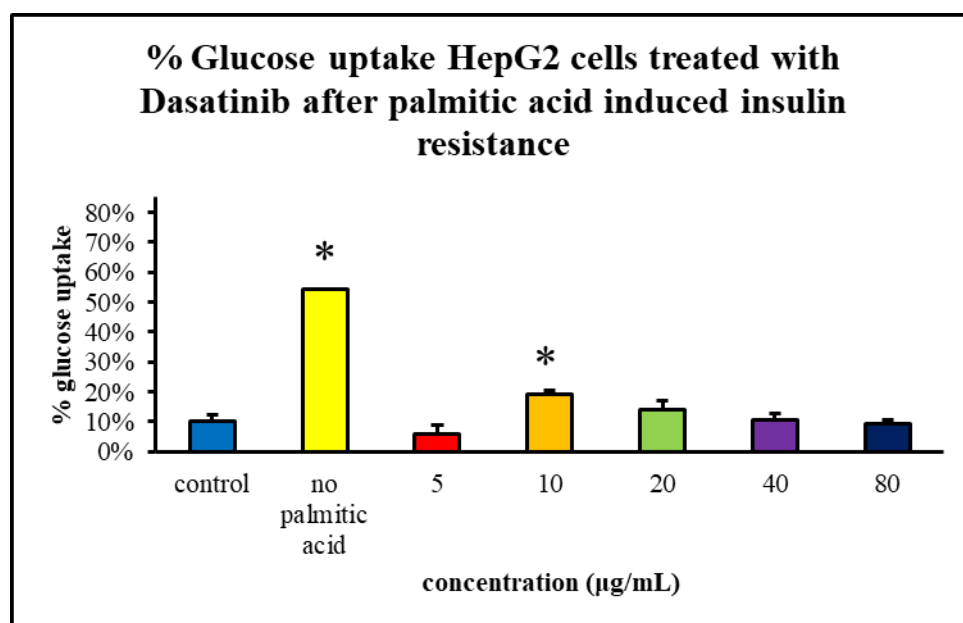


Figure 3. 20: Percentage glucose uptake in HepG2 cells treated with dasatinib at different concentrations 5, 10, 20, 40, and 80µg/mL after palmitic acid-induced insulin resistance. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.4.3 Effect of Dasatinib on GLUT4 translocation in C2C12 in the presence of PI3K-inhibitor (Wortmannin)

The translocation of GLUT4 in C2C12 cells was investigated following the coadministration of dasatinib and wortmannin as depicted by Figure 3.20. The C2C12 cells that were not exposed to dasatinib or wortmannin served as the control. The wortmannin bar represents the C2C12 cells that were exclusively exposed to wortmannin. The C2C12 cells exclusively treated with wortmannin were observed to show a percentage translocation of GLUT4 that was slightly higher than the control. Initially, at 5 $\mu\text{g/mL}$, the percentage translocation of GLUT4 is greater than the control. However, as the concentration of dasatinib increased a decrease in percentage translocation was observed. The C2C12 cells concurrently exposed to wortmannin and dasatinib at concentrations of 5 and 10 $\mu\text{g/mL}$ exhibited greater GLUT4 translocation compared to cells treated with wortmannin alone. However, at concentrations between 20 and 80 $\mu\text{g/mL}$, GLUT4 translocation was comparable between cells exposed to both dasatinib and wortmannin, and those treated with wortmannin alone.

C2C12 cell line

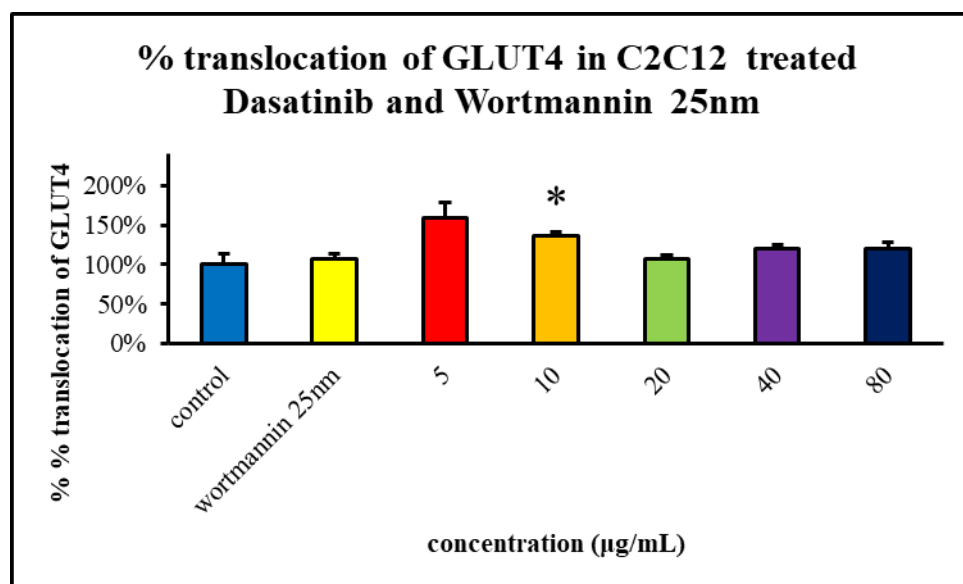


Figure 3. 21: Percentage translocation of GLUT4 in C2C12 cells concurrently treated with wortmannin and dasatinib at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, (n = 3), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.4.4 Effect of dasatinib on GLUT4 translocation in palmitic-induced insulin resistance

The percentage translocation of GLUT4 in C2C12 cells following exposure to palmitic acid to induce insulin resistance and then treated with dasatinib were examined as depicted in Figure 3.21. C2C12 cells that were not exposed to palmitic acid served as the baseline control (yellow bar), while C2C12 cells that were there were palmitic acid-induced insulin resistance served as the control. C2C12 cells that were not exposed to palmitic were observed to demonstrate a percentage GLUT4 translocation that was higher than the control with statistical significance. At 10 $\mu\text{g/mL}$, a statistically significant increase in GLUT4 compared to the control was observed. However, at 5, 20, 40, and 80 $\mu\text{g/mL}$ the percentage of GLUT4 translocation was

observed to be only slightly higher than the control. C2C12 cells not exposed to palmitic acid were observed to have higher GLUT4 translocation in comparison to C2C12 cells that were treated with dasatinib, with the exception of 10 $\mu\text{g/mL}$.

C2C12 cell line

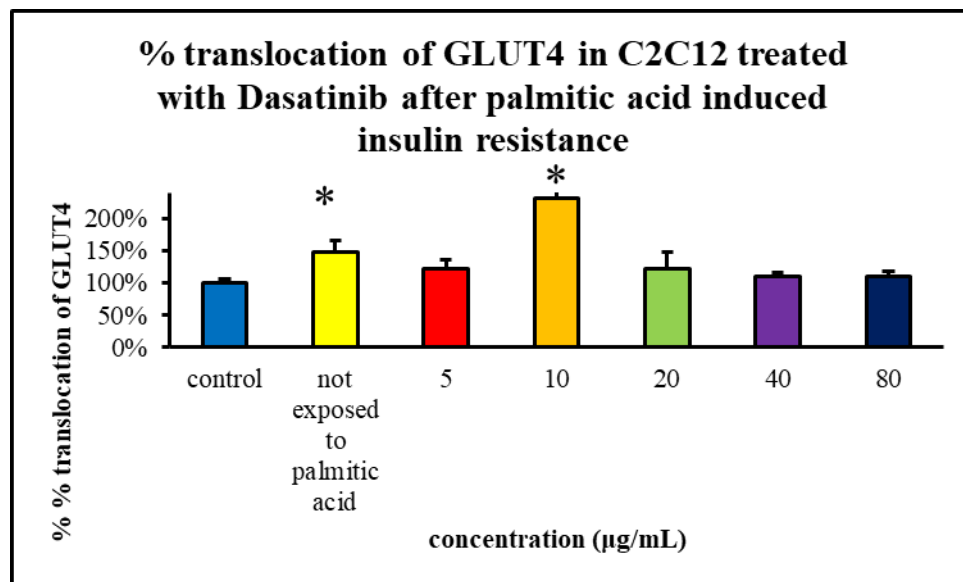


Figure 3. 22: Percentage translocation of GLUT4 in C2C12 cells treated with dasatinib at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$ after palmitic acid induced insulin resistance. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.5 Enzyme inhibition assays

3.5.1 Alpha-amylase inhibition assay

The percentage of alpha-amylase inhibition by acarbose (standard reference drug) and small-molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$ are presented in Figure 3.22 and Figure 3.23. The control is considered to exhibit 0% inhibition of alpha-amylase.

It was observed that as the concentration of acarbose increased the percentage inhibition of amylase was observed to increase in comparison to the control (not exposed to acarbose) as depicted in Figure 3.22. Statistical significance was observed at 20, 40, and 80 $\mu\text{g/mL}$.

As presented in Figure 3.23 none of the small-molecule tyrosine kinase inhibitors demonstrated a percentage inhibition higher than 35% while acarbose at maximum concentration(80 $\mu\text{g/mL}$) used showed a percentage inhibition of approximately 60%. As concentrations of erlotinib and imatinib increased from 5 to 20 $\mu\text{g/mL}$ and 10 to 40 $\mu\text{g/mL}$ respectively an increase in percentage inhibition was observed.

Acarbose

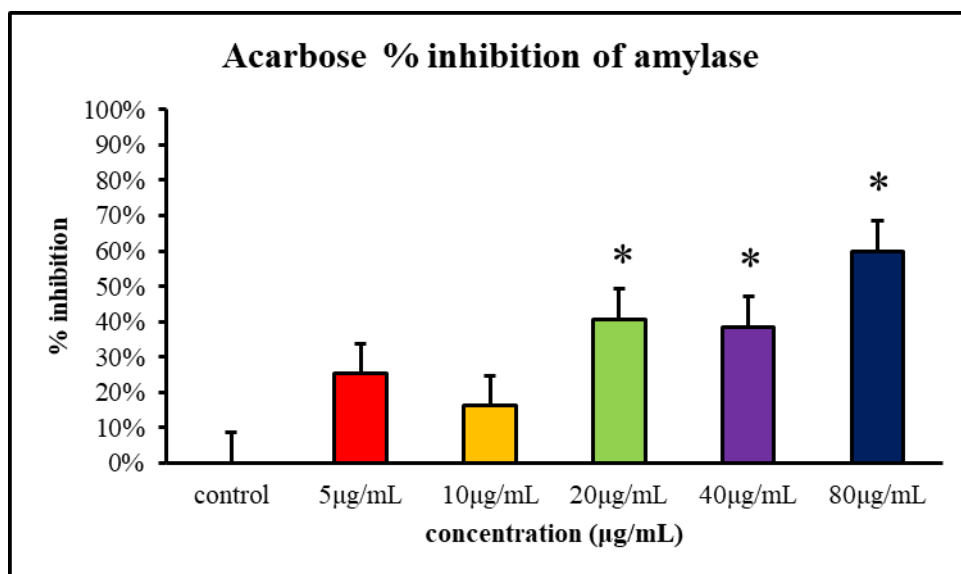


Figure 3. 23: Percentage inhibition of α -amylase by acarbose at 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

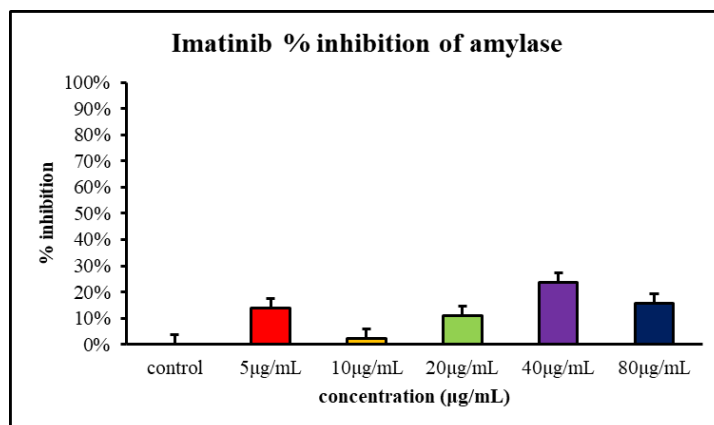
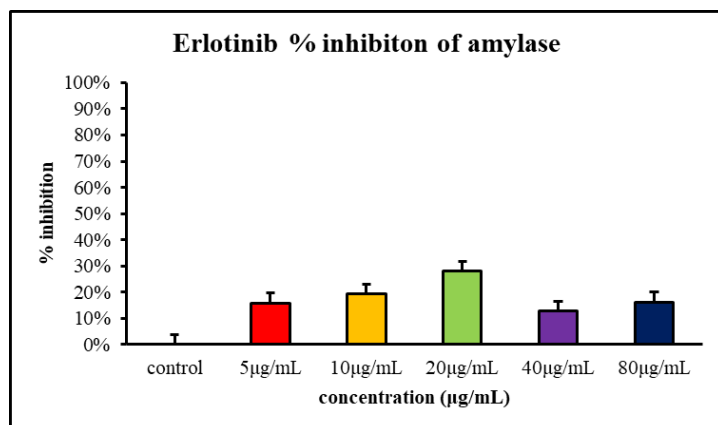
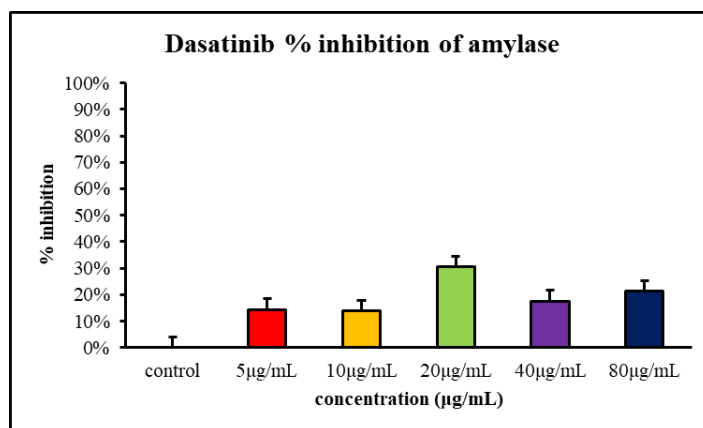
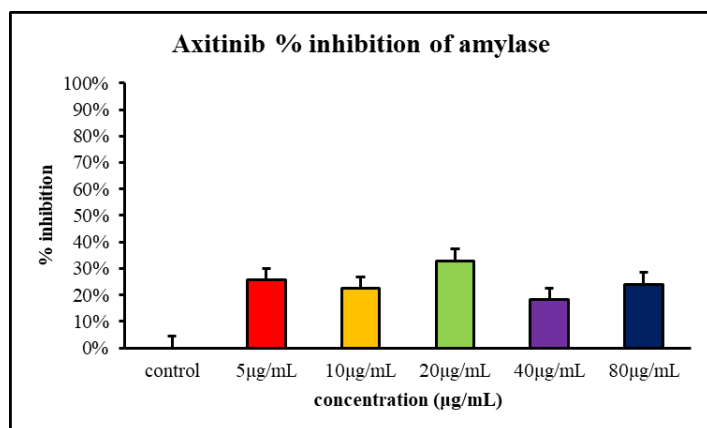


Figure 3. 24: Percentage inhibition of α -amylase by small molecule tyrosine kinase inhibitors (axitinib, dasatinib, erlotinib, and imatinib) at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and

the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

3.5.2 Alpha-glucosidase inhibition assay

The inhibitory effect of acarbose (standard reference drug) and small-molecule tyrosine kinase inhibitors axitinib, dasatinib, erlotinib, and imatinib at different concentrations 5, 10, 20, 40, and 80 $\mu\text{g/mL}$ on alpha-glucosidase was investigated, Figure 3.24 and Figure 3.25. As demonstrated in Figure 3.24 as the concentration of acarbose increased the percentage inhibition of amylase was observed to increase in comparison to the control (not exposed to acarbose). It was observed in Figure 3.25 that axitinib and dasatinib had elevated percentage inhibition of alpha-glucosidase enzyme with statistical significance in comparison to the control. Erlotinib and imatinib on the other hand had percentage inhibition of alpha-glucosidase that was comparable to the control.

Acarbose

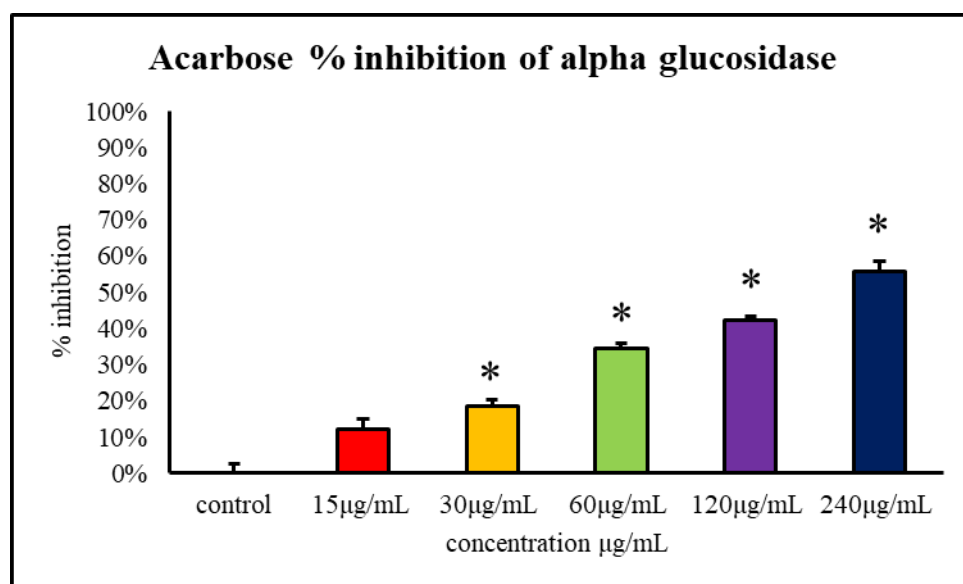


Figure 3. 25: Percentage inhibition of α -amylase by acarbose at 15, 30, 60, 120, and 240 $\mu\text{g/mL}$. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

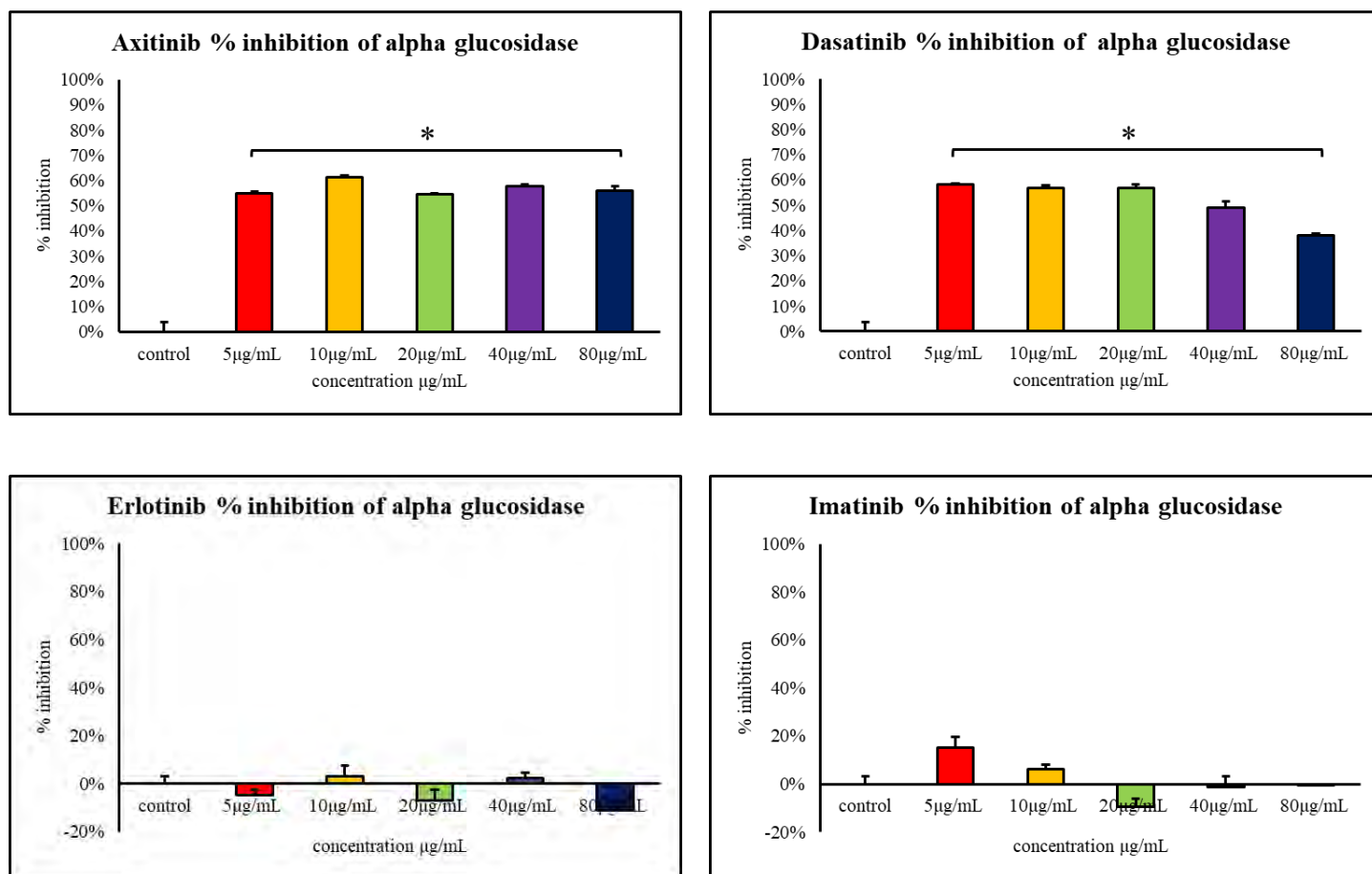


Figure 3. 26: Percentage inhibition of α -amylase of small molecule tyrosine kinase inhibitors at different concentrations (axitinib, dasatinib, erlotinib, and imatinib) 5, 10, 20, 40, and 80 µg/mL. The data is presented as mean $\pm$ SD represented with error bars, ($n = 3$), and the asterisk (*) represents the statistical difference between the test compounds and the control at ($p < 0.05$).

CHAPTER 4: DISCUSSION

Studies have reported that a class of anticancer drugs known as small molecule tyrosine kinase inhibitors show mixed alterations of glucose metabolism in patients with cancer. Some small molecule tyrosine kinase inhibitor generations have been observed to be associated with hyperglycaemia while others have been reported to be associated with hypoglycaemia. This study seeks to establish the underlying mechanisms through which small molecule tyrosine kinase inhibitors alter glucose metabolism *in vivo*. The lowering of blood glucose concentration in patients with cancer being treated with axitinib, dasatinib, erlotinib, and imatinib has been highlighted in numerous studies (162,273,298,299). In some cases, the treatment with these particular small molecule tyrosine kinase inhibitors led to discontinuation of antidiabetic medication by patients due to hypoglycaemic episodes (160,200,240,260). Given the dysregulation of glucose metabolism observed clinically in patients with cancer, the underlying mechanisms are yet to be outlined. In the preceding sections, some of the leading proposed mechanisms through which small molecule tyrosine kinase inhibitors are believed to alter glucose metabolism were briefly highlighted. In this study, four small molecule tyrosine kinase inhibitors namely axitinib, dasatinib, erlotinib, and imatinib were investigated.

C2C12 and HepG2 are insulin-sensitive cell lines that are used *in vitro* as examination models to study glucose uptake mechanisms of various compounds, and most importantly, the development of insulin resistance (276,300,301). Li et al. (2016) investigated the effect of simvastatin on glucose metabolism through *in-vitro* studies on C2C12 cells where they assessed glucose uptake and GLUT4 translocation (302). Sun et al. (2018) investigated the effect of atorvastatin on glucose metabolism in the C2C12 cell line (303). Docrat et al. (2018) investigated the potential impact on the PI3K/Akt signalling pathway by investigating atorvastatin-induced MicroRNA-145 expression in HepG2 cells (304). The HepG2 cell line has been extensively utilized in cancer and toxicology studies (305,306). Skeletal muscle metabolism is mediated by insulin, particularly glucose uptake and the synthesis of glycogen linked with the PI3K/Akt signalling pathway (277). Skeletal muscles stimulated by insulin result in a series of phosphorylation and activation of PI3K/Akt. Activated Akt phosphorylates AS160 resulting in AS160 inactivation which leads to GLUT4 translocation to the cell membrane (277,307). Activated Akt inhibits GSK-3 β enhancing glycogen synthesis through stabilizing glycogen synthase (307). An altered PI3K/Akt signalling pathway leads to disruptions in glucose transport and glycogen synthesis which is directly linked to insulin resistance and type 2 diabetes mellitus. The role of hepatocytes in gluconeogenesis has been investigated in diabetes mellitus due to their dysregulation in insulin resistance conditions, with the HepG2 cell line widely utilized in such studies because of its key role in gluconeogenesis (308). Insulin binds to the insulin receptor initiating the liver's insulin signalling pathway through autophosphorylation of the tyrosine residue in the insulin receptor. This is followed by the activation of downstream signalling molecules such as IR-1 and IR-2, PI3K, and Akt/PKB (308,309). Activation of Akt inhibits the transcription factor FOXO1 which down-regulates PEPCK and inhibits glucogenesis. Akt activation also plays a role in inhibiting GSK-3 β in the inactivation of GS. Active state GS promotes glycogen synthesis (309).

Cytotoxicity studies using the MTT assay were performed as preliminary studies to determine the cell viability of the C2C12 and HepG2 cell line following treatment with small molecule tyrosine kinase inhibitors. The four investigated small molecule tyrosine kinase inhibitors

(axitinib, dasatinib, erlotinib, and imatinib) exhibited low toxicity at concentrations of 5, 10, 20, 40, and 80µg/mL. In this study 80µg/mL was the maximum small molecule tyrosine kinase inhibitor concentration used for analysis, as this was the concentration where a decline in cell viability was observed. Hence concentrations higher than 80µg/mL were not utilized in this study. A cell viability percentage of less than 80% was considered to represent unacceptable cytotoxicity. In this study concentrations with less than 80% cell viability were not considered for further *in-vitro* analyses. Small molecule tyrosine kinase inhibitors inhibit phosphorylation of the receptor tyrosine kinase domain via competitive ATP inhibition. These tyrosine kinase inhibitors disrupt signal transduction pathways of protein kinases. Inhibition of phosphorylation of the receptor at the tyrosine kinase domain by small molecule tyrosine kinase inhibitors interferes with cell differentiation, proliferation, migration, and survival, and they induce cell apoptosis. Small molecule tyrosine kinase inhibitors do not cure cancer, but they put cancer into long-term remission, as they control how fast cells grow and divide. Their effect is on the downstream signalling pathways associated with cell proliferation and differentiation. Hence, we expected the cell viability of C2C12 and HepG2 cell lines not to be drastically affected by exposure to small molecule tyrosine kinase inhibitors for 24 hours. Therefore, these were the concentrations that were used for further analysis.

Observations from this study suggested that exposure of the C2C12 cell line to axitinib induced the translocation of GLUT4 and had a limited effect of the expression of GLUT4 in the absence of insulin, however in the presence of insulin axitinib treated C2C12 cells showed elevated GLUT4 expression. In the absence of insulin C2C12 and HepG2 cell lines exposure to axitinib were observed to express lower levels of Akt compared to the control. In the C2C12 cell line exposed to axitinib in the presence of insulin showed elevated Akt expression. This may indicate a potential synergistic effect of axitinib on insulin activity in C2C12 and HepG2 cell lines. The observations from this study are in support of the clinical observations made by Tyrell and Pwint, who observed that 5mg twice daily resulted in decreased blood glucose concentration and HbA1c levels (199). However, *in vitro*, axitinib had minimal to no effect on glucose uptake in C2C12 and HepG2 cell lines, highlighting the limitations of cell models in accurately replicating the complex metabolic dysfunction present in the human body (310). The C2C12 cell line has unique myosin content and glycogen storage which better suits it for exercise or stress response analysis. Observations from this study highlight that C2C12 cells treated with axitinib had elevated IL-6 expression, however, this was not the case in the HepG2 cell line or harvested media samples. Studies have linked IL-6 to the development of insulin resistance and the pathogenesis of type 2 diabetes mellitus. IL-6 is thought to impair the phosphorylation of the insulin receptor and insulin receptor substrate-1 by promoting the expression of SOCS-3 which is believed to be a potential inhibitor of insulin signalling this all accumulates to insulin resistance (311–313). The findings of this study underscore the potential mechanisms through which axitinib alters glucose metabolism through GLUT4 Akt expression and GLUT4 translocation.

This study targeted the insulin signalling pathway. The insulin signalling pathway is responsible for cellular metabolism and energy regulation. This pathway is integral to understanding the metabolic impact of small molecule tyrosine kinase inhibitors like erlotinib on glucose homeostasis, providing insights that could help guide their clinical use and patient management. Erlotinib is believed to reduce insulin resistance through inhibition of TNF- α (261). Inflammation has been identified as the link between obesity and the development of

insulin resistance (314). This study investigated erlotinib's effect on IL-6 to uncover erlotinib's effect on inflammation *in vitro*. In this study erlotinib's effect on IL-6 was explored to clarify its impact on inflammation *in vitro*, given that inflammation is strongly associated with diabetes mellitus and insulin resistance. IL-6 is a prominent pro-inflammatory cytokine, often elevated in metabolic disorders, assessing its levels helps us understand how small molecule tyrosine kinase inhibitors like erlotinib may modulate inflammatory pathways. By investigating IL-6 levels in C2C12 and HepG2 cells, the study aimed to elucidate the broader anti-inflammatory potential of erlotinib and its possible influence on insulin sensitivity, which could offer new insights into managing insulin resistance in clinical contexts. This study shows that the C2C12 cell line when exposed to erlotinib demonstrated elevated GLUT-4 translocation in comparison to the control. However, the glucose uptake in C2C12 cells treated with erlotinib was similar or comparable to the control. This is potentially due to the presence of GLUT1 in the C2C12 cell line which plays a role in basal glucose uptake, resulting in similar glucose uptake levels in cells treated with erlotinib and the control after 24 hours. Skeletal muscle expresses both GLUT4 and GLUT1 transporters. GLUT1 according to Marette et al. (1992) accounts for 5-10% of total glucose carriers in rat skeletal muscle (315,316). This contributes to steady glucose uptake. A lower expression of GLUT-4 in C2C12 cells treated with erlotinib in the absence of insulin was observed while in the presence of insulin, an increase in GLUT-4 expression was noted. Studies have shown that GLUT4 gene expression is regulated by numerous factors in skeletal muscle for example thyroid hormone, MEF2, NF1, MyoD myogenic, and others (317). Erlotinib may potentially interact with these factors or act directly as a factor. Increases in p38 MAPK and AMPK phosphorylation and subsequent effects on their downstream targets following exercise have been recognised as a potential mechanism for increase in GLUT4 gene expression (318). With regards to IL-6, as the concentration of erlotinib increased the percentage of IL-6 in media harvested from the HepG2 cell line was observed to decrease. An increase in IL-6 in C2C12 cells as well as their media was observed with the increase of erlotinib concentration. The C2C12 cells exposed to small molecule tyrosine kinase inhibitors had elevated IL-6 expression across all concentrations except at 5µg/mL. Cytokines are produced in response to stress, hence this study sought to uncover the effect of small molecule tyrosine kinase inhibitors of IL-6 in C2C12 and HepG2 cells (319). Elevated IL-6 observed in this study supports the antidiabetic properties demonstrated by small molecule tyrosine kinase inhibitors in other studies. Wallenius et al. (2002) demonstrated that IL-6 deficient mice developed glucose intolerance and obesity but when treated with IL-6 chronically, attenuation of these metabolic perturbations was observed.

As mentioned in the erlotinib section above, this study targeted the insulin signalling pathway and pro-inflammatory cytokine IL-6. It has been suggested that imatinib promotes β -cell survival in response to pro-inflammatory cytokines and toxins. Imatinib has also been shown to inhibit Akt which is part of the insulin signaling pathway (320–322). Imatinib by inhibiting the phosphorylation of certain proteins potentially leads to better signalling and/or improved functioning of effectors which then improves insulin sensitivity. Typically, the phosphorylation of insulin signalling proteins leads to glucose uptake (183). Hägerkvist et al. (2006) observed that imatinib had the ability to reduce the effect of several different apoptotic-promoting substances including the pro-apoptotic MAP kinase JNK (323). As stated above, inflammation has been proven to be closely linked to the pathogenesis of type 2 diabetes mellitus. Interestingly, the IL-6 levels in the C2C12 cell line were observed to have increased as the concentration of imatinib increased. The same observation was made in media that was

harvested from the C2C12 cell line. An increase in IL-6 has been linked with a reduction in obesity and glucose intolerance (324). Huang et al. (2009) demonstrated a reduction in inflammation by imatinib in mdx mice (325). They highlighted a suppression of IL-1 β and TNF- α expression. In some studies, imatinib demonstrated efficacy in the treatment of immune-related diseases namely inflammatory bowel disease, autoimmune diabetes, rheumatoid arthritis and multiple sclerosis (326,327). An increase in glucose uptake with increasing concentrations of imatinib in the C2C12 cell line was observed. The *in vitro* observations made were in agreement with observations made in numerous retrospective studies where patients on imatinib therapy were noted to demonstrate a reduction in blood glucose concentration, with some patients discontinuing their antidiabetic medication while on imatinib therapy. Interestingly, we noted that as the concentration of imatinib increased (5 to 20 μ g/mL particularly) a decrease in GLUT-4 translocation in the absence of insulin was observed in the C2C12 cell line. Typically, it is expected that an increase in glucose uptake is coupled with an increase in GLUT4 translocation in skeletal muscle cells. This potentially means that glucose uptake was promoted through the other transporter, GLUT1. In the presence of insulin however, a gradual increase in GLUT-4 translocation was observed with increasing imatinib concentrations (5 to 20 μ g/mL in particular). However, the maximum GLUT-4 translocation observed in C2C12 cells treated with imatinib was comparable to the control. When the C2C12 cell line was treated with imatinib in the absence of insulin an elevated GLUT-4 expression was observed, the same was not observed in the presence of insulin. In the presence of insulin, we observed a slight increase in GLUT-4 expression at high concentrations. We propose that imatinib may activate a pathway that promotes GLUT4 expression. Given the elevated GLUT4 translocation levels in C2C12 cells treated with imatinib and decreasing trend with increasing imatinib concentrations, the link between imatinib's effect on GLUT4 expression and translocation which leads to increased glucose uptake needs to be closely investigated. This may mean that imatinib has the potential to promote the expression of GLUT-4 independent of insulin. The C2C12 cells treated with imatinib in the absence of insulin demonstrated an increase in Akt expression whereas in the HepG2 cell line, a decrease (5 to 20 μ g/mL) and then an increase (20 to 80 μ g/mL) was observed.

This study suggests that dasatinib plays a role in the insulin signalling pathway in a way that may potentially enhance glucose uptake. This is in support of literature that shows the clinical hypoglycaemic properties of dasatinib in patients. Figures 3.5 and 3.6 depict that treatment with dasatinib resulted in enhanced GLUT4 translocation in the C2C12 cell line exposed to insulin and in C2C12 cells where insulin was absent. The enhanced translocation of GLUT4 was more pronounced in the absence of insulin. The elevated GLUT4 translocation in the C2C12 cell line treated with dasatinib, in particular in the absence of insulin may be an indication that dasatinib might stimulate GLUT4 translocation through an alternative signalling pathway, perhaps the AMPK. An insulin-independent pathway such as the AMPK pathway can facilitate GLUT4 translocation. AMPK is an energy sensor that regulates cellular metabolism namely glucose and lipid. When activated it promotes glucose uptake and lipid oxidation to produce energy (328). In skeletal muscle when insulin binds to its cell-surface receptor it triggers the insulin signalling pathway leading to the translocation of GLUT4 via activation of the PI3K/Akt pathway (329,330). GLUT4 is primarily responsible for increased glucose uptake as a response to insulin in peripheral tissues namely adipose and muscle (331,332). In terms of expression of GLUT4 in the C2C12 cell line, increased levels of GLUT4 were only observed in C2C12 cells that were exposed to insulin. In the presence or absence of

insulin, the C2C12 cell line treated with dasatinib resulted in elevated levels of Akt expression. As stated in the results section, dasatinib was selected for further analysis due to the mixed reports in the literature. In this study dasatinib was the only compound to show percentage translocation of GLUT4 that was elevated and above the control in the C2C12 cell line exposure to insulin and in the absence of insulin. These observations necessitated the need to uncover whether dasatinib is capable of promoting GLUT4 translocation and expression independent of the insulin receptor. To investigate this hypothesis dasatinib was analysed further. One of the assays analysed co-administration of dasatinib with wortmannin, a known potent PI3K inhibitor. As the concentration of dasatinib increased, an increase in glucose uptake in C2C12 and HepG2 cells was observed, despite the inhibition of PI3K by wortmannin. This suggests that dasatinib could potentially promote glucose uptake independently of the insulin receptor. The impact dasatinib has on insulin-resistant cell models was examined further, by investigating whether dasatinib promotes glucose uptake under conditions mimicking insulin resistance where the conventional pathways that enhance glucose uptake are impaired. The results suggest that insulin-resistant induced HepG2 cells treated with dasatinib demonstrated higher glucose uptake at 10 and 20 µg/mL compared to the control (insulin-resistant HepG2 cells not treated with dasatinib). Interestingly, dasatinib had no effect on insulin-resistant induced C2C12 cells as depicted by Figure 3.18. Co-administration of dasatinib and wortmannin had a minimal effect on the translocation of GLUT-4 in the C2C12 cell line, particularly at higher concentrations. The same was observed for insulin-resistant C2C12 cells. As depicted in Figure 3.3 treatment of C2C12 cells with dasatinib led to slightly elevated glucose uptake. The observations made in this study strongly support the blood-lowering properties of dasatinib that have been reported.

Our findings suggest that dasatinib may act through a mechanism alternative to the insulin receptor signalling pathway. Dasatinib potentially elicits the observed effects through signalling pathways similar to those activated by PPAR γ agonists such as glitazones. PPAR γ is a glucose and lipid metabolism regulator, its synthetic ligands like glitazones have been proven to improve glucose and insulin parameters and increase insulin sensitivity (333). PPAR γ agonists such as thiazolidinediones from which glitazones are derived enhance insulin sensitivity and reduce gluconeogenesis in the liver which leads to a reduction in the fasting plasma glucose levels (334,335). PPAR γ agonists thiazolidinediones are regarded as the only true insulin sensitizers that reduce postprandial glucose levels (336–338). The precise mechanism through which dasatinib lowers blood glucose concentration in patients is unknown. Findings from our study support studies that reported dasatinib to exhibit hypoglycaemic effects in patients. If it is proven that dasatinib has PPAR γ agonist (ligand) properties, then dasatinib has the ability to augment glucose uptake and it could be a potential treatment for insulin resistance especially where traditional insulin sensitizers have failed. summary, the findings from this study support the clinical findings reported in studies, which state that small molecule tyrosine kinase inhibitors alter glucose metabolism (154,298,339,340). This can be seen in the elevated Akt and GLUT4 translocations depicted in Figures 3.9 and 3.5 respectively. Imatinib in Figure 3.3 showed elevated glucose uptake compared to the control and cells treated with insulin, the standard drug. From the findings, small molecule tyrosine kinase inhibitors potentially alter glucose metabolism through an alternative pathway to the insulin receptor signalling pathway. Potentially through their role in AMPK and/or PPAR γ activation. The elevated translocation of GLUT4 in C2C12 cells treated with small molecule tyrosine kinase inhibitors in the absence of insulin is a key observation in

support of this hypothesis. There is need to elucidate small molecule tyrosine kinase inhibitors effect on gene transcription of GLUT4. Another potential mechanism to consider is their effect on GLUT1. Coupling the findings with the reports from retrospective clinical studies, patients with diabetes on small molecule tyrosine kinase inhibitors therapy need to have their blood glucose constantly monitored. The studies need to be extended to non-diabetic patients. In conclusion, the findings from this study support the clinical findings reported in studies, which state that small molecule tyrosine kinase inhibitors alter glucose metabolism .

Dasatinib is an orally administered small molecule tyrosine kinase inhibitor used in the treatment of chronic myeloid leukaemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukaemia. Dasatinib is ingested as a once-daily dose of 100mg for CML and 140mg for Philadelphia chromosome-positive acute lymphoblastic leukaemia. It was modelled to inhibit SRC and ABL, it also shows activity in multiple kinase names and PDGFR- α , PDGFR- β , and c-Kit (269,341). Concerning the effects of dasatinib on glucose metabolism in patients there have been mixed observations from studies. Lu Yu et al. (2019) in a retrospective study of 370 patients with chronic myeloid leukaemia highlighted that the mean fasting glucose level significantly increased in patients that underwent dasatinib therapy for 3 or more months (270). Some studies have suggested that dasatinib can lower blood glucose concentration in patients undergoing treatment (240,243). In a case report by Katsumi Iizuka et al., a 63-year-old man on dasatinib treatment showed improvements in their glycaemic index (to less than 6), an improvement in insulin sensitivity, increased plasma levels of adiponectin and leptin (242). The clinical antidiabetic properties of dasatinib observed in the studies mentioned above have led to the proposal of dasatinib as a novel diabetes mellitus therapy. The mechanism through which dasatinib alters glucose metabolism however remains unclear. The mixed hypoglycaemia or hyperglycaemia induced by dasatinib treatment as reported in studies can be attributed to comorbidities, different age populations, genetics, and ethnicity.

Of the four small molecule tyrosine kinase inhibitors investigated, dasatinib showed the most potential with regards to repurposing them for diabetes therapy. Dasatinib was the only compound that demonstrated translocation of GLUT4 that was increasing and above the control in C2C12 that were exposed to insulin. This potentially means that dasatinib has an added stimulus effect on the translocation of GLUT4 and subsequently the uptake of glucose or promotes GLUT4 translocation through an alternative pathway, like the AMPK pathway. Dasatinib was not able to counteract the effect of wortmannin. However, compared to the cells (both C2C12 and HepG2) that were only exposed to wortmannin, cells treated with dasatinib showed increasing percentage glucose uptake with increasing concentrations of dasatinib. It was noted however that despite this increasing trend glucose uptake was still lower than in cells not exposed to either dasatinib and wortmannin or just wortmannin.

There are two major factors to consider when it comes to the repurposing of small molecule tyrosine kinase inhibitors, such as the cost and side effects. It has been reported that nearly all patients experience treatment related adverse events while on small molecule kinase inhibitor treatment (342–344). One study stated that up to 70% of patients undergoing small molecule tyrosine kinase inhibitors therapy experienced adverse events (345). Caldemeyer et al. reported that 60% of the patients on imatinib treatment experienced oedema, 50% nausea, 49% muscle cramps, and 47% musculoskeletal pain. However, overall small molecule tyrosine kinase inhibitors appear to be well tolerated with patients experiencing mild or moderate adverse events. Some common adverse events include skin toxicity, diarrhoea, and fatigue

(154,345,346). Adverse events led to discontinuation of imatinib in only 4% of patients in some study by Kim et al., (2018) (347). Small molecule tyrosine kinase inhibitors share the same mechanism of action of competitive inhibition of ATP at the catalytic site of tyrosine kinase. However, they differ in the specific tyrosine kinase they target, hence they elicit the varied pharmacological responses and side effects. This difference opens the possibility for targeted therapy and minimal side effects with regard to their use in DM management.

The estimated cost of small molecule tyrosine kinase inhibitors for chronic myeloid leukaemia ranges between \$ 3 632 and \$8 429 (348). While according to drugs.com the price for 30 metformin 500mg oral tablets is approximately \$13,15 (349). According to drugs.com, 30 tablets of 100 mg oral imatinib tablets and 28 oral tablets of 12,5 mg sunitinib have an approximate cost of \$219 and \$5 198–\$5 492 respectively (350). For chronic use in the management of DM, the cost-benefit ratio is not in favour of small molecule kinase inhibitors when compared to current antidiabetic agents which already have healthcare budgets across the globe stretched. The cost of small molecule tyrosine kinase inhibitors is a major obstacle that would limit their use in DM. Another concern to highlight is that the current price of small molecule tyrosine kinase inhibitors is a direct contributing factor to non-adherence mortality from certain cancers.

The findings from this study coupled with further extensive studies will be a valuable tool for physicians, prescribers, and other healthcare professionals. For example, diabetic patients with cancer being treated by small molecule tyrosine kinase inhibitors may need to be assigned to those that are known to improve blood glucose concentration. This may have potential benefits including a reduction in polypharmacy which is associate with adverse events. The line of thought highlighted in this study can be backed by studies which indicated that some patients on small molecule tyrosine kinase inhibitors required dose reductions in their diabetic medication with some case patients discontinuing diabetes treatment while under a small molecule kinase inhibitor therapy. With the opposite being true as well, patients at risk of hyperglycaemia complications including neuropathy or kidney damage may need to be kept away from small molecule tyrosine kinase inhibitors known to induce hyperglycaemia.

This study highlights the significance of continuous therapeutic drug monitoring in patients with cancer on small molecule tyrosine kinase inhibitors as well the need to channel efforts into the repurposing of promising small molecule tyrosine kinase inhibitors as diabetes mellitus treatment.

CHAPTER 5: CONCLUSIONS

The development of small molecule tyrosine kinase inhibitors has revolutionized the cancer treatment landscape, offering hope for targeted therapy. Small molecule tyrosine kinase inhibitors have however been associated with glycaemic dysregulation in patients with cancer, with some small molecule tyrosine kinase inhibitors improving hyperglycaemia. This study aimed to uncover the effect of small molecule tyrosine kinase inhibitors on glycaemic control particularly focusing on the insulin signalling pathway. The ultimate goal is to improve patient health outcomes and prognosis. The findings suggest that axitinib, dasatinib, erlotinib, and imatinib play a role in glycaemic dysregulation. They all showed the potential to reduce blood glucose levels; this was deduced from their effect on GLUT-4 and Akt expression as well as GLUT-4 translocation observed in this study. Dasatinib showed promising potential with

regard to antidiabetic capabilities. The findings from this study are in accordance with the case studies and retrospective studies conducted on the effect of small molecule tyrosine kinase inhibitors on blood glucose concentrations. While these compounds have shown promise in modulating glucose metabolism and insulin signalling pathways the high cost associated with small molecule tyrosine kinase inhibitors presents a significant barrier to their potential application in the management of diabetes mellitus.

CHAPTER 6: LIMITATIONS AND FUTURE STUDIES

This study has potential limitations. Cell-based models are extensively used to study the pathogenesis of insulin resistance and diabetes mellitus. Using *in-vitro* cell culture in a laboratory setting is a cost-effective and straightforward approach to addressing the 3R principles (reduction, refinement, and replacement) in animal studies. Due to similarities between animals and humans at a biological level, animal studies are widely utilized. It is widely accepted that animal studies are essential in designing novel and effective treatments for metabolic diseases the likes of diabetes mellitus. The use of animal models in preclinical studies is limited due to being time-consuming and the high cost of maintenance. When working with animal models, ethical considerations pertaining to the amoral procedure of handling animals need to be considered.

The expression of GLUT1 was not analysed due to the financial burden of procuring GLUT1 antibodies. GLUT1 Colorimetric Cell-Based ELISA kits are costly, with one kit containing one 96-well plate. The estimated cost of one kit is \$480 (as of 01/11/2024). Gene expression studies on the expression of GLUT4, GLUT1, and Akt in C2C12 and HepG2 cell lines treated with small molecule tyrosine inhibitors needs to be explored. Due to financial limitations, this study only investigated the C2C12 and HepG2 cell lines. There is a need to investigate the impact of small molecule tyrosine inhibitors on pancreatic and adipose cell lines. Studies have suggested that some small molecule tyrosine inhibitors such as imatinib preserve β -cell viability and mass, hence the need for further investigation on pancreatic cell models. The effect of small molecule tyrosine inhibitors on the pancreas in particular their effect on insulin secretion and the mass of the pancreas in *in vivo* studies. This will provide insight into the mechanisms through which small molecule tyrosine inhibitors potentially improve glycaemic control in patients. AMPK is an enzyme that helps to regulate cellular energy balance by sensing and responding to low energy levels. Cancerous cells are energy-demanding which potentially stimulates this pathway. This necessitates the need to measure the activity of AMPK, which in the context of our study was not performed. In particular, there is a need to investigate the effect of small molecule tyrosine kinase inhibitors particularly dasatinib on AMPK pathway via experiments measuring AMPK activation for example phosphorylation. Future investigations could help uncover whether dasatinib directly or indirectly influences PPAR γ pathways. This will help reconcile the elevated GLUT4 translocation findings highlighted in our study.

The long-term effects of the altered glucose metabolism by small molecule tyrosine kinase inhibitors are poorly documented. There is a necessity to elucidate whether the cases of glycaemic dysregulation in patients are isolated events that are due to particular conditions. For instance, certain morbidities render patients more susceptible to phenomena which individuals

where these morbidities are absent are not as susceptible. This calls for large-scale follow-up studies and not just limiting our understanding to retrospective case studies. This would help uncover if the effects elicited by the small molecule tyrosine kinase inhibitors are limited to a particular demographic. If the effects can be expected in the general population, the relevant healthcare professionals, especially oncologists need to be informed accordingly for the safe and optimum use of these compounds.

Indeed, understanding the mechanism of action at the molecular level is a starting point, which should then be followed by elucidating the structure-activity relationships. A deeper understanding of structure-activity relationships will allow these compounds to be modified accordingly. As mentioned earlier, the mechanism of action of small molecule tyrosine kinase inhibitors is largely the same (ATP competitive inhibition), while the differences are conferred by the spectrum of targeted tyrosine kinases. Elucidating why certain small molecule tyrosine kinase inhibitors have a higher affinity for specific tyrosine kinase as compared to others is key. Small molecule tyrosine kinase inhibitors' effect on glucose metabolism is strongly linked to the tyrosine kinase they interact with. Structural modification of small molecule tyrosine kinase inhibitors paves the way for specificity in terms of the tyrosine kinase they will interact strongly with. This in turn may be utilized to optimise their role in glucose metabolism.

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APPENDICES

Appendix 1: Abstract of a review article published in the Frontiers in Pharmacology.

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Front. Pharmacol., 01 February 2024
Sec: Experimental Pharmacology and Drug Discovery
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In light of the expected increase in the prevalence of diabetes mellitus due to an aging population, sedentary lifestyles, an increase in obesity, and unhealthy diets, there is a need to identify potential pharmacological agents that can heighten the risk of developing diabetes. Similarly, it is equally important to also identify those agents that show blood glucose-lowering properties. Amongst these agents are tyrosine kinase inhibitors used to treat certain types of cancers. Over the last two decades, there has been an increase in the use of targeted chemotherapy for cancers such as renal cell carcinoma, chronic leukaemia, and gastrointestinal stromal tumours. Small molecule tyrosine kinase inhibitors have been at the forefront of targeted chemotherapy. Studies have shown that small molecule tyrosine kinase inhibitors can alter glycaemic control and glucose metabolism, with some demonstrating hypoglycaemic activities whilst others showing hyperglycaemic properties. The mechanism by which small molecule tyrosine kinase inhibitors cause glycaemic dysregulation is not well understood, therefore, the clinical significance of these chemotherapeutic agents on glucose handling is also poorly documented. In this review, the effort is directed at mapping mechanistic insights into the effect of various small molecule tyrosine kinase inhibitors on glycaemic dysregulation envisaged to provide a deeper understanding of these chemotherapeutic agents on glucose metabolism. Small molecule tyrosine kinase inhibitors may elicit these observed glycaemic effects through preservation of β -cell function, improving insulin sensitivity and insulin secretion. These compounds bind to a spectrum of receptors and proteins implicated in glucose regulation for example, non-receptor tyrosine kinase SRC and ABL. These receptor tyrosine kinase EGFR, PDGFR, and FGFR.

Appendix 2: Abstract for a presentation that was presented during the South African Society for Basic and Clinical Pharmacology at Rhodes University on the 24th - 27th August 2023 Africa, 31- 3 September 2023



Certificate of Participation

This is to confirm that

Mr Jakudzwa Mugiya

Has attended the 2023 Conference
of
The South African Society for Basic and Clinical Pharmacology
at Rhodes University
on the 24th - 27th August 2023
(accredited for 18 CPD Points)



"Pharmacology celebrating resilience: Current and Future strategies"

Prof Mamza Mothibe
Chairperson: 2023 Organising Committee

Ms Tanusha Dukhan
Secretary: 2023 Organising Committee

Appendix 3: Abstract for a presentation that was presented during the Faculty of Pharmacy postgraduate research day at Rhodes University on the 20th of October 2023.

Takudzwa Mugiya

UNDERSTANDING THE ROLE OF SMALL MOLECULE KINASE INHIBITORS ON GLUCOSE METABOLISM IN KEY INSULIN TARGET TISSUES IN-VITRO

Takudzwa Mugiya, Ntethelelo Sibiya
Pharmacology Division,

Background: The prevalence of diabetes mellitus due to an aging population, sedentary lifestyles, an increase in obesity, and unhealthy diets, continues to pose a threat to the world population. This necessitates the identification of pharmacotherapy agents that can heighten the risk of developing diabetes. Amongst, these agents are the drugs used for cancer as chemotherapy including tyrosine kinase inhibitors. Tyrosine kinase inhibitors have been reported to alter glycaemic control and glucose metabolism, with some demonstrating hypoglycaemia whilst others show hyperglycaemic properties. However, the mechanism by which tyrosine kinase inhibitors alter glycaemic control is not well understood, hence, this study seeks to elucidate the effect of various tyrosine kinase inhibitors on glucose handling in hepatic (HEPG-2) and skeletal (C2C12) cells.

Methods: In this study, both cell lines were sub-divided according to their treatment and control groups. The treatments groups were exposed to small kinase inhibitors imatinib and erlotinib for 24 hours. Thereafter, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability after treatment. while, cell culture glucometer was used to assess the glucose uptake in the cell culture medium post treatment.

Results: The results revealed that neither imatinib nor erlotinib significantly affected cell viability compared to the control group. Both drugs resulted in a decrease in glucose utilization was observed in the cell culture media compared to the control, indicating a potential impact on glucose metabolism. The minimum concentration was 5 ug/ml and the maximum concentration was 80/ug/ml. Notably, this elevation in glucose levels was observed across all drug concentrations tested, suggesting that the effect is independent of drug concentration. lower concentrations might be ineffective and higher might be toxic.

Conclusion: The results demonstrate that these inhibitors do not affect cell viability but induce a concentration-independent reduction in glucose utilization/uptake in the cell culture media. These findings highlight the need for further investigation into the underlying mechanisms of altered glucose metabolism caused by kinase inhibitors, potentially contributing to a better understanding of their effects on metabolic homeostasis and providing a basis for future therapeutic strategies.

Appendix 4: Abstract for a presentation that was presented during the RU Postgraduate Conference at Rhodes University, 8-9 September 2023.

Mugiya Takudzwa
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Pharmacy
Topic: Understanding the Role of Small Molecule Kinase Inhibitors on Glucose Metabolism in Key Insulin Target Tissues In-Vitro
Understanding the Role of Small Molecule Kinase Inhibitors on Glucose Metabolism in Key Insulin Target Tissues In-Vitro Takudzwa Mugiya, Ntethelelo Sibiya Pharmacology Division, Background: The prevalence of diabetes mellitus due to an aging population, sedentary lifestyles, an increase in obesity, and unhealthy diets, continues to pose a threat to the world population. This necessitates the identification of pharmacotherapy agents that can heighten the risk of developing diabetes. Amongst, these agents are the drugs used for cancer as chemotherapy including tyrosine kinase inhibitors. Tyrosine kinase inhibitors have been reported to alter glycaemic control and glucose metabolism, with some demonstrating hypoglycaemia whilst others show hyperglycaemic properties. However, the mechanism by which tyrosine kinase inhibitors alter glycaemic control is not well understood, hence, this study seeks to elucidate the effect of various tyrosine kinase inhibitors on glucose handling in hepatic (HEPG-2) and skeletal (C2C12) cells. Methods: In this study, both cell lines were sub-divided according to their treatment and control groups. The treatment groups were exposed to small kinase inhibitors imatinib and erlotinib for 24 hours. Thereafter, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability after treatment. While, cell culture glucometer was used to assess the glucose uptake in the cell culture medium post treatment. Results: The results revealed that neither imatinib nor erlotinib significantly affected cell viability compared to the control group. Both drugs resulted in a decrease in glucose utilization was observed in the cell culture media compared to the control, indicating a potential impact on glucose metabolism. The minimum concentration was 5 ug/ml and the maximum concentration was 80 ug/ml. Notably, this elevation in glucose levels was observed across all drug concentrations tested, suggesting that the effect is independent of drug concentration. Lower concentrations might be ineffective and higher might be toxic.

Appendix 5: Abstract for a presentation that was presented during the South African Society for Basic and Clinical Pharmacology at Sefako Makgatho Health Science University on the 15th - 17th September 2024

Examining the Impact of Small Molecule Tyrosine Kinase Inhibitors on Glucose Metabolism in Primary Insulin Target Tissues In-Vitro

TC Mugiya

Rhodes University, Pharmacology Department

Introduction: Diabetes mellitus is rising due to aging, sedentary lifestyles, obesity, and unhealthy diets, posing a global health threat. Identifying pharmacotherapy, such as small molecule tyrosine kinase inhibitors (SMTKIs), is crucial. These drugs, used in cancer chemotherapy, can affect glycaemic control and metabolism variably. This study aims to clarify how different SMTKIs impact glucose regulation in hepatic (HepG2) and skeletal muscle (C2C12) cells.

Aim and Objectives: This study aims to elucidate how SMTKIs affect glucose metabolism in C2C12 and HepG2 cells in vitro, including their impact on glucose uptake, AKT, GLUT-4, and IL-6 expression, GLUT-4 translocation, and alpha-amylase and alpha-glucosidase activity.

Methods: In this study, C2C12 and HepG2 cells were seeded in well plates and the initial media glucose concentration was recorded. Cells were treated with small molecule tyrosine kinase inhibitors; imatinib, dasatinib, axitinib, and erlotinib for 24 hours. Thereafter, the effect of the test drugs was assessed on cell viability, glucose uptake, expression of AKT GLUT-4 and IL-6, and translocation of GLUT-4.

Results and Discussion: Cells treated with SMTKIs were viable after 24 hours. A concentration-dependent decrease in C2C12 cells treated with imatinib was observed. SMTKIs demonstrated an increase in GLUT4 translocation in the absence of insulin. It was also observed that these cells showed lowered levels of GLUT-4 in comparison to the control. SMTKIs showed an increase in AKT expression. SMTKIs demonstrated inhibition of alpha-amylase, while only dasatinib and axitinib showed inhibition of alpha-glucosidase.

Conclusion: SMTKIs impact glucose metabolism in C2C12 and HepG2 cells via their effect on GLUT-4 translocation and expression and AKT expression. Further research is needed to better understand these mechanisms' effects on metabolic homeostasis and inform future therapeutic strategies.

Appendix 6: Certificate for presentation at the Rhodes University Postgraduate Conference Building Bridges, 13-14 September 2024



**Investigating the Impact of Small Molecule Tyrosine Kinase
Inhibitors on Glucose Metabolism in Primary Insulin Target Tissues
In-Vitro**

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Background information: In light of the expected increase in the prevalence of diabetes mellitus due to an aging population, sedentary lifestyles, an increase in obesity, and unhealthy diets, there is a need to identify potential pharmacological agents that can heighten the risk of developing diabetes. Similarly, it is equally important to also identify those agents that show blood glucose-lowering properties. Amongst these agents are tyrosine kinase inhibitors used to treat certain types of cancers.

Purpose: This study aims to elucidate how SMTKIs affect glucose metabolism in C2C12 and HepG2 cells in vitro, including their impact on glucose uptake, AKT, GLUT-4, and IL-6 expression, GLUT-4 translocation, and alpha-amylase and alpha-glucosidase activity.

Methods: In this study, C2C12 and HepG2 cells were seeded in well plates and the initial media glucose concentration was recorded. Cells were treated with small molecule tyrosine kinase inhibitors; imatinib, dasatinib, axitinib, and erlotinib for 24 hours. Thereafter, the effect of the test drugs was assessed on cell viability, glucose uptake, expression of AKT GLUT-4 and IL-6, and translocation of GLUT-4.

Results: Cells treated with SMTKIs were viable after 24 hours. A concentration-dependent decrease in C2C12 cells treated with imatinib was observed. SMTKIs demonstrated an increase in GLUT4 translocation in the absence of insulin. It was also observed that these cells showed lowered levels of GLUT-4 in comparison to the control. SMTKIs showed an increase in AKT expression. SMTKIs demonstrated inhibition of alpha-amylase, while only dasatinib and axitinib showed inhibition of alpha-glucosidase. Of the 4 SMTKIs, dasatinib showed potential for repurposing for diabetes treatment.

Conclusions: SMTKIs impact glucose metabolism in C2C12 and HepG2 cells via GLUT-4 translocation and expression and alter AKT expression. Further research is needed to better understand these mechanisms' effects on metabolic homeostasis and inform future therapeutic strategies.