The influence of newly synthesized hybrid Thiazolidinedione (TZD)-methoxy on the expression and acetylation of diabetes related genes

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DECLARATION

I, **Bonolo Betty Masilo**, wish to declare that this dissertation is my own work unless where acknowledged. It has not been submitted to any institution for the purpose of obtaining a qualification.

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Signature of candidate

Signed at MEADOWLANDS..... on this ...07.... day of.....APRIL....., 2018

Prof E Mukwevho

(Supervisor)

Signed at ..... on this .....09..... day of.....APRIL....., 2018

DEDICATION

This work is dedicated to my late father who taught me that I can do anything I put my mind to. I would also like to devote this work to my entire family (especially my mother) for always believing in me, keeping me motivated and supporting my dreams.

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ABSTRACT

Type 2 diabetes mellitus is a metabolic illness that is categorized by fasting plasma glucose levels above 7 mmol/l. It is preceded by insulin resistance which is triggered by increased dietary fats and sedentary lifestyle. Type 2 diabetes is often associated with the down regulation of critical genes that confer protection against the disease, especially genes involved in glucose transport and lipid oxidation (*NRF-1*, *GLUT4*, *MEF2A*). Furthermore, diabetes is also associated with decreased expression and activity of various histone acetyl transferases (*pCAF/p300*, *GCN5*) involved in acetylation of these gene promoters. Therefore, finding the means to up-regulate genes that confer protection against diabetes can be a positive step towards the discovery of new and effective therapeutic modalities in the treatment of type 2 diabetes. Though metformin has been widely used as the most potent drug in treating and managing type 2 diabetes, it still does not cure the disease and is associated with some side effects. Therefore, this study was aimed at investigating whether the newly synthesized Thiosemicarbazone-triazole hybrid compound can be a better therapeutic modality in the treatment of type 2 diabetes than metformin.

This study was conducted in C2C12 myotubes, which are mouse embryonic stem cells. Palmitate was introduced in this cell line to induce insulin resistance. A cell viability test was done using MTT assay kit to establish an optimal Thiazolidinedione (TZD) concentration to be used in these cells. There were five treatment groups in this study, namely, control (normal), palmitate (to induce insulin resistance), palmitate + metformin, palmitate + TZD and TZD only. Complementary Deoxyribonucleic acid (cDNA) was synthesized from total ribonucleic acid (RNA) extracted from myotubes and amplified

using Quantitative Polymerase Chain Reaction (qPCR). To study the influence of TZD on transcriptional factor binding to their respective *cis*-element DNA binding domains, Chromatin Immunoprecipitation (ChIP) was used to investigate the binding extent of *MEF2A* to the *GLUT4* promoter. Furthermore, the effect of this TZD on antioxidant properties was also assessed. In this respect, Trolox Equivalent Antioxidant Capacity (TEAC) was used to measure radical scavenging activity while Ferric Reducing Antioxidant Power (FRAP) assay was used to measure the ferric reducing antioxidant power of the hybrid compound. To study the breakdown of glucose in the cells, glucose oxidase assay was conducted according to the manufacturer's instructions. Adenosine triphosphate (ATP) synthesis was also examined using a standard ATP assay kit as per the manufacturer's protocol.

The results in this study showed that most of the genes involved in glucose transport and lipid metabolism assessed (*NRF-1, GLUT4, MEF2A*) and those involved in acetylation (*pCAF/p300*, *GCN5*) were upregulated by the hybrid compound 2d' higher than metformin. Furthermore, regarding protein-DNA complex interactions, the TZD increased *MEF2A-GLUT4* binding. The TZD also showed high antioxidant activity at different degrees. As a result, TZD has potential in deterring the commencement and development of diseases caused by free radical etiology such as type 2 diabetes mellitus. The TZD treatments led to increased glucose oxidase activity while the ATP content was found to be even higher. This study demonstrated that our newly synthesized TZD upregulated and increased gene expression and acetylation of diabetes related genes. These findings are a crucial step towards the development of new drugs that may be critical in the better management and treatment of diabetes.

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LIST OF ABBREVIATIONS

ACC-1: Acetyl- CoA Carboxylase

ADP: Adenine diphosphate

Alas: Aminolevulinate synthase

AMPK: 5' Adenosine Monophosphate-activated Protein Kinase

ATP: Adenosine Triphosphate

β-cell: Any of the lymphocytes that develop into plasma cells in the presence of a specific antigen

BSA: Bovine Serum Albumen

C2C12: Mouse muscle cell line

CaMK: Ca2+/calmodulin dependent protein kinase

ChIP: Chromatin Immunoprecipitation

Co-A: Co-Enzyme-A

COX: Cytochrome oxidase

Cpt-1: Carnitine palmitoyltransferase I

DMEM: Dulbecco's modified Eagle's medium

dNTP: Deoxyribonucleotide triphosphate

EDTA: Ethylene Diamine Tetra Acetic Acid

FBS: Fetal bovine serum

FCS: Fetal calf serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GEF: GLUT4 enhancer factor

GLUT4: Glucose Transporter Type 4

HATs: Histone Acetyltransferase

HDACs: Histone Deacetylates

HIV: Human Immunodeficiency Virus

IRS: Insulin Receptor Substrate

MAPK: Mitogen-activated protein kinases

MEF2A: Myocyte Enhancer Factor 2A

mRNA: messenger Ribonucleic acid

$NADP^+$: Nicotinamide Adenine Dinucleotide Phosphate

NRF-1: Nuclear Respiratory Factor-1

PAGE: Polyacrylamide gel electrophoresis

PCAF: p300/CBP-associated factor

PDH: Pyruvate dehydrogenase

PDK1: Phosphoinositide-dependent Protein Kinase-1

PGC-1: Proliferator-activated Receptor-gamma Coactivator-1

PIP_3: Phosphatidylinositol 3,4,5-tridiphosphate

PPARλ: Peroxisome Proliferator-activated receptor gamma

qPCR: quantitative Polymerase Chain Reaction

SDS: Sodium Dodecyl Sulphate

SH2: Src homology 2

TB: Tuberculosis

TFAM: Mitochondrial Transcription Factor

TZD: Thiazolidinedione

DEFINITION OF CONCEPTS

ACC-1: Acetyl- CoA carboxylase is a biotin-dependent enzyme that catalyzes the irreversible carboxylation of Acetyl-CoA to produce malonyl-CoA through its two catalytic activities, biotin carboxylase (BC) and carboxyltransferase (Stenbit *et al.*).

Acetylation: It is the process of introducing an acetyl group (resulting in an acetoxy group) into a compound, namely the substitution of an acetyl group for an active hydrogen atom.

Adipose: (especially of body tissue) used for the storage of fat.

C2C12: A mouse embryonic myoblast cell line.

Diabetes mellitus: A group of metabolic diseases with symptoms that result from a deficiency in insulin production or utilization; characterized by a failure in glucose transport from the blood into cells at normal glucose concentration.

Fatty acid: Molecules that are long chains of lipid-carboxylic acid found in fats and oils and in cell membranes as a component of phospholipids and glycolipids.

Glucagon: A hormone formed in the pancreas which promotes the breakdown of glycogen to glucose in the liver.

Glucose transport: The carrying of glucose over a plasma membrane.

Glycine: The simplest naturally occurring amino acid, which is a constituent of most proteins.

Glycolysis: A series of enzymatically catalyzed reactions by which glucose and other sugars are broken down to yield lactic acid (anaerobic glycolysis) or pyruvic acid (aerobic glycolysis). The breakdown releases energy in the form of adenosine triphosphate (ATP).

Hyperglycemia: An excess of glucose in the bloodstream, often associated with diabetes mellitus.

Hypoglycemia: It is a condition characterized by an abnormally low level of blood sugar (glucose), your body's main energy source.

Incretins: A group of metabolic hormones that stimulate a decrease in blood glucose levels.

Insulin: A hormone produced in the pancreas by the islets of Langerhans, which regulates the amount of glucose in the blood.

Lipolysis: The breakdown of fats and other lipids by hydrolysis to release fatty acids.

MEF2A: A gene that encodes a DNA-binding transcription factor, which activates an array of muscle-specific, growth factor-induced and stress-induced genes. It plays a role in muscle development, neuronal differentiation, cell growth control and apoptosis.

Methylation: It is a process by which methyl groups are added to DNA. Methylation modifies the function of the DNA. When located in a gene promoter, DNA methylation typically acts to repress gene transcription.

Metformin: An antidiabetic drug used in the form of its hydrochloride $C_4H_{11}N_5 \cdot HCl$ specially to treat type 2 diabetes in patients unresponsive to or intolerant of approved sulfonylurea drugs.

Myoblast: Any of the cells derived from the mesoderm in the vertebrate embryo that develops into muscle tissue.

Nephropathy: It is a damage to the kidneys which can be due to diabetes.

Neuropathy: Disease or dysfunction of one or more peripheral nerves, typically causing numbness or weakness.

NRF-1: (nuclear respiratory factor 1) is a transcriptional activator of certain key metabolic genes regulating cellular growth, as well as nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication.

Obesity: The state of being grossly fat or overweight.

Oxidation: The chemical combination of a substance with oxygen.

Phosphorylation: A biochemical process that involves the addition of phosphate to an organic compound.

Prokaryotic: Any of the typically unicellular microorganisms that lack a distinct nucleus and membrane-bound organelles.

Thiazolidinediones: A class of medications used in the treatment of type 2 diabetes mellitus.

CHAPTER 1

1.0 Introduction and Problem statement

1.1 General Introduction

Diabetes mellitus is a metabolic disorder that is characterized by the continuous presence of fasting plasma glucose levels greater than 7 mmol/l, with allied instabilities in the uptake of carbohydrates, fats and proteins (Page, 2012). It is a complex syndrome mainly caused by β-cell dysfunction associated with a variable degree of insulin resistance (Shaat *et al.*, 2017). There are two main types of diabetes namely Type 1 and Type 2 diabetes. Type 1 diabetes is an auto-immune genomic ailment in which the pancreas produces little or no insulin, a hormone required to permit glucose to enter cells to produce energy (Atkinson *et al.*, 2014). Type 2 diabetes arises when the cells develop resistance to insulin or when the pancreas does not produce sufficient insulin.

Type 2 diabetes is responsible for more than 90% of all diabetes cases (Szkudelski & Szkudelska, 2011) and was thus the focus of this study. Insulin resistance (particularly of the skeletal muscles) is an important aspect of the etiology of type 2 diabetes and is also linked to an extensive array of other pathophysiologic abnormalities such as hypertension, hyperlipidaemia, atherosclerosis (that is the metabolic condition, or syndrome X) (Zhang *et al.*, 2016). This is because the skeletal muscle is the predominant site of insulin-mediated glucose uptake in the postprandial state (Bouche *et al.*, 2010). Diet and the amount of food a person consumes have a direct impact on the changes in blood glucose levels (Zhang *et al.*, 2016). Since insulin resistance is accompanied by defects in insulin secretion and action, healthy eating is of vital importance in preventing and managing the disease (Samuel *et al.*, 2010). Plants,

exercise and pharmacological means have also been used to treat the disease. Although the latter have many side effects, treatments such as Metformin, Sulfonylureas, Meglitinides, and Thiazolidinediones are available for the management of type 2 diabetes (McDermott, 2015). Metformin is a favoured pharmacological therapy for patients with type 2 diabetes mellitus because of its auspicious general profile, as well as its glucose-reducing capacity, weight-neutral properties, and truncated risk of hypoglycaemia, although gastrointestinal (GI) intolerance can limit use in a few individuals (Jabbour & Ziring, 2011). Therefore, this study focused on the influence of Thiazolidinediones, also known as Glitazones which are a class of oral hypoglycemics, with respect to gene expression and acetylation.

Thiazolidinediones mode of action occurs through the activation of the nuclear peroxisome proliferator-activated receptor-gamma (PPAR-gamma) which in turn improves insulin action and lowers blood glucose concentrations through the increase of various insulin-sensitive genes (He *et al.*, 2015). They diminish insulin resistance in adipose tissue, muscle and the liver (Lomonaco *et al.*, 2012). Proglitazone is the main TZD in use for the treatment of type 2 diabetes at present (Bailey, 2015). Our laboratory has synthesized ten new hybrid TZD compounds to assess their influence on diabetes pathways. Initial findings have shown positive results, and this has necessitated further investigations especially in gene expression and acetylation which became the focus of the study. Even though there are still various unknowns around the mechanism of action of the TZD (Shannon *et al.*, 2017), it is evident that these agents are likely to improve the full 'insulin resistance syndrome' related to type 2 diabetes.

There are various genes that confer protection against diabetes, for example the Glucose transporter type 4 (*GLUT4*) gene, which is the major glucose transporter in

skeletal muscle (Kinfe *et al.*, 2013). Of late, it has been shown that this gene can also be indirectly regulated by nuclear respiratory factor *NRF-1*, a mitochondrial transcription factor involved in lipid oxidation (Cherry & Piantadosi, 2015) . *NRF-1* regulates *GLUT4* indirectly by controlling Myocyte-specific enhancer factor-2A (*MEF2A*) *(Ramachandran et al., 2008)*. The influence of TZDs on the regulation of this transcriptional cascade *NRF-1-MEF2A-GLUT4* has not been studied yet.

It is well established that physical exercise adjusts the capacity of numerous physiological systems (dos Santos *et al.*, 2015). Many of these progressions happen through epigenetic alterations to DNA; for example, histone modification (Ntanasis-Stathopoulos *et al.*, 2013). DNA acetylation is regulated by two enzymes, namely Histone acetyltransferases (HATs) and Histone deactylates (HDACs) (Khan & Khan, 2010). HATs such as *pCAF/p300* and *GCN5* add acetyl groups to DNA while the HDACs (for example *HDAC4*) remove them (Bassett & Barnett, 2014). The balance between HATs and HDACs determines the level of histone acetylation and subsequently, the level of transcription (Beharry & Judge, 2015). Targeting these genes through chromatin remodelling can therefore be a therapeutic intervention.

Since diabetes is a consequence of metabolic errors, it is important that we study metabolic networks that are accountable for the production of energy and the utilization of nutrients (Rani *et al.*, 2016). Examples of such networks include glycolysis, the Krebs and tricarboxylic acid (TCA) cycles. A fundamental metabolite at the intersection of carbohydrate, fatty acid, and amino acid oxidation, Acetyl Co-enzyme A (CoA), applies an enormous impact on cell signalling (DeBerardinis & Thompson, 2012).

Acetyl- CoA is used in many biochemical reactions and is thus an important molecule in metabolism (Galdieri *et al.*, 2014). Acetylation has long been associated with gene

transcription (Alamdari *et al.*, 2013). In diabetes, most genes are down regulated, thus indicating a direct association of genes and the diseases (Wu *et al.*, 2017). Acetyl CoA donates acetyl groups during acetylation which directly relates to increased gene transcription (Wolfe, 2005). For example, *NRF-1* is also regulated by posttranslational modifications such as acetylation by *p300/pCAF* resulting in increased binding to its target genes such as *MEF2A*. On the other hand, *MEF2A*, the transcription factor of the *GLUT4* promoter, is also regulated through acetylation by *p300* resulting in increased expression. Accordingly, there is a pressing need to describe the complex pathophysiology of the sickness, to recognize and target particular systems with a specific end goal to back off the overall diabetes epidemic (Gonzalez-Franquesa *et al.*, 2012). Furthermore, antioxidants aid in scavenging reactive oxygen species (ROS) which attack vital cellular macromolecules such as DNA. New therapeutic approaches support the use of these ROS scavengers for the treatment of diseases.

1.2 Problem Statement

Type 2 diabetes is growing rapidly to epidemic levels, and to date has no cure. There is growing evidence that this disease is also caused by disturbances in genes involved in energy metabolism, especially pathways in glucose and fatty acid metabolism. Understanding the behaviour of genes involved in both lipid and glucose metabolism or energy metabolism is crucial in the pathogenesis of the disease. Current drugs do not cure the disease, as such necessitating the development of new therapeutic modalities that can better manage or cure the disease. Therefore, this study was aimed at investigating the influence of our newly synthesized hybrid TZD on gene expression and their associated acetylation in C2C12 cells.

1.3 Research Aim and Objectives

1.3.1 Aim

The aim of this study was to assess the influence of hybrid TZD on genes expression and associated acetylation.

1.3.2 Objectives

The objectives of this study were:

1. To evaluate the influence of the TZD on the expression of:
   - Glucose transport genes (*NRF-1*, *GLUT4* and *MEF2A*).
   - Genes associated with the acetylation of glucose transport genes [HATs (*pCAF*, *p300*, *GCN5*)].
2. To analyse protein-DNA interactions (*MEF2A*→ *GLUT4*) within the chromatin of the cells after the various treatments.
3. To determine the effect of the TZD on antioxidant activity using TEAC and FRAP assays.
4. To evaluate the oxidation of glucose and to measure the ATP content of the cells after various treatments.

CHAPTER 2

2.0 Literature Review

2.1 Diabetes

Type 2 diabetes mellitus (T2DM) is a potentially incapacitating and severe illness, which if left untreated, can lead to the continuous development of a series of other illnesses. These diseases include retinopathy which leads to blindness, nephropathy leading to renal failure, and/or neuropathy that may cause a higher risk of the development of foot ulcers, limb amputation, and autonomic and sexual dysfunction (Forbes & Cooper, 2013). T2DM is identified by hyperglycaemia, insulin resistance, and relative shortage of insulin (Gonzalez-Franquesa *et al.*, 2012). People with type 2 diabetes are also vulnerable to cardiovascular disease, peripheral vascular disease and to having cerebrovascular accident (Huo *et al.*, 2016). Type 2 diabetes is established once fasting blood glucose levels are above 7.0 mM or the two-hour blood glucose levels higher than 200 mg/dL (11.1 mM) soon as a glucose tolerance test is done (Gonzalez-Franquesa *et al.*, 2012). It is therefore important to sustain plasma glucose levels at about 5 mM (Sjøberg *et al.*, 2017). The symptoms of diabetes include extreme thirst and excessive urination, weakness, loss of weight, unclear vision, slow-healing sores or recurring infections, itchy hands and feet and red, swollen, painful gums (Savage *et al.*, 2007).

Two types of diabetes exist namely Type 1 (formerly known as juvenile-onset diabetes) , which usually arises in younger patients as a result of β-cell death following viral/autoimmune attack (Vogelauer *et al.*, 2012) and type 2. There is total failure of insulin production and emission in type 1 diabetes (Atkinson *et al.*, 2014). Treatment is through insulin replacement therapy. Type 2 diabetes (also named age-related or maturity onset diabetes) on the other hand, is widespread in mid or later life and can be

managed by pills or just by exercise (DeFronzo *et al.*, 2014). It is hypothesized that the biochemical origin of type 2 diabetes variations is the relatively ineffective insulin produced (Vogelauer *et al.*, 2012). Hazard elemental variables for type 2 diabetes include hereditary predisposition as well as ecological elements, including unfavourable intrauterine environment, dormancy, eating routine, obesity and aging (Selvin *et al.*, 2014).

Type 2 diabetes is a major challenge to world health (Hu, 2011), and this is as a result of lifestyle changes and adaptations. T2DM is presently thought to be a worldwide epidemic with noteworthy societal and commercial consequences both at the individual and public levels (Gonzalez-Franquesa *et al.*, 2012). Globally, 387 million people are diagnosed with type 2 diabetes with the International Diabetes Federation (IDF) predicting an increase to 592 million by 2035 (Selvin *et al.*, 2014). According to the Centre for Diabetes and Endocrinology in Johannesburg, 3.5 million South Africans (~6% of the population) suffer from type 2 diabetes and there are many more who are undiagnosed (Meier *et al.*, 2016) . The most elevated pervasiveness of diabetes is among the Indian populace in South Africa (11-13%) as this group has a solid hereditary inclination for diabetes. This is followed by 8-10% in the coloured group, 5-8% among blacks and 4% among whites (Selvin *et al.*, 2014). The World Diabetes Foundation (WDF, 2016) states that the interactions between diabetes, HIV/Aids and TB are higher in Africa than in any other continent. Patients who obtain anti-retroviral drugs are at an increased risk of developing diabetes, as glucose intolerance is one of the side effects caused by the drugs (Quin, 2014). Individuals who suffer from diabetes have a greater vulnerability to tuberculosis because diabetes decreases the body's immunity (Koo, 2013). In addition, the interaction between drugs that treat diabetes and

TB reduces the efficiency of both drugs, thus making it difficult to control both diseases (Koo, 2013).

A state investigation for individuals aged 20 years or older disclosed that the occurrence of type 2 diabetes in the United States (2010 to 2012) was 7.6% in non-Hispanic whites, 9.0% in Asian Americans, 12.8% in Hispanics, 13.2% in non-Hispanic blacks, and 15.9% in American Indians/Alaska Natives (Selvin *et al.*, 2014). Outside the US, type 2 diabetes is most widespread in Polynesia and other Pacific islands (~ 25%) (Collins *et al.*, 1994). In 2010 a cross-sectional survey of approximately 100000 adults in China revealed that the incidence of diabetes was estimated at 11.6%, while the prevalence of prediabetes (impaired glucose tolerance), impaired fasting glucose [IFG], or A1C between 5.7 and 6.4 was 50% (Xu *et al.*, 2013).

In the underlying phases of advancement of type 2 diabetes, insulin is not ready to accurately invigorate skeletal muscle glucose uptake after carbohydrate admission, prompting postprandial hyperglycaemia (Lambadiari *et al.*, 2015). In adipose tissue, the significant fat storing tissue in vertebrates, insulin resistance results in expanded lipolysis and unsaturated fat discharge (Morigny *et al.*, 2016). Expanded circling unsaturated fats diminish the capacity of insulin to overpower hepatic glucose creation and permit a consistent increment in unsaturated fat combination (Boden *et al.*, 2014). Amid the main phases of the advancement of the sickness, pancreatic β-cells can make up for insulin resistance by expanding basal and postprandial insulin secretion to counteract hyperglycaemia (Cerf, 2013). At the point when pancreatic β-cells can no longer compensate, they are unable to react appropriately to glucose levels (Cerf, 2013). This pancreatic β-cell distress prompts the crumbling of balances in glucose and the advancement of T2DM (Chang *et al.*, 2015).

Moreover, strange discharge and control of incretins in the gastrointestinal tract, hyperglucagonemia, glucose reabsorption in the kidneys, and adjusted balance of the central nervous system pathways are a vital part in the progression of type 2 diabetes (Nishi *et al.*, 2015). This complex pathophysiology makes it hard to distinguish the essential events in charge of the advancement of type 2 diabetes (Gonzalez-Franquesa *et al.*, 2012). An additional type of diabetes is gestational diabetes, which develops at any time during pregnancy in women who do not have diabetes and often leaves after the delivery of the baby (Kralisch *et al.*, 2017). Pregnancy usually induces a state of insulin resistance due to the increased demand on pancreatic β-cell function (Baz *et al.*, 2016).

Skeletal muscle is the biggest insulin-delicate organ in people representing more than 80% of insulin invigorated glucose transfer (Gallagher & LeRoith, 2015). In this way, insulin resistance in this tissue has significant outcomes on entire body homeostasis (Gonzalez-Franquesa *et al.*, 2012). The mechanism through which obesity causes insulin resistance in skeletal muscle is by all accounts connected with the accumulation of unsaturated fats in the myocytes (Dubé *et al.*, 2011). Expanded free unsaturated fat accessibility obstructs glucose usage through the hindrance of key catalysts required in glucose digestion system that is, Pyruvate Dehydrogenase (PDH), glycogen and hexokinase II (Jung & Choi, 2014). Insulin resistance induced by unsaturated fats is basically connected with disabled glucose uptake as opposed to glucose gathering (Dotzert *et al.*, 2016). Inflammation has likewise been proposed as a potential component required in the improvement of restricted insulin affectability (Gonzalez-Franquesa *et al.*, 2012). Unsaturated fats actuate provocative signs by advancing emission of professional inflammable cytokines (Dotzert *et al.*, 2016). This initiation

prompts a diminishing in insulin activity because of the phosphorylation of Insulin Receptor Substrate-1 IRS-1 (Cheng *et al.*, 2010).

A few confirmations have connected mitochondrial deformities to insulin resistance and type 2 diabetes, proposing that these organelles are key players in keeping up energy homeostasis (Patti & Corvera, 2010). Mitochondria are two fold film organelles that constitute the real site for oxidative energy creation in the cell (Cherry & Piantadosi, 2015). Not only do they produce the greater part of cellular adenosine triphosphate (ATP) by means of oxidative phosphorylation (OXPHOS), but they additionally generate various metabolites by means of the tricarboxylic (TCA) cycle, breakdown of amino acids and unsaturated fats, combine ketone bodies, ornithine cycle (otherwise called the urea cycle), control cytoplasmic reticulum and calcium signalling, and repair DNA (Murgia *et al.*, 2009).

Mitochondrial biogenesis is characterized as the era of more mitochondrial mass and happens because of expanded energy request (Liesa *et al.*, 2009). A calibrate control of mitochondrial biogenesis and elements is important to get and keep up functional mitochondria (Zorzano *et al.*, 2009). A few translational components have appeared to manage articulation of qualities required in the respiratory chain and mitochondrial breakdown system. However, just a couple are viewed as the significant interpretation elements critical for mitochondrial biogenesis (Gonzalez-Franquesa *et al.*, 2012). One such transcription factor is known as the Nuclear Respiratory Factor-1 (*NRF-1*). *NRF-1* is a transcription factor for mitochondrial biogenesis which is shown to be involved in many other pathways, particularly in glucose transport and lipid metabolism (Joseph *et al.*, 2017). It has been confirmed that this gene regulates *GLUT4* expression by controlling *MEF2A* (Ramachandran *et al.*, 2008). Therefore, *NRF-1* is an important

gene in the design of therapeutic modalities much required to cure or better manage type 2 diabetes.

2.2 Nuclear Respiratory Factor-1

Nuclear Respiratory Factor-1 translates a protein that operates as a transcription factor that triggers the countenance of some important metabolic genes responsible for cellular development and nuclear genes compulsory for mitochondrial respiration, DNA transcription and duplication (Bugno *et al.*, 2015). *NRF-1* and *NRF-2* mediate the biogenomic coordination, amid nuclear and mitochondrial genomes by directly modifying the expression of numerous nuclear-encoded electron transport chain (ETC) proteins, and indirectly regulating the three mitochondrial-encoded cytochrome c oxidase (COX) subunit genes by stimulating mitochondrial transcription factors TFA, TFB1, and TFB2 (Cherry & Piantadosi, 2015). The *NRF* proteins are also vital for the upregulation of antioxidant and xenobiotic-metabolizing enzymes during oxidative stress (Library, 2002). Interruption of the *NRF-1* quality in mouse models results in mitochondrial DNA exhaustion and debilitated mitochondrial membrane potential with an early embryonic deadly phenotype (Huo & Scarpulla, 2001).

NRF-1 is a transcriptional factor that regulates mitochondrial biogenesis (Evans & Scarpulla, 1990). Mitochondrial biogenesis encompasses the collaboration of two genomes; that is, those situated in the nucleus and those contained in the mitochondria (Liesa *et al.*, 2009). Mitochondrial genome codes for 13 respiratory chain polypeptides, 2 ribosomal RNAs and 22 transfer RNAs (Gonzalez-Franquesa *et al.*, 2012). The rest of the proteins essential for a comprehensive conservation and expression of mitochondria are encoded by the nuclear genome (Mukwevho, 2010). *NRF-1* works with other transcription factors to act on nuclear genes that express the proteins involved in heme

biosynthesis, oxidative phosphorylation, mitochondrial gene transcription and replication, importation of protein into the mitochondria, and several respiratory chain subunits that take part in cellular respiration (Evans & Scarpulla, 1990). Regulation of the expression of this group of genes by these transcription factors ends in a well synchronized program of events in the nucleus and in the mitochondria, subsequently resulting in mitochondrial biogenesis (Lenka *et al.*, 1998). The *NRF-1* activity must match the ATP demand in the cells because the mitochondrion is vital in producing ATP from substrates (Patti *et al.*, 2003). Thus, *NRF-1* can be viewed as a critical element of the energy detecting machinery in mammalian cells which alters physiological signals that arise during energy demand conditions, for example physical activity, into improved ability for energy generation (Li *et al.*, 1999).

Mitochondrial dysfunction is related to a broad band of diseases and has been linked with insulin resistance in skeletal muscle and the development of type 2 diabetes mellitus (Gonzalez-Franquesa *et al.*, 2012). Patients with type 2 diabetes mellitus have a reduced expression of oxidative phosphorylation genes, most of which are regulated by *NRF-1* dependent transcription (Bugno *et al.*, 2015). Furthermore, a growing body of evidence proposes that there is a cordial association between *NRF-1* and Myocyte Enhancer factor 2A *MEF2A* whereby *NRF-1* regulates the expression of *MEF2A*, which in turn regulates other genes (Baar *et al.*, 2003). In *NRF-1* transgenic mice, *GLUT4*, *MEF2A* and mitochondrial proteins are all amplified (Baar *et al.*, 2003). The fact that *NRF-1* controls the *MEF2A* gene to induce the expression of respiratory subunits (Ramachandran *et al.*, 2008) suggests that it may also induce the expression of *GLUT4* since *GLUT4* is also regulated by *MEF2A* (Wright *et al.*, 2007). *NRF-1* can be regulated through posttranslational modifications. Therefore, genetic modifications of the *NRF-1*

gene could bring about a breakthrough in the management and treatment of type 2 diabetes mellitus.

2.3 Epigenetics

Epigenetic modifications are lengthy adjustments on the epi-gene that modifies the transcriptional potential of a cell (dos Santos *et al.*, 2015). This includes alterations of DNA, DNA binding proteins, and histones that modify the structure of chromatin without altering the DNA nucleotide sequence, as well as non-coding RNA (ncRNA) molecules such as microRNAs (miRNAs), which can change regulation of gene expression (Sommese *et al.*, 2017). Studies have publicised that epigenetic modifications play a chief role in many developed chronic diseases such as type 2 diabetes, as changes in the epigenome can vary cell phenotype resulting in the manifestation of the disease (Tewari *et al.*, 2012).

There are three major epigenetic mechanisms known to regulate gene expression namely DNA methylation, histone modifications and ncRNA activity (Kowluru *et al.*, 2013). DNA methylation characterizes a major epigenetic mechanism that modifies the availability of gene promoters, thus causing transcriptional repression (Sommese *et al.*, 2017). A methyl group binds to the 5' position of cytosine residues in a dinucleotide Cytosine-phosphate-Guanine (CpG) via the DNA methyltransferases (DNMTs, including DNMT1, DNMT3A, and DNMT3B) (dos Santos *et al.*, 2015). Demethylases of the translocation (TET) family of DNA dioxygenases (TET1/2/3) also regulate the methylation status of the genome, by eliminating the methyl group from the methylated cytosines (Ito & Kuraoka, 2015). S-adenosylmethionine (SAM) donates the methyl group in the DNMT-catalysed reactions, while Fe (II)-, a-ketoglutarate- (a-KG), ascorbate-, and O_2 are cofactors of the TET-catalysed oxidation of 5-methylcytosine

(5mC) to 5-hydroxymethylcytosine (5hmC) (Sommese *et al.*, 2017). Unlike methylation, demethylation of DNA usually activates transcription. Posttranslational histone modifications (PTHMs) include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation of specific amino acid residues of histones (Khan & Khan, 2010). PTHMs adjust chromatin condensation and consequently inducing either gene activation, gene silencing, or both (Polo, 2015). While acetylation of histone lysine residues increases chromatin accessibility and activates gene expression, the ultimate outcome of histone methylation varies according to the specific methylated residue and the quantity of added methyl groups (Ito & Kuraoka, 2015). Histone acetylation and deacetylation are catalysed by 2 classes of enzymes, Histone acetyltransferases (HATs) and Histone deacetylates (HDACs), respectively (Bannister & Kouzarides, 2011). The organized regulation of HATs and HDACs launches the level of histone acetylation and, hence, contributes to the regulation of gene expression (Gray & De Meyts, 2005). HAT inhibitors are considered as novel possible epidrugs to treat diabetes (Sommese *et al.*, 2017). For example, garcinol, from garcinia fruit rinds, is an effective HAT inhibitor whose targets include *pCAF* and *p300* (Balasubramanyam *et al.*, 2004). HDAC inhibitors have the potential anti-diabetic activity arising from their anti-inflammatory effect, enhancement of insulin sensitivity/secretion, induction of β-cell differentiation, and prevention of inflammatory damage of β-cells (Christensen *et al.*, 2011). ncRNAs can also regulate gene expression by manipulating the protein biosynthetic machinery at both the posttranscriptional and translational levels (Barnett M.P.. *et al.*, 2013). miRNAs decrease stability or inhibit translation of target transcripts by binding to their 30-untranslated regions (UTRs). Remarkably, many genes can be targeted by a single miRNA, and a single gene can be targeted by many miRNAs (Barnett M.P.. *et al.*, 2013). Long ncRNAs are expressed in a tissue- and time-

specific manner and consist of different types of functional RNAs that comprise enhancer-associated RNAs (eRNAs), sense- and anti-sense lncRNAs, and intergenic-lncRNAs (Sommese *et al.*, 2017). They can recruit DNMTs and histone modifiers to their target genes and, particularly eRNAs, facilitate promoter-enhancer looping, hence promoting the countenance of nearby genes (Arnes & Sussel, 2015). Even though there is an indication of regulation of islet-specific gene expression by lncRNAs, their role in pancreas development and β-cell function awaits conclusive experimental evidence (Raghuraman *et al.*, 2016). miRNAs are considered potential pharmacological agents for the treatment of diabetes (Broderick & Zamore, 2011). Two main miRNA-based therapeutic approaches have been developed for diabetes. These are overexpression of miRNAs using chemically synthesized miRNA mimics and inhibition of miRNAs by specific inhibitors (Kolfschoten *et al.*, 2009). On the basis of the accumulating evidence, it is believed that epigenetic mechanisms could provide a significant role in fighting the diabetes epidemics (Sommese *et al.*, 2017).

2.4 Histone deacetylates (HDACs)

Histone deacetylases are a class of enzymes that eliminate acetyl groups ($O=C-CH_3$) from an ε-N-acetyl lysine amino acid on a histone, permitting the histones to wrap the DNA more tightly (Bassett & Barnett, 2014). Histone deacetylases function in an extensive variety of molecular procedures, including gene expression, and are of significant interest as therapeutic targets (Vogelauer *et al.*, 2012). There are 18 HDACs in humans which are grouped into 5 classes based on sequence homologies to the yeast orthologues RPD3, HDA1 and SIR2 (class I, IIa, IIb, III and IV) (Clocchiatti *et al.*, 2013). *HDAC4* (a member of the class IIa subfamily) was studied in this project. Like other class IIa members (*HDAC5, HDAC7* and *HDAC9*), *HDAC4* is exposed to intense regulation by diverse signal transduction pathways in order to link variations in gene

expression to the improved environmental conditions (Haberland *et al.*, 2009). Class IIa HDACs are recruited on specific genomic regions succeeding interactions with DNA-binding transcription factors because they cannot bind to DNA in a sequence specific manner (Clocchiatti *et al.*, 2013).

MEF2 (myocyte enhance factor) transcription factors are the greatest characterized partners of class IIa HDACs (Yang & Seto, 2008). HDACs cause the condensation of chromatin by the elimination of negative charges, hereafter concealing the MEF2 sites and blocking entrance for transcription regulators (MEF2_HDAC complex) (Mukwevho, 2010). The phosphorylation of HDAC by *CAMKII* can separate this complex resulting in the localization of HDAC from the nucleus to the cytoplasm and then promoting MEF2-binding to histone acetyltransferases (HATs) thus relaxing chromatin (Mukwevho *et al.*, 2008). A growth in the binding of transcription factor to DNA intensifies gene transcription and HATs such as *p300*, likewise acetylate MEF2 to intensify binding and transcriptional activity (Beharry & Judge, 2015). HDACs are notorious for suppressing gene transcription and the transfer of HDAC out of the nucleus favours the binding of HAT thus increasing gene transcription and *GLUT4* expression (Smith *et al.*, 2008). Studies have shown that Ca^{2+} from the cytosol regulates this expression through *CAMK* signalling which increases the binding between the *GLUT4* gene and *MEF2A* and therefore triggering *GLUT4* protein production (Smith *et al.*, 2007). This intensifies the expression of *NRF-1* and thus increases the binding of *NRF-1* to *MEF2A* (transcriptional cascade *NRF-1—MEF2A—GLUT4*), eventually increasing *GLUT4* transport (Mukwevho, 2010).

Even though their local structures, subcellular limitation, and enrolment systems to chromatin, have been broadly focused, significantly less is thought about whether the

enzymatic action of non-sirtuin HDACs can be directed by normal metabolites (Vogelauer *et al.*, 2012) . A few coenzyme A derivatives, for example, acetyl-CoA, butyl-CoA, HMG-CoA and malonyl-CoA, and in addition NADPH, go about as allosteric activators of recombinant *HDAC1* and *HDAC2 in vitro*, taking after a blended initiation dynamic (Shimazu *et al.*, 2013). Interestingly, free CoA, as unconjugated butyrate, restrains HDAC movement in vitro (Vogelauer *et al.*, 2012). An analysis of a large number of engineered *HDAC1* mutants suggests that the HDAC activity can potentially be decoupled from being activated by the CoA derivatives (Vogelauer *et al.*, 2012).

The Histone Code is a broadly utilized theory in which successive adjustments to the histones in chromatin lead to managed translation of genes, one of the adjustments utilized as a part of the histone code being acetylation (Bannister & Kouzarides, 2011). This is probably the categorized amendment used in the histones, which is carried out under the control of HATs and HDACs (Zilio *et al.*, 2014). Cumulative evidence links possible deregulation of these mechanisms in the pathogenesis of type 2 diabetes, with important therapeutic consequences (Vogelauer *et al.*, 2012).

2.5 Histone acetyltransferases (HATs)

Histone acetyltransferases are catalysts that change chromatin histones and are key players in the epigenetic variety of quality transcription programs (Luan *et al.*, 2015). These compounds cooperate with HDACs to institutionalize a wide and complex cluster of physiological procedures; for example, cell multiplication, separation, senescence, apoptosis and digestive systems (Bai *et al.*, 2008). The enzymes require two substrates which are peptide or protein to be acetylated and acetyl coenzyme A as the acetyl donor (You *et al.*, 2014). A general property of HATs is that in spite of the fact that they

can acetylate no less than one type of histone, they can just acetylate histones in nucleosomes as individuals from HAT complexes (Alamdari *et al.*, 2013).

HATs are arranged into two groups as indicated by the instrument of catalysis and on cell localization, that is HAT A and HAT B (Zhou *et al.*, 2012). The members of the HAT A family are situated in the nucleus where they eliminate the acetyl group from Acetyl-CoA an ε-NH2 group of N-tails after gathering into nucleosomes (Peserico & Simone, 2011). This family is further divided into three subclasses according to their homology with yeast proteins: *GCN5*-related N-acetyltransferase (GNAT) superfamily (*GCN5*, *p300*/CREB1-binding protein (CBP) associated factor (*pCAF*), Elp3 and Hpa2), MYST family (*Tip60, p300/CBP, MORF, MO2*, and *HBO1*) and nuclear receptor co-activators (*SRC-1, ACTR*, and *TIF2*) (Dengler *et al.*, 2014). The members of the HAT B family, on the other hand, are found in the cytoplasm and transmits the acetyl group from Acetyl-CoA to an ε-NH_2 group of free histones before their establishment in the DNA (Peserico & Simone, 2011).

The HAT enzyme, *pCAF*, is formerly recognized as a *p300/CBP*-binding protein and has a critical capacity in the regulation of myofilament contractile activity, myogenic program, and adipocyte proliferation (Cherasse *et al.*, 2007). Oxidative stress can activate *p300* HAT and result in an improved level of histone acetylation that normalizes gene expression (Sundar *et al.*, 2013). Transcriptional coactivator *PGC1-α* is able to organize the expression of a variety of glucose and fatty acid metabolism genes through its acetylation state (Dominy *et al.*, 2010). The acetyltransferase General Control Non-repressed Protein 5 (*GCN5*) is a far more effective acetyltransferase for *PGC-1* than *p300 in vivo* (Dominy *et al.*, 2010). *GCN5* might be a pharmacological target to govern

the activity of *PGC-1α*, providing a possible therapy for metabolic disorders in which hepatic glucose production is dysregulated (Dominy *et al.*, 2010).

Acetylation of lysine residues on the N-terminal tolls of histone proteins is a key aspect in chromatin dynamics and gene expression (Bannister & Kouzarides, 2011). The acetyl CoA groups used to alter histones are principally produced by acetyl CoA synthetase in yeast and ATP citrate lyase in mammalian cells, which both enter the nucleus to yield a localized acetyl CoA pool (DeBerardinis & Thompson, 2012). Loss of function of these enzymes lessens histone acetylation with global consequences on gene expression (Siudeja *et al.*, 2011). Subsequently, variations in acetylation status have repercussions for protein stability, protein-protein interactions, and protein-DNA relations (Alamdari *et al.*, 2013). Studying the impact of the TZD on the transcription of HATs might assist in the development of mechanisms through which gene expression may be improved. Enhancing the gene expression of HATs will increase acetylation and thus reduce the impact of type 2 diabetes which is often associated with the down regulation of genes.

2.6 Acetyl Coenzyme A

Acetyl coenzyme A or Acetyl-CoA is an essential molecule in metabolism and is involved in a variety of cellular processes. Its main function is to transport the carbon atoms inside the acetyl group to the citric acid cycle (Krebs cycle)/ tricarboxylic acid (TCA) cycle to be oxidized for energy to be produced (Mapanga, 2013). It is a substrate for protein acetylation, which plays a role in the supervision of enzyme function and DNA transcription (Krivoruchko *et al.*, 2015). In chemical structure, acetyl-CoA is the thioester of Co-enzyme A (a thiol) and acetic acid (an acyl group carrier). Acetyl-CoA is formed in the second step of aerobic cellular respiration, pyruvate decarboxylation,

which transpires in the matrix of the mitochondria (Krivoruchko *et al.*, 2015). It then enters the citric acid cycle in a step catalysed by citrate synthase(CS) (Nelson *et al.*, 2008). This molecule also functions as a precursor for many molecules of biological relevance, and this results in drain of acetyl-CoA, potentially compromising cellular function (Krivoruchko *et al.*, 2015).

2.6.1 Acetyl-CoA in the central carbon metabolism

Under aerobic glucose restricted conditions, the TCA cycle plays a vital role in catabolism and acetyl-CoA produced by the pyruvate dehydrogenase (PDH) complex from pyruvate is the main substrate procedure (Krivoruchko *et al.*, 2015). CS together with oxaloacetate catalyses the condensation of acetyl-CoA yielding citrate (Pietrocola *et al.*, 2015).

Acetyl-CoA synthetase (ACS) is an alternative important source for acetyl-CoA (Kozak *et al.*, 2014). The reaction catalysed by ACS consumes ATP to transform acetate to acetyl-CoA; AMP is then formed in two steps. In the first step, the enzyme binds ATP and then acetate, producing enzyme-bound acetyl-AMP and releasing pyrophosphate (PPi) (Liang *et al.*, 2016). In the second step, ACS binds CoA and converts the intermediate to acetyl-CoA and AMP (Mayer *et al.*, 2012). The two ACS enzymes are members of the AMP-forming ACS family, which was found to be post-translationally regulated by acetylation of lysine in a preserved region (Bannister & Kouzarides, 2011). Nonetheless, the detailed regulatory indicators and mechanisms for acetylation and deacetylation of ACS are still unclear (Krivoruchko *et al.*, 2015).

2.6.2 Protein acetylation

Acetylation of lysines is an ancestral posttranslational adjustment that can impact the activity of several proteins including histones, the molecular blocks on which DNA wraps around (Clocchiatti *et al.*, 2013). Histone acetylation is a widespread epigenetic modification that marks active expression, by weakening the interaction of their positively charged tails with the negative backbone of DNA (Clocchiatti *et al.*, 2013). This occurs under the control of two competing enzymatic activities, that is the HATs and HDACs (Yang & Seto, 2007).

In both eukaryotes and prokaryotes, protein acetylation at α- or ε-amino groups during post translational modification progressions has been found to be important for regulation (Jones & O'Connor, 2011). Histone acetylation influences chromatin structure and directs gene transcription through various interactions (Bannister & Kouzarides, 2011). Non-histone protein acetylation regulates cell motioning at numerous levels, for example, mRNA steadiness, protein limitation, protein collaboration, protein degradation or protein capacity (Spange *et al.*, 2009). Various HATs and HDACs have been distinguished in *S. cerevisiae*, which are in charge of the acetylation and deacetylation of histone and non-histone proteins; however for a large portion of them, their capacities as transcriptional controllers are still being scrutinized (Wang *et al.*, 2012).

Acetyl-CoA has essential stimulus in protein acetylation as an acetyl benefactor. The nucleocytosolic acetyl-CoA abundance straightforwardly directs the dynamic acetylation and deacetylation of proteins (Krivoruchko *et al.*, 2015). Diminished movement of

Acc1p, which devours acetyl-CoA for *de novo* synthesis of unsaturated fats appears to bring about expanded histone acetylation and adjusted transcriptional control (Galdieri & Vancura, 2012). Acetyl-CoA initiates the transcriptional development program by advancing the acetylation of histones at development related qualities in yeast, and it is anticipated that intracellular acetyl-CoA variances may speak to an unmistakable gage of cell metabolic states that could be decoded by methods for elemental acetylation and deacetylation responses (Cai & Tu, 2011).

Lastly, by manipulating the acetylation profile of some proteins, plus histones, acetyl-CoA manages key cellular processes, as well as energy metabolism, mitosis, and autophagy, both directly and through the epigenetic control of gene expression (Pietrocola *et al.*, 2015). Thus, acetyl-CoA governs the stability between cellular catabolism and anabolism by operating as a metabolic intermediate and as a subordinate messenger at the same time (Jankowska-Kulawy *et al.*, 2010a). Along these lines, extra research endeavours must be centred on how to ideally increment metabolic flux towards acetyl-CoA formation (Krivoruchko *et al.*, 2015).

2.7 Insulin Signalling Pathway

Type 2 diabetes is a lifelong condition that may be caused by insulin resistance. Insulin resistance is a ailment in which cells are incapable of using insulin effectively which consequently leads to high blood glucose levels (Cerf, 2013). Insulin is a hormone that aids cells in glucose uptake from the bloodstream. It also encourages the synthesis of lipids and prevents their degradation (Guilherme *et al.*, 2008). Together with the restricting hormone glucagon, insulin is in charge of keeping up glucose homeostasis,

which is important to guarantee legitimate capacity and existence of all organs (Gonzalez-Franquesa *et al.*, 2012). The essential focuses of insulin activity to keep up glucose homeostasis are skeletal muscle, liver and adipose tissue as they are the predominant site of insulin-mediated glucose uptake (Samuel *et al.*, 2010). The hormone binds to its receptor on the major insulin responsive tissues of the body (Wajchenberg & Cohen, 2014). The insulin receptor is a tyrosine kinase that catalyses the phosphorylation of some intracellular substrates together with the insulin receptor substrate (IRS) proteins, GAB-1, Shc, APS, p60DOK, SIRPS and c-Cbl (Boucher *et al.*, 2014). The purpose of each of these substrates is to recruit a diverse set of signalling proteins comprising Src homology 2 (SH2), which specifically cooperate with sequences adjacent to the phosphate residue (Saltiel & Pessin, 2002). In addition to tyrosine phosphorylation, the insulin receptor and IRS protein undergo serine phosphorylation, which may reduce signalling by decreasing insulin-stimulated tyrosine phosphorylation and endorse interaction with 14-3-3 proteins (De Meyts & Whittaker, 2002). This inhibitory phosphorylation delivers negative feedback to insulin signalling and acts as a mechanism for cross-talk from other pathways that yield insulin resistance (Boucher *et al.*, 2014).

The activated receptor then recruits and phosphorylates a panel of substrate molecules. Among these, IRS1 and IRS2 appear to be the adapter molecules playing a major role in the coupling to the P13K-PKB and MAPK downstream kinases (White, 2002). Tyrosine phosphorylated IRS1/2 assembles the heterodimeric p85/p110 P13K at the plasma membrane, where it forms the lipid second messenger PIP_3 which then recruits a serine/threonine phosphorylation cascade of PH-domain containing proteins. PIP_3 targets consist of PDK1, the serine/threonine protein kinase B(PKB)/AKt, and the atypical protein kinase isoforms (Boucher *et al.*, 2014). Mechanistically, PDK1, PKB and

aPKCs, which all contain a PH-domain, are recruited at the plasma membrane by binding to PIP_3 (Tobias *et al.*, 2016). PDK1 then phosphorylates PKB and aPKCs on a threonine residue located in the activation loop of the catalytic domain, causing their activation (Scheid *et al.*, 2005). Major targets of activated PKB are GSk-3 and AS160 which upon PKB-mediated phosphorylation protein phosphatase-1 (PP1) activation, releases the inhibiting phosphorylation of Glycogen synthase GS which becomes activated and promotes glycogen synthesis (Beeson *et al.*, 2003). PKB also controls the insulin-stimulated translocation of the glucose transporter *GLUT4* to the plasma membrane, subsequently improving glucose uptake (Fröjdö *et al.*, 2009).

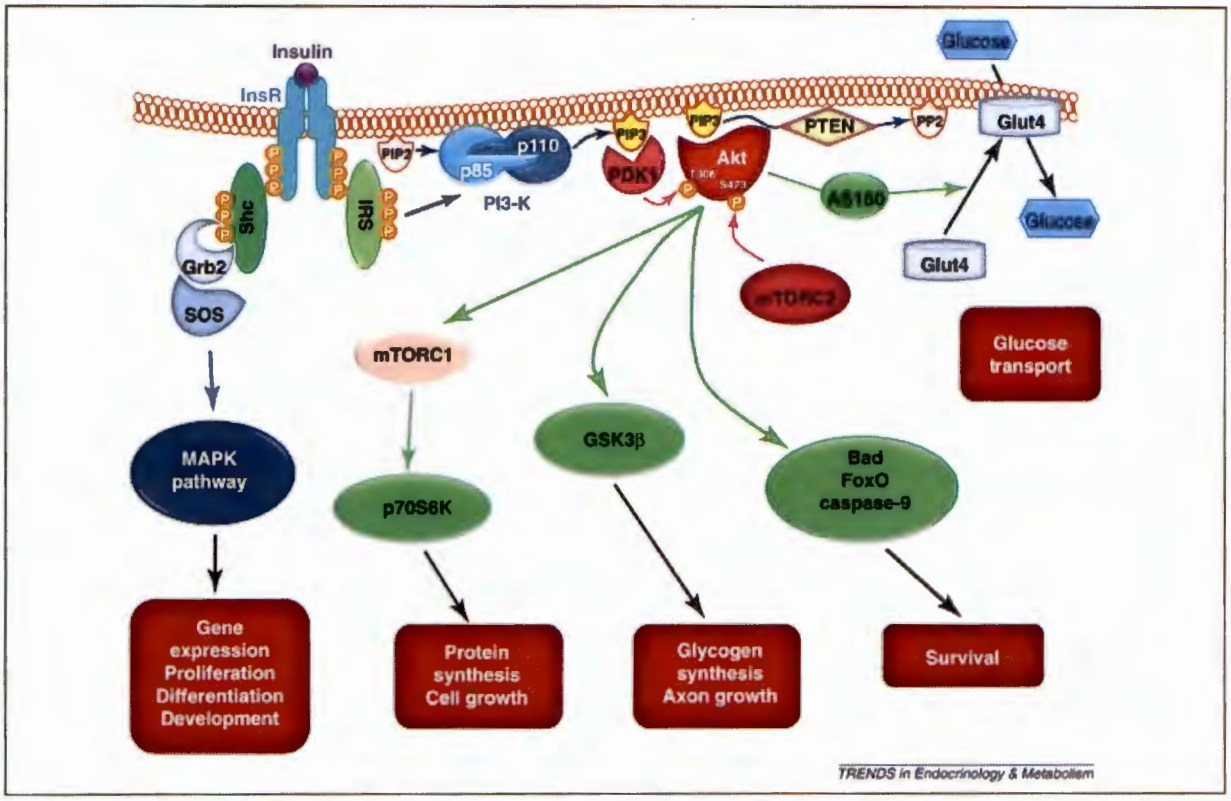


Figure 2.1: A summary of the insulin signalling pathway. The binding of insulin to InsR stimulates autophosphorylation of the receptor and recruits docking proteins Shc and IRS. IRS phosphorylation stimulates PI3K activation, and this leads to the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP2). PDK1 and Akt are recruited to the plasma membrane by binding to PIP3 through their PH domains. PDK1 phosphorylates a threonine residue in the catalytic domain of Akt. Full activation of Akt requires a second phosphorylation of a serine residue by the mTORC2 complex. Alternatively, phosphorylated Shc recruits Grb2/SOS, which stimulates the MAPK signalling pathway. Substrates activated by the MAPK and PI3K/Akt pathway mediate various downstream biological responses of insulin, including cell survival and glucose metabolism (Bhumsoo & Feldman, 2012).

The confirmation that insulin acts by binding to a plasma membrane receptor proposed that the adjusted affectability to insulin as seen in obesity, insulin resistance and type 2 diabetes might be reliant on reduced receptor level or frail attachment to its agonist (Islam *et al.*, 2014). From that point forward, the intracellular signalling apparatus conveying the metabolic activities of insulin has been clarified to a decent degree, and a

few of the chemicals taking part in insulin movement have appeared to experience adjustments of their physiological capacity in the insulin safe state (Fröjdö *et al.*, 2009). The disclosure of the intracellular strides of insulin signalling has prepared escalated examination for understanding the atomic system in charge of the foundation of the insulin resistant state, both on rat models and in people (Li *et al.*, 2010). Insulin resistance is the hallmark of type 2 diabetes mellitus. Understanding the insulin signalling pathway as seen in figure 2.1 is, therefore, important in the pathogenesis of the disease and the progression of mechanisms that can increase insulin sensitivity and subsequently, cellular glucose uptake.

2.8 Glucose transport

Glucose is a vital energy source that is needed by all cells and organs of our bodies (Rosen & Spiegelman, 2006). It must cross the plasma membrane into the cell. This occurs by encouraged dispersion intervened by a group of proteins known as glucose transporters (GLUT), which are circulated in various types of cells (Gonzalez-Franquesa *et al.*, 2012). Muscle cells and adipocytes, which are sensitive to insulin, have a transporter known as *GLUT4* (Richter & Hargreaves, 2013). Due to insulin, these are selected from vesicles inside the cell to the plasma layer, where glucose uptake builds up (Edelman, 2008). It should be noted that transporters in the liver, *GLUT2* and *GLUT1*, are constitutively situated in the plasma membrane and thus do not need insulin to be active (Vogelauer *et al.*, 2012).

Insulin stimulated cellular glucose uptake is principally mediated by the facilitative transporter *GLUT4*, an individual from the family related transporters that are vastly expressed in adipose tissue and skeletal and cardiac muscle (Saltiel & Pessin, 2002). The *GLUT4* transporter is the entryway for glucose to enter the skeletal muscle and fat

tissue (Landowne, 2006) as seen in figure 2.2. Insulin is the enrolling sergeant that assembles the intracellular vesicles containing *GLUT4* and adds them into the plasma membrane (sarcolemma on account of muscle) (Holten *et al.*, 2004b). However, since vanadate or phenylasine oxide (Sommese *et al.*), which are protein tyrosine phosphatase inhibitors, can re-establish ordinary glucose transport. The confirming that the deficiency is in the signalling procedure rather than in the *GLUT4* particle itself (Vogelauer *et al.*, 2012).

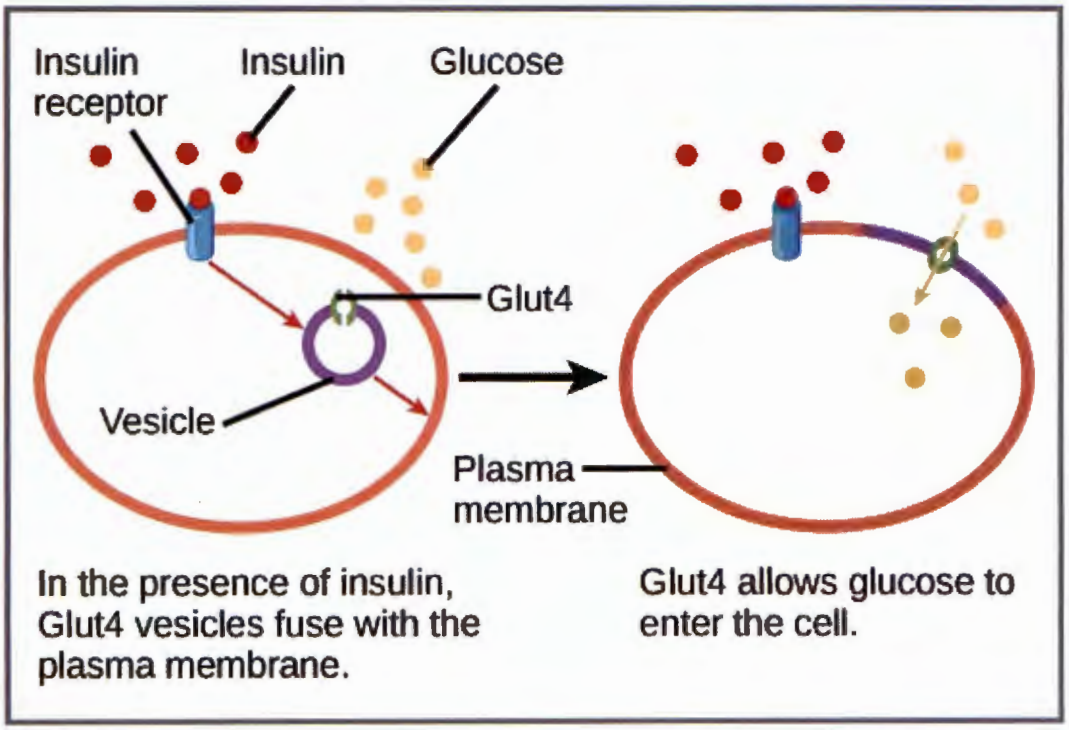


Figure 2.2: Brief outline of the glucose transport process. A cascade of events that occurs upon insulin binding to a receptor in the plasma membrane cause GLUT-containing vesicles to fuse with the plasma membrane so that glucose may be transported into the cell. (Boundless, 2016).

Myocyte enhancer factor 2 (MEF2) and *GLUT4* enhancer factor (*GEF*) are key for the control of *GLUT4* expression at a transcriptional level (Hayashi *et al.*, 2000). Mutations in the DNA binding areas for both of these proteins result in removal of transgene

GLUT- expression (Dohm, 2002). These outcomes incited a study in 2005 which demonstrated that 5'AMP-activated protein kinase (*AMPK*) straightforwardly phosphorylates *GEF*, yet it does not appear to specifically stimulate MEF2 (Lee *et al.*, 2014).

A large body of evidence from diverse studies supports the observation that a noticeable growth in glucose transport and *GLUT4* protein expression arises in skeletal muscle in response to exercise (Holmes & Dohm, 2004). Impairments in insulin stimulated *GLUT4* expression and translocation in muscle cells are significant causes of insulin resistance and type 2 diabetes (Mukwevho, 2010). Exercise increases *GLUT4* content, *GLUT4* translocation to the plasma membrane, and glucose uptake in type 2 diabetes patients thus improving whole body glucose disposal and insulin sensitivity (Richter & Hargreaves, 2013). Exercise is therefore, an important therapeutic modality for insulin resistance and type 2 diabetes (Holten *et al.*, 2004a). Studies such as this one, which seek to assess the impact of the TZD on genes associated with diabetes and acetylation could identify molecular targets for drugs to treat type 2 diabetes and insulin resistance.

2.9 Type 2 diabetes and Exercise

There is no lone intervention with better effectiveness than physical exercise to decrease the risk of nearly all long-lasting diseases concurrently (Bird & Hawley, 2012). Proper exercises are clinically confirmed to interrupt and in many situations avert the health problems accompanying diabetes in humans and animals (Gibala *et al.*, 2012). Exercise is therefore an important component in the management of type 2 diabetes (Hayashino *et al.*, 2012).

The reduction in the risk of type 2 diabetes due to exercise is as a result of increased glucose transport capacity and insulin sensitivity (Mukwevho, 2010). Although the molecular mechanisms are not fully understood, epidemiological studies indicate that individuals who uphold regular physical movement are less likely to develop type 2 diabetes than physically inactive individuals (Booth *et al.*, 2012). These studies provide a basis for the existing support of regular physical activity as a way to protect people from this disease (dos Santos *et al.*, 2015).

Although exercise and weight reduction enhance insulin resistance and may now and again avert or defer onset of the disease, treatment that combats insulin resistance in the individuals who neglect to change their way of life is required (Yki-Jarvinen, 2004). Furthermore, some individuals are already obese by the time they are diagnosed with type 2 diabetes and hence struggle to exercise. It is for this reason that a medical hybrid compound (TZD) rather than physical exercise was used as the basis of this study.

2.10 Thiazolidinediones

To validate our use of the TZD, it is important to note that metformin is most frequently used to increase insulin sensitivity in diabetic patients. It reduces hepatic glucose synthesis and increases glucose uptake in skeletal muscle (Meier *et al.*, 2016). Metformin is considered by the World Health Organization as an indispensable medicine satisfying the conditions of the public health relevance, evidence on efficiency and safety, and relative cost effectiveness (Jabbour & Ziring, 2011). Its mechanism of insulin sensitization comprises activation of hepatic and muscle *AMPK*, which results in destruction of fatty acid synthesis, simulation of fatty acid oxidation in liver and increase in muscle glucose uptake (Inzucchi *et al.*, 2015). *AMPK* also reduces the expression of sterol-regulatory element-binding-protein 1(SREBP-1), a transcription factor involved in

adipocyte differentiation and pathogenesis of insulin resistance, dyslipidaemia and diabetes (Meier *et al.*, 2016). Unfortunately, the drug has many side effects (muscle and stomach pain, nausea, weakness etc) and is still unable to cure the disease. Metformin has a long-standing evidence base for efficacy and safety, is inexpensive, and is regarded by most as the primary first-line treatment for type 2 diabetes. When metformin fails to achieve or maintain glycaemic goals, another agent needs to be used. We therefore designed various TZD hybrid compounds in our quest to finding new and effective therapeutic modalities to treat or better manage diabetes and investigated their influence on genes that confer protection against diabetes.

Thiazolidinediones (TZDs), otherwise called glitazones, are a class of insulin sensitizing drugs which include Avandia (rosiglitazones), Actos (pioglitazones), Rezulin (troglitazone) and ciglitazone (Zaidi *et al.*) (Chinthala *et al.*, 2013). They were first discovered in 1975 by Takeda Pharmaceuticals in Japan and are the main current antidiabetic agents that function essentially by increasing insulin sensitivity (Soccio *et al.*, 2014). These drugs are additionally effective in treating a few patients with halfway lipodystrophy, including HIV-infected people (Hadigan *et al.*, 2004).

Thiazolidinediones empower stem cells that are too immature to differentiate in adipocytes (Tang *et al.*, 2011) and may build lipid oxidation and energy use in fat (Soccio *et al.*, 2014). These medications have likewise been accounted for binding mitochondrial membranes (Feinstein *et al.*, 2005), and the pyruvate transporters MCP1 and MCP2 have been firmly entangled as the mitochondrial targets of TZDs (mTOTs) (Colca *et al.*, 2013b). TZDs have appeared to reduce fat build up in the liver in patients with type 2 diabetes (Carey *et al.*, 2002). Other bioactivities displayed by these medications include antimicrobial (Liu *et al.*, 2011), analgesic (Garg *et al.*, 2011), anti-

inflammatory (Jain *et al.*, 2013) and anticancer activities (Galli *et al.*, 2010). Various studies demonstrated better adequacy of TZDs over other accessible antidiabetic treatments in the control of diabetic hyperglycaemia (Bailey, 2015) .

TZDs increase insulin sensitivity by means of action of peroxisome proliferator-activated receptors (PPARs) (Meier *et al.*, 2016). PPARs are a subfamily of the 48-part nuclear receptor subfamily and manage gene expression through ligand binding (Chawla *et al.*, 2001). Following ligand binding, PPARs experience particular conformational changes that take into consideration the recruitment of one co-activator protein or more (Willson *et al.*, 2001).

Thiazolidinediones have been observed to be ligands for the nuclear receptor transcriptional factor PPARɣ (Ahmadian *et al.*, 2013). PPARɣ is a nuclear hormone receptor that upgrades various genes encoding catalysts required in glucose and fat metabolic systems (Aronson, 2016). This receptor is communicated at abnormal states in fat tissue (Tontonoz & Spiegelman, 2008) and is crucial for typical adipocyte differentiation and unsaturated fat uptake and storage (Yki-Jarvinen, 2004). Late advances in DNA sequencing innovation have permitted more itemized testing of TZD impacts (Soccio *et al.*, 2014). Chromatin immunoprecipitation followed by greatly parallel sequencing (CHiP-seq) has uncovered a huge number of restricting destinations for PPARɣ through the entire genome, with improvement close to the normal adipocyte metabolic genes (Lefterova *et al.*, 2014). Information from knockout-mouse models suggest that fat tissue is the most imperative site for TZD activity if there are regular measures of fat tissue (Yki-Jarvinen, 2004).

Adiponectin, an adipocytokine delivered only by fat tissue (Maeda *et al.*, 2002), has both insulin-sensitizing and antiatherogenic properties in mice (Matsuda *et al.*, 2002). PPARɣ

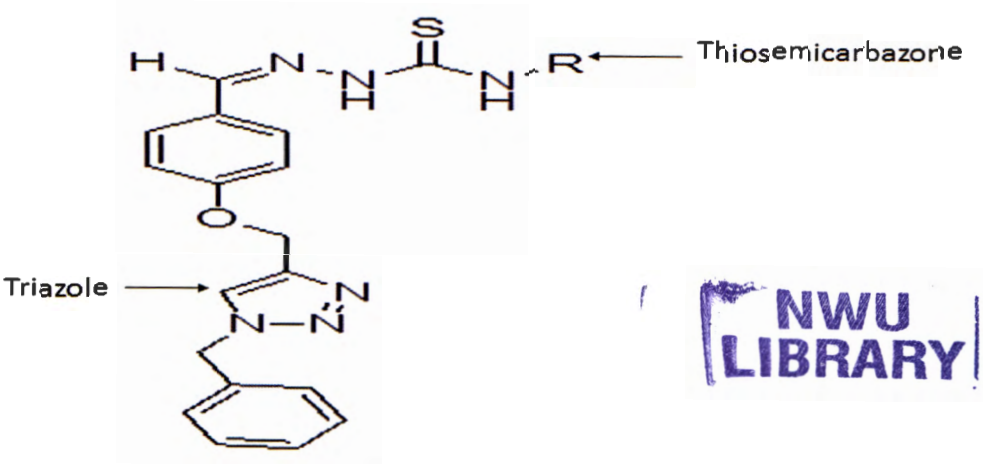
agonists raise adiponectin expression *in vitro* in adipose tissue (Maeda *et al.*, 2001). Adiponectin levels are low in patients with obesity and type 2 diabetes (Hotta *et al.*, 2000) and also in patients with lipodystrophy (Haque *et al.*, 2002). *In vitro* treatment with TZDs extraordinarily increases circulating concentrations of adiponectin (Knights *et al.*, 2014). The numerous impacts of thiazolidinediones in different tissues make it difficult to characterize the precise mechanisms fundamental to their insulin-sensitizing effects *in vivo* (Yki-Jarvinen, 2004).

Ciglitazone and troglitazone have been pulled back due to unfavourable side effects influencing the liver (Aronson, 2016). Rosiglitazone and pioglitazone are presently affirmed in many nations for the treatment of hyperglycaemia in patients with type 2 diabetes either as monotherapy or in blend with sulfonylureas or metformin (Bailey, 2015). Rosiglitazone is a TZD that is accepted for the treatment of hyperglycaemia in patients with conventional type 2 diabetes (Kahn & McGraw, 2010). The medication stimulates peroxisome proliferator-activated gamma receptors, builds hepatic and fringe insulin sensitivity, conserves insulin emission, and might advance pancreatic β-cell wellbeing (Cerf, 2013). These properties, together with information from trials with troglitazone propose that rosiglitazone could diminish the recurrence of diabetes in high risk people (Bailey, 2015). An online article by Drugwatch (2013) stated that Avandia use was seriously limited by the FDA in 2010 after it was associated with a huge number of heart attacks, strokes and heart failures (Tucker, 2016). The FDA expelled the confinements in late 2013 (Tucker, 2016). The FDA directed the outcomes from the Rosiglitazone Evaluated for Cardiovascular Outcomes and control of Glycaemia in Diabetes (RECORD) trial which demonstrated that rosiglitazone posed no elevated cardiovascular risk (Bae, 2016). After the tempest of debate and bad publicity,

it is unlikely that rosiglitazone will ever be largely utilized again as there is no novel advantage for this medication over pioglitazone (Soccio *et al.*, 2014).

Pioglitazone is a thiazolidinedione whose mechanism is like that of rosiglitazone (Al-Majed *et al.*, 2016). The medication relies on the presence of insulin for its mechanism of action (Russo *et al.*, 2003). It might be utilized alone or with different drugs, for example, insulin, metformin, or sulfonylurea operators (Al-Majed *et al.*, 2016). Pioglitazone is utilized together with a balanced diet and exercise to control glucose levels (Giles *et al.*, 2008). It diminishes insulin resistance in the periphery and in the liver, bringing about expanded insulin-dependent glucose transfer and reduced hepatic glucose yield (Al-Majed *et al.*, 2016). In spite of the fact that pioglitazones reduces hyperglycaemia, hyperinsulinemia and hypertriglyceridemia, it displays a few disadvantages including poor solubility and low bioavailability due to broad breakdown system in the liver (Uchiyama *et al.*, 2010), hepatoxicity (Floyd *et al.*, 2009), related cardiac irregularities (Giles *et al.*, 2008) and weight increase because of liquid retention (Nesto *et al.*, 2003). In pharmacological carcinogenicity studies, pioglitazone amplified urothelial bladder disease in male rats (Tseng & Tseng, 2012), but not in female rats, thus prompting a few studies in humans showing that pioglitazone increases bladder cancer risk (Soccio *et al.*, 2014). Interestingly, pioglitazone use is linked with significantly lower risk of death and lesser number of myocardial infarction and stroke incidence (Lincoff *et al.*, 2007), demonstrating that cardiovascular effects of TZDs are not a drug class effect, but rather precisely associated with TZD type (Meier *et al.*, 2016).

The TZD that was used in this study is a hybrid compound which is made up of two or more drug pharmacophores in one molecule with the hope of applying multi-drug action



(Kinfe *et al.*, 2013). The compound comprises a thiosemicarbazone and a triazole as seen in figure 2.3. Thiosemicarbazone is an analogue of a semicarbazone (condensation of semicarbazide and aldehyde/ketone) containing a Sulphur atom in place of the oxygen atom (Ayeleso *et al.*, 2017). Numerous thiosemicarbazone subordinates have appeared to have a wide range of natural impacts including antidiabetic activities (Kadowaki *et al.*, 2013). Triazoles are heterocyclic complexes containing a five-member ring of two carbon atoms and three nitrogen atoms as part of the aromatic five-member ring with molecular formula $C_2H_3N_3$ (Wakale *et al.*, 2013). They too, like thiosemicarbazones, have been reported to have antidiabetic benefits (Kadowaki *et al.*, 2013).

Figure 2.3: Chemical structure of the thiosemicarbazone-triazole hybrid compounds (Kinfe *et al.*, 2013). R- represents the various alkyl and aryl groups listed in Table 1.

The hybrids 1a-h were manufactured as per a published procedure outlined in Scheme 1 (Kinfe *et al.*, 2013; Ayeleso *et al.*, 2017). In summary, the synthesis began with alkylation of commercially available 4-hydroxybenzaldehyde with propargyl bromide in the presence of K_2CO_3 to give **4** which incorporates an alkynyl group which is required for click chemistry. Compound **4** was exposed to click chemistry with newly prepared benzyl azide to give 1,4-disubstituted triazole **5**. Methylhydrazinecarbodithioate **7**, that was prepared in a one pot synthesis from the condensation of hydrazine monohydrate and methyl iodide reacted with triazole **5** under Schiff's base condensation reaction conditions to produce compound **8**. This is shown in figure 2.4.

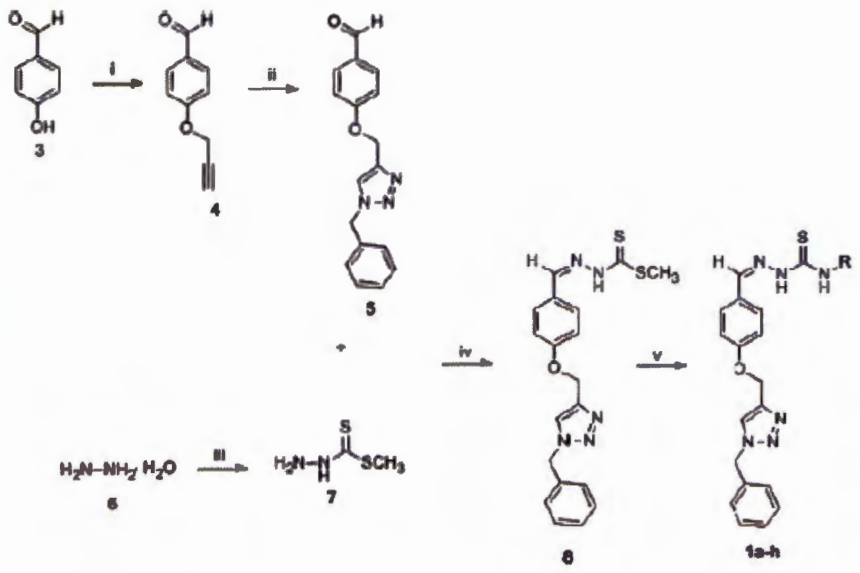


Figure 2.4: The scheme used in the synthesis of the hybrid compounds. Compound **8** went through nucleophilic substitution reactions with a series of primary amines to produce hybrid compounds 1a-h (Kinfe *et al.*, 2013).

The reagents and conditions that were involved in the synthesis of the hybrid compounds include:

i. K_2CO_3, propargyl bromide acetone, reflux, 2.5 hours, 92%;
ii. BnN_3, $CuSO_4.5H_2O$, sodium ascorbate, DMF:H_2O (4:1), 60°C, 3 hours, 80%;
iii. CS_2, KOH, CH_3I, H_2O: isopropanol (1:1), room temperature, 4 hours, 90%;
iv. 5,7, Methanol, reflux, overnight, 89%; and
v. RNH_2, Methanol, reflux, 24 hours, 62-85%.

The hybrid compounds were labelled 1a-1h as shown in the table 1:

Table 1: The hybrid compounds and the amines that were used to prepare them (Kinfe *et al.*, 2013).

Hybrid Compounds	**Amines (R-NH_2)**
1a	$PhCH_2NH_2$
1b	$CH_3CH_2CH_2CH_2CH_2NH_2$
1c	$PhCH_2CH_2NH_2$
1d	$(CH_3)_2NCH_2CH_2NH_2$
1e	$HOCH_2CH_2CH_2CH_2CH_2CH_2NH_2$
1f	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2NH_2$
1g	$CH_3CH(CH_3)CH_2NH_2$
1h	$HOCH_2CH_2NH_2$

All the eight compounds synthesized in table 1 (1a-1h) were further modified by adding a methoxy group in each compound to enhance the solubility and functionality thus producing compounds (2a-2h). In the previous findings (Ayeleso *et al.*, 2017) compound

1d had significantly upregulated the glucose transport genes hence compound 2d was used in this study. A methoxy group was added to compound 1d to produce compound 2d' and to study if it could enhance the results obtained using compound 1d. Understanding how TZDs work and efficiently harnessing the fundamental mechanisms with fewer side effects would be a welcome improvement in the collection of antidiabetic drugs (Kahn & McGraw, 2010; Meier *et al.*, 2016).

2.11 Antioxidants

Free radicals belong to a group of reactive oxygen species (ROS) (Ayeleso *et al.*, 2017). Not only are they unstable, but they also have one or more unpaired electrons and are transitory (Aruoma, 1996). They damage the cell and attack important cellular macromolecules such as protein, lipid and DNA (Lobo *et al.*, 2010). Free radicals in the body are scavenged by antioxidants (Goswami & Chatterjee, 2014). Molecular trails such as insulin signalling pathways are negatively affected in biological systems intensively exposed to ROS (Incir *et al.*, 2016). Aerobic organisms possess a defence mechanism constituting of antioxidants which specifically deal with ROS (Lobo *et al.*, 2010). Antioxidants inhibit initiation and generation steps and this terminates the chain reaction and delays the process of oxidation (Khalighi-Sigaroodi *et al.*, 2012). Antioxidant activity is exhibited by the donation of hydrogen atoms or the transfer of a single electron to a radical (Berczyński *et al.*, 2013). Both enzymatic and non-enzymatic antioxidant systems keep ROS levels constant as an imbalance of antioxidants (that is too much or too less) results in oxidative stress (Li *et al.*, 2013) and may also lead to the malfunction of several metabolic pathways such as mitochondrial biogenesis (Cherry & Piantadosi, 2015).

The increased coupling of mitochondria plays a significant role in the generation of reactive oxygen species, resulting in the increment of oxidative stress in the liver and skeletal muscles (Cherry & Piantadosi, 2015). Heart tissue is relatively vulnerable to oxidative damage because it has great oxidative capacity and its antioxidant enzyme capacity is poor compared to other organs (Thirunavukkarasu *et al.*, 2004). Metabolic rates of tissues and organs differ and so does their oxygen consumption, making their antioxidants levels and capacity distinguishable from tissue to tissue and organ to organ (Limon-Pacheco & Gonsebatt, 2009). Oxidative stress has been implicated in the pathogenesis of many chronic diseases including diabetes (Ayeleso *et al.*, 2017). Oxidative stress plays a critical role in the progression of diabetes complications, both microvascular and cardiovascular (Cherry & Piantadosi, 2015). The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, and in the myocardium (Oboh *et al.*, 2014). Both prokaryotic and eukaryotic organisms have developed an antioxidant defence mechanism for survival which assists in the removal of toxic oxygen by-products produced in living organisms (Lobo *et al.*, 2010).

Furthermore, antioxidants have been reported to slow down the aging process and to fight diseases such as diabetes mellitus, hypertension and cancer (Oboh *et al.*, 2014). The hybrid compound used in this study has been shown to have antioxidant potentials and could therefore play important protective roles in counteracting the onset and progression of type 2 diabetes mellitus (Ayeleso *et al.*, 2017).

CHAPTER 3

3.0 Materials and Methods

3.1 Cell Culture

C2C12 myocytes were cultured in an incubator (5% CO_2) at 37°C in petri-dishes. The growth medium contained 89% of Dulbecco's modified eagles' medium (DMEM) containing 4500 mg/L glucose, 10% of fetal bovine serum (FBS) and 1% antibiotic mixture containing streptomycin, penicillin and fungizone. The cells were maintained in a continuous passage by trypsinization of sub-confluent cultures with 0.25% trypsin versene. Myoblast were then achieved by the introduction of a medium containing 2% heat-inactivated horse serum when myocytes were ~80% confluent. The cells were kept in this medium for 5-10 days until myotubes formed. The C2C12 cells used in this study were obtained from the American Tissue Culture Collection (ATCC).

This study was conducted with 5 experimental groups namely control (normal), palmitate, palmitate + metformin, palmitate + TZD and TZD. The TZD used in this study was compound 2d' which was derived from a primary compound 1d (found to be effective in previous studies). Palmitate is a saturated fatty acid. Elevated levels of free fatty acids in the plasma is often associated with insulin resistance in diabetic patients. An impairment of glucose use and insulin sensitivity has been observed in experimental studies with high administration of free fatty acids. Hence palmitate was used in this study. The 5 groups underwent the following experiments: MTT for cell viability, qPCR for gene expression, ChIP assay to measure protein-DNA interactions, antioxidant assays to measure antioxidant activity and enzymatic assays to measure glucose activity and ATP synthesis.

3.2.1 MTT assay

An MTT assay was performed using the Vybrant ® MTT Cell Proliferation Assay Kit (Life technologies). Growth of C2C12 cells (treated with different concentrations of the TZD) was quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. At the end of 72 hours of incubation, the medium in each well was replaced by MTT solution (20 cell/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for 4 hours under 5% CO_2 and 95% air at 37°C. The MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in 100 µl of DMSO and gently shaken. The absorbance was then determined by Multiskan Spectrum plate reader (Thermo Fischer Scientific, USA) at 540 nm. (Nemati *et al.*, 2013) as seen in figure 3.1 below.

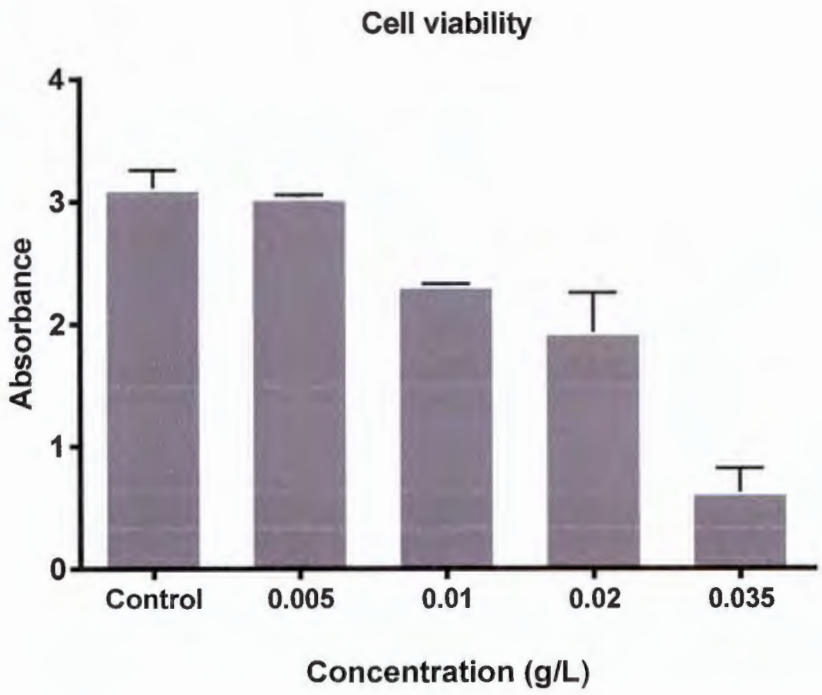


Figure 3.1: Graph showing cell viability as related to the absorbance of cells treated with different concentrations of the TZD. The 0.005 g/L concentration was optimal.

3.2.2 RNA extraction

Cells were plated (as described in section 3.1) and the palmitate groups were treated by a protocol outlined by a previous study (Mazibuko *et al.*, 2013). Briefly, a 75 mM stock solution was prepared by dissolving 0.209 g of Sodium palmitate in 10 ml of ethanol. For the working solution, 100 µl of the stock solution was mixed with 9900µl of DMEM with 2% BSA. This gave a final volume of 10 ml yielding a final palmitate concentration of 0.75 mM which was used to treat the cells in each plate and then incubated for 16 hours. The metformin treatment required an extra 4 hours during which 1 uM of metformin was added to the plate. The TZD treatment on the other hand was left for an extra 24 hours where 5 µl of hybrid compound 2d' (10 mg/ml in DMSO) was added to the plate as described by a previous study (Kinfe *et al.*, 2013). After several preliminary studies, we established that the metformin treated cells began to die when left for the same treatment hours as that of the TZD, hence the metformin treatment hours were reduced to what seemed to be an optimal incubation period. There were 5 treatment groups namely control, palmitate, palmitate + metformin, palmitate + TZD and TZD. RNA was extracted from the cells using RNeasy mini kit, quick-start protocol respectively. A maximum of 1 x 10^7 cells was harvested by direct lysis in the vessel. A volume of 500 µl of RLT buffer was added to the cells. After this; 1 ml of 70% ethanol was added to the lysate. The sample was then transferred to an RNeasy Mini spin column which was recruited in a 2 ml collection tube. The tube was then centrifuged for 15 seconds at 8000 x g. The flow through was discarded and 700 µl of buffer RW1 was added to the RNeasy spin column. The tube was again centrifuged for 15 seconds at 8000 x g. After discarding the flow through, 500 µl of buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at 8000 x g. The previous step was repeated, but this time centrifugation was for 2 minutes at 8000 x g. The RNeasy spin

column was placed in a new 1.5 ml collection tube and 30 µl of RNase-free water was added directly to the spin column membrane. The tube was then centrifuged for 1 minute at 8000 x g to elute the RNA.

Gel electrophoresis was then performed to determine the presence and integrity of the RNA. A 1% gel was prepared by weighing 1 g agarose into a 100 ml bottle. Into this bottle, 100 ml of TAE buffer was added and mixed well. The mixture was then heated in a microwave until the agarose powder was completely dissolved (~60 seconds). The mixture was cooled to 50°C. Once cooled, 1.5 µl of ethidium bromide was added to the mixture to allow fluorescence and then shaken well. The mixture was then poured into a gel tray to which combs were added and then left to solidify. Once ready, 6 µl of loading dye and 4 µl of RNA were loaded into the wells. The gel was run at 60 volts for 50 minutes.

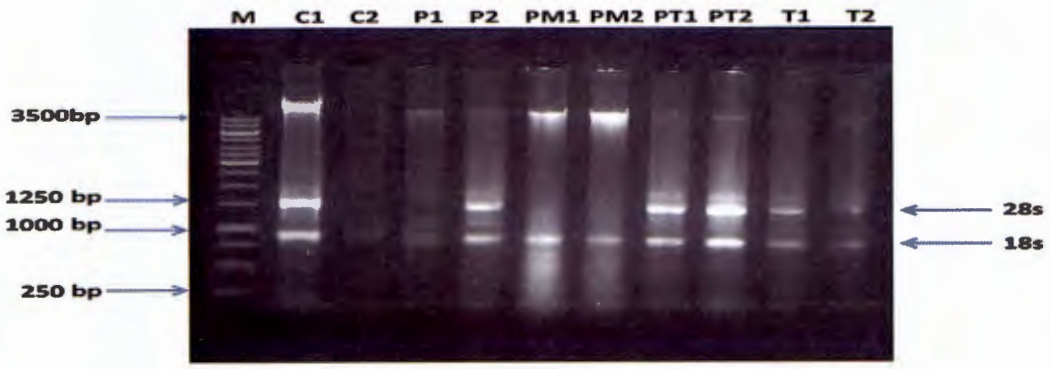


Figure 3.2: Agarose gel (1% w/v) image depicting RNA extracted from 8 treated cells and 2 control cells. Lane M= 1kb RNA marker, Lane 2= Control 1, Lane 3= Control 2, Lane 4= Palmitate1, Lane 5= Palmitate 2, Lane 6= Palmitate + Metformin 1, Lane 7= Palmitate + Metformin 2, Lane 8= Palmitate + TZD 1, Lane 9= Palmitate + TZD 2, Lane 10= TZD 1 and Lane 11= TZD 2.

3.3 Quantitative Polymerase Chain Reaction (qPCR) of diabetes and acetylation related genes.

Double stranded cDNA was synthesized from approximately 3 µg of total RNA by SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA) using the manufacturer's protocol. The following reagents were added to 2.5 µg of RNA: 4 µl of 5X VILO Reaction Mix, 2 µl of 10X SuperScript Enzyme Mix and made up to 20 µl using DEPC-treated water. The contents of the tube were mixed gently and incubated at 25°C for 10 minutes and then incubated again at 42°C for 60 minutes. The reaction was terminated at 85°C for 5 minutes.

Real time PCR was performed in triplicate using a StepOnePlus Real Time PCR machine (Applied Biosystems) together with the PowerUp SYBR Green Master Mix reagent (life technologies) and cycling was per the manufacturer's instructions. The following reagents were mixed in 0.1 ml tubes: 5 µl of PowerUp SYBR Green Master Mix, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of cDNA and 1 µl of RNase-free water to make up a reaction volume of 10 µl. A standard was prepared by adding 3 µl of each sample. The standard was then diluted by means of serial dilution to prepare other standards. Actin was used as the reference gene. The samples and standards were then run on the StepOnePlus Real Time PCR machine. The machine program was set according to the manufacturer's protocol, as follows:

UDG Activation 50°C for 2 minutes on hold, AmpliTaq Fast DNA Polymerase, UP Activation 95°C for 2 minutes on hold, Denaturation 95°C for 1 second at 40 cycles and Annealing/ Extension at 60°C for 30 seconds at 40 cycles.

The expression ratio was calculated by using relative standard method. The calculated concentrations (CC) of the reference and target genes were collected. The CC value of

the target gene was divided by that of the reference gene. The average was then calculated. The expression value was obtained by dividing all the values with control value. By plotting fluorescence against the cycle number, the real-time PCR instrument generated an amplification plot that represents the accumulation of product over the duration of the entire qPCR reaction. qPCR was used to assess *NRF-1*, *MEF2A*, *GLUT4*, *pCAF*, *p300*, *GCN5* and *HDAC4* for gene transcription. *GAPDH* served as the reference gene. The primers used in each qPCR analysis are listed in Table 3.1.

Table 3.1: List of primers for the genes used in the qPCR analysis.

Gene type	Primer type	Primer sequence (Mukwevho, 2010)
GAPDH	Forward	5'-GCA CAG TGA AGG CCG AGA AT-3'
	Reverse	5'-GCC TCTC TCC ATG GTG GTG AA-3'
HDAC 4	Forward	5'-GAG AGA ATT CTG CTA GCA ATG AGC TCC CAA-3'
	Reverse	5'-GAG ACT CGA GCT ATG CAG GTT CCA AGG GCA GTGA-3'
MEF2A	Forward	5'-GTG TAC TCA GCA ATG CCG AC-3'
	Reverse	5'-AAC CCT GAG ATA ACT GCC CTC-3'
GLUT4	Forward	5'-GTG GGT TGT GGC ATG GAG TC-3'
	Reverse	5'-AAG ATG GCC ACG GAG AGA G-3'
pCAF	Forward	5'-CCG TGA AGA AAG CGC AAC TAC-3'
	Reverse	5'-AGA CTC CTC GCG CTT GCA-3'
p300	Forward	5'-GGG ACT AAC CAA TGG TGG TG-3'
	Reverse	5'-ATT GG GAGA AGT CAA GCC TG-3'
GCN5	Forward	5'-CTG GTG CCT GAG AAG AGG AC-3'
	Reverse	5'-CTC CGA AGG TGG CAT GGT GAA G-3'

All primers were purchased at Integrated DNA Technologies, except for those of the *pCAF*, *p300* and *GCN5* which were purchased at Inqaba Biotec. The primers were reconstituted to 100 mM stock solution by dissolving in a specific amount of distilled water (varies with each primer). To prepare the working solution, the stock solution was diluted at a ratio of 1:10 yielding a concentration of 10 µM.

3.4 Chromatin Immunoprecipitation (ChIP) assay

ChIP assays was performed to analyze *MEF2A—GLUT4* binding. C2C12 cells were cultured as mentioned in section 3.1, differentiated into myotubes and treated with the newly synthesized TZD. The newly synthesized TZD compound was chemically modified by the addition of a methoxy side chain to the already existing or known TZD. After treatment, cells were incubated in a medium containing 1% formaldehyde at 37°C to cross-link protein-DNA and protein-protein interactions. Rotation was done gently at 37°C for 10 minutes. Cross-linking was stopped by adding 2.5 M glycine to each plate bringing the reaction mix to a final concentration of 0.125 M through the addition of 500 µl for every 10 ml. Rotation was done gently at an incubation of 37°C. Cells were washed thrice with cold 1X PBS and lysed with 250 µl of SDS lysis buffer on ice for 10 minutes, mixing every 5 minutes. Cell lysates were scrapped and transferred to micro tubes for sonication.

3.4.1 Sonication

Sonication was done on ice using a sonicator set at 6 watts at 10 x 15 second bursts (1minute rest on ice) at 33% maximum. After sonication, lysates were centrifuged at 4°C for 10 minutes at 13000 rpm. The supernatants containing the cross-linked DNA-protein fragments were collected into 1.5 ml tubes. Total protein concentration for each sample

was determined using a nanodrop. Proteins were immunoprecipitated by taking 100 µl of the supernatant and diluting it 9-fold with ChIP dilution buffer to make 1 ml.

3.4.2 Immunoprecipitation

The remaining supernatant was mixed with 30 µl of 50% slurry agarose beads/ salmon sperm (SCBT). The sample was incubated at 4°C on a rotating platform for 1-2 hours. The beads were pelleted at 2000 rpm for 2 minutes at 4°C and the supernatant transferred to a new tube. Antibodies for *MEF2A* were added. Immunoprecipitation was allowed for 36-48 hours at 4°C rotating gently. After incubation, 40 µl of SCBT beads were added to the samples and rotated for 4-6 hours at 4°C. The beads were pelleted at 5000 rpm for 1 min at 4°C. The pellet was washed and centrifuged at 2000 rpm, 1 ml 1 x 3-5 minutes, at 4°C with the following buffers respectively, low salt wash buffer, high salt wash buffer, LiCl wash buffer and 1ml TE buffer 2 x 3-5 minutes at room temperature. The beads were resuspended in fresh elution buffer. To the 30 µl input sample, 90 µl of elution buffer and 7.2 µl 5M NaCl were added. Crosslinking was reversed at 65°C overnight. The following reagents were then added to the sample, 5 µl EDTA (0.5M), 20 µL Tris-HCl (1 M, Ph 6.5) and 1 µl proteinase K (stock 10 µg/µl) which was then incubated at 45°C for 1 hour. For immunoprecipitation, 150 µl of elution buffer was added to the sample which was rotated at room temperature for 15 minutes and then centrifuged at 2000 rpm. The supernatant was transferred to a new micro tube. The 2 previous steps were repeated, but this time the supernatant was added to the existing 150 µl sample to make 300 µl to which 18 µl 5 M NaCl was added. Crosslinking was reversed at 65°C overnight. The following reagents were then added to the sample, 5 µl EDTA (0.5 M), 20 µL Tris-HCl (1 M, pH 6.5) and 1 µl proteinase K (stock 10 µg/µl) which was then incubated at 450°C, using a modular heat block for 1 hour to destroy the protein in the sample. The DNA was purified using the Chloroform/Phenol

procedure. Briefly, 300 µl was added to each sample and rotated for 5 minutes at room temperature. The samples were then centrifuged for 1 minute at 13000 rpm. The aqueous phase was transferred to a new tube and the phenol tube was discarded. After this, 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to each tube which was then vortexed and rotated for 5 minutes at room temperature. The samples were again centrifuged at 13000 rpm for 1 minute. The aqueous phase was carefully transferred to a new tube and the old tubes discarded. To the DNA tube 3 µl of glycogen (that is 2 µg/µl), 30 µl 3 M sodium acetate (pH 5.2) and 750 µl of 100% ethanol were added. The samples were incubated overnight at -20°C. The DNA tube was then centrifuged at 13000 rpm for 5 minutes. The pellet was washed with 70% ethanol. The sample was again centrifuged for 5 minutes at 13000 rpm. The supernatant was decanted and the pellet air dried. The pellet was then resuspended in 20 µl of distilled water. The samples were then stored at -87°C. Conventional PCR was performed by mixing 3 µl of Taq polymerase, 0.6 µl of forward and reverse primers, 3 µl of $MgCl_2$, 3 µl of buffer, 2.4 µl of dNTPs and 20.1 µl of DEPC water to a final volume of 30 µl. Lastly 1.5 µl of DNA sample was added to the mixture. The PCR mixture was denatured at 94°C for 4 minutes & 94°C for 30 seconds, annealed at 63.5°C for 30 seconds and extended at 72°C for 1 minute & 5 minutes for 35 cycles. The primers used in this reaction are listed in the table 3.2.

Table 3.2: Primers for the genes used in the ChIP assay.

Designed Primer name	**Amplicon size**	**Primer type**	**Primer sequence (Mukwevho, 2010)**
GLUT4-MEF2A (+ve)	268bp	Forward	5'-CAG GCA TGG TCT CCA CAT ACA C-3'
		Reverse	5'-GGT AAC TCC AGC AGG ATG ACA-3'

The primers pairs (forward and reverse) were designed to span the *MEF2* site on the *GLUT4* promoter. These are referred to as positive primers in this study. The primers were selected based on least potential to form inter- or intra primer dimmers and hairpins, GC content between 50-66%, the melting temperature between 55-70°C, 18-24 bp nucleotides and finally, transcript size between 250-340 bp.

3.5 Antioxidant activity

3.5.1 ABTS radical scavenging activity/ Trolox equivalent antioxidant capacity (TEAC)

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity was done according to a method described by (Re *et al.*, 1999). ABTS solution was prepared 24 hours prior to the experiment, by mixing 8 mM of ABTS salt with 3 mM of potassium persulfate and stored in the dark. Before use, the ABTS solution was further diluted with distilled water and 300 µl mixed with 25 µl of the sample in a 96-well clear microplate. The plate was incubated for 30 minutes at room temperature and read in a Multiskan Spectrum plate reader (Thermo Fischer Scientific, USA) at 734 nm. Trolox was used as the standard and results expressed as µmol TE/g sample.

3.5.2 Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out using the method described by Benzie and Strain (Benzie & Strain, 1996). The FRAP reagent was prepared by mixing (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ), (10 mM in 40 mM HCl) and $FeCl_36H_2O$ (20 mM). Then 300µl of the FRAP reagent was mixed with 10 µl of the sample in triplicates. After incubation at room temperature for 30 minutes, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fischer

Scientific, USA). Ascorbic acid (AA) was used as the standard and the result expressed as µmol AAE/g sample.

3.6 Enzymatic assays

3.6.1 Glucose oxidase assay

A glucose oxidase assay was performed using a Glucose Oxidase Activity Assay Kit (Sigma-Aldrich). H_2O_2 standards for Colorimetric detection were prepared by diluting 10 µl of the 0.88 M H_2O_2 with 870 µl of water to prepare a 10 mM standard solution. The 10 mM was further diluted to 0.5 mM by diluting 50 µl of the standard solution with 950µl of GOx Assay buffer. Next, 0, 2, 4, 6, 8 and 10µl of the 0.5 mM standard solution were added into a 96-well plate, generating 0 (Blankenship *et al.*), 1, 2, 3, 4, and 5 nM/ well standards. To bring the volume to 50 µl, GOx assay buffer was added to each well. H_2O_2 standards for Fluorometric detection were prepared by diluting 10 µl of the standard solution with 90 µl of the GOx assay buffer to make a 50 µM standard solution. Then, 0, 2, 4, 6, 8 and 10 µl of the 50 µM standard solution was added into a 96-well plate, generating 0 (Blankenship *et al.*), 0.1, 0.2, 0.3, 0.4, and 0.5 nM well standards. To bring the volume to 50 µl, GOx assay buffer was added to each well. Then, 15 µl of the samples were mixed with 35 µl of GOx assay buffer. The reaction mixture was prepared by adding 36 µl of GOx assay buffer, 2 µl of GOx developer, 2 µl of Fluorescent Peroxidase Substrate and 10 µl of GOx substrate respectively. The same reaction mixture was prepared for the blank but without the GOx substrate. After this, 50 µl of the appropriate reaction mixture was added to each well and mixed by pipetting. After 5 minutes, the initial measurement ($T_{initial}$) was taken. For colorimetric assays, the absorbance was measured at 570 nm $(A_{570})_{initial}$. For fluorometric assays, the fluorescence intensity was measured ($FLU_{initial}$, λex = 535/ λem = 585 nm). The plate was incubated at 37°C and measurements were taken every 5 minutes. The

measurements were taken until the value of the most active sample was greater than the value of the most active standard. The H_2O_2 standard curve was plotted.

3.6.2 ATP assay

An ATP assay was carried out using an ATP Determination Kit Invitrogen (Thermo Fischer Scientific). Firstly1 mL of 1X reaction buffer was made by adding 50 µL of 20X reaction buffer to 950 µL of deionized water. This volume was sufficient to make 1 mL of 10 mM D-luciferin stock solution. Next, 1 mL of a 10 mM D-luciferin stock solution was prepared by adding 1 mL of 1X Reaction Buffer to one vial of D-luciferin. The mixture was protected from light until use. A 100 mM DTT stock solution was prepared by adding 1.62 mL of dH_2O to the bottle containing 25 mg of DTT. The solution was aliquoted into ten 160 µL volumes and stored frozen at ≤ -20°C. Low-concentration ATP standard solutions were prepared by diluting the 5 mM ATP solution in dH_2O. The components of the reaction were combined as follows to make 10 mL of a standard reaction solution: 8.9 mL dH_2O, 0.5 mL 20X Reaction Buffer, 0.1 mL 0.1 M DTT, 0.5 mL of 10 mM D-luciferin, and 2.5 µL of firefly luciferase 5 mg/mL stock solution. The tubes were inverted gently to mix, and not vortexed as the firefly luciferase enzyme is easily denatured. An appropriate volume of the standard reaction solution was placed in the luminometer and background luminescence was measured. The reaction was started by adding the desired amount of dilute ATP standard solution and the luminescence read. The volume of the dilute ATP standard solution that was added to the standard assay solution was no more than 10% of the total assay volume. The background luminescence was subtracted. A standard curve was generated for a series of ATP concentrations.

3.7 Statistics Analysis

Data were presented as means ± Standard Deviation of three replicates. Statistical differences between treatments were determined using a one-way ANOVA. Significance was accepted at $p < 0.05$. When the ANOVA test showed a significant difference, the Dunnett's multiple comparison test was conducted. Graph Prism 7 software was used for these analyses. The p values were represented as seen in table 3.3.

Table 3.3: Description of the statistical analysis symbols

SYMBOL	MEANING
Blank	$p > 0.05$
*	$p \leq 0.05$
**	$p \leq 0.01$
***	$p \leq 0.001$
****	$p \leq 0.0001$

CHAPTER 4

4.0 Results

4.1 Gene transcription of glucose transport and acetylation related genes

Since most glucose transport and acetylation genes are down regulated in diabetes, this experiment aimed to determine if the TZD would avert the down regulation of genes under insulin resistance conditions induced by palmitate. Insulin resistance is known to precede diabetes. The experiments below indicate how this newly synthesized hybrid compound influenced expression of these genes and its significance thereof towards finding new and effective therapeutic means to better manage or cure diabetes.

4.1.1 Gene transcription of *NRF-1*

The five treatments were tested for their expression of the *NRF-1* gene, which is a transcription factor involved in glucose transport. The results in figure 4.1 show that the TZD treatment increased the expression of the *NRF-1* gene ~5-fold compared to the untreated (control) cells. When palmitate only was used to treat the cells, the *NRF-1* expression was reduced significantly lower than the control cells, indicating that insulin resistance was effective. When palmitate was used together with the TZD, the expression of *NRF-1* was increased ~3.8-fold, whereas it only increased ~2.5 fold when used with metformin. The TZD treatments appear to have better upregulated the *NRF-1* gene expression than the palmitate + metformin.

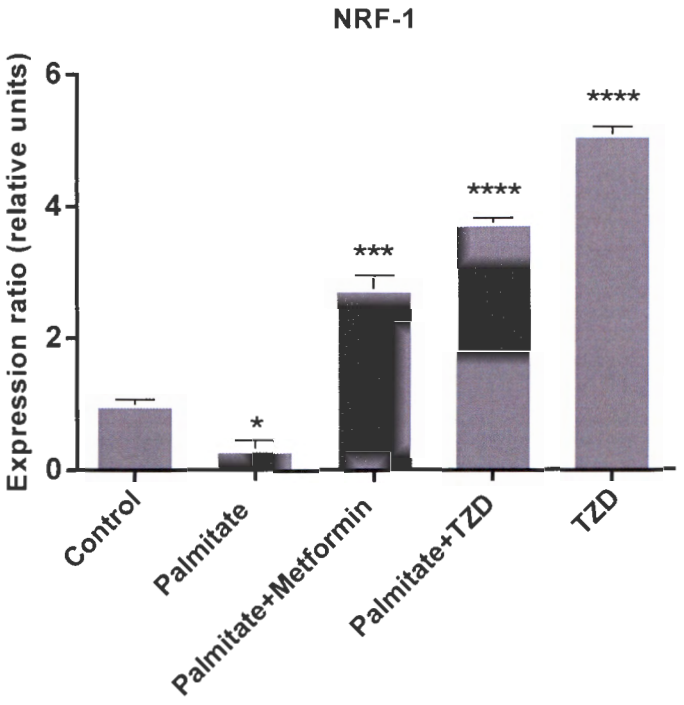


Figure 4.1: *NRF-1* gene expression in response to a variety of treatments in C2C12 myotubes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.2 Gene transcription of *MEF2A*

Having observed increased *NRF-1* gene expression, it was therefore logical to assess its target gene, *MEF2A*. *MEF2A* is a transcription factor for *GLUT4*, whose expression is controlled by *NRF-1.* Using similar treatments performed above with *NRF-1*, the effect of the hybrid TZD on the expression of the *MEF2A* gene was also investigated. The results in figure 4.2 indicate that *MEF2A* gene was increased by ~2.5 fold when treated with the TZD only compared to the control group. Again, palmitate only almost obliterated its expression. However, when palmitate was used together with either TZD or metformin, the expression of *MEF2A* did increase moderately above the palmitate only group.

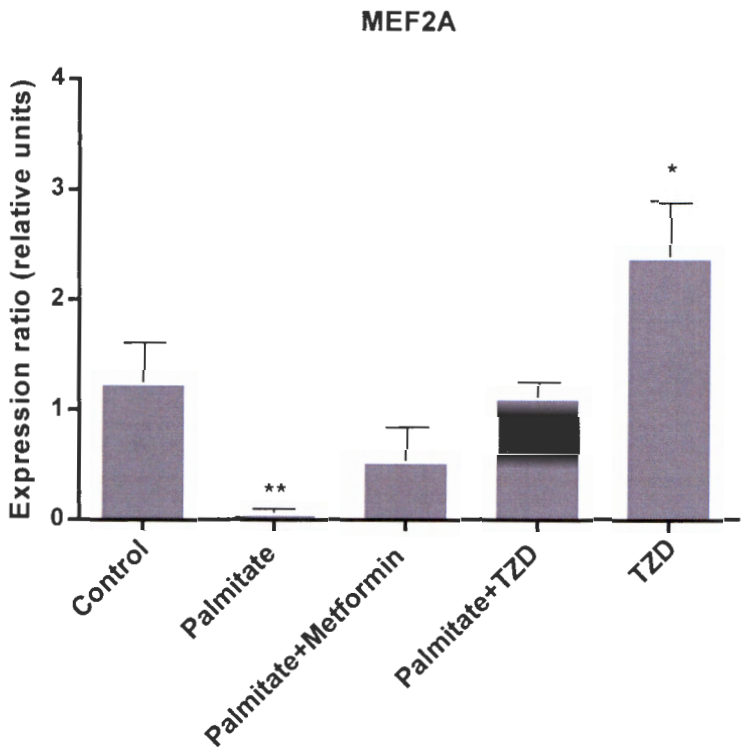


Figure 4.2: *MEF2A* gene expression in response to a variety of treatments in C2C12 myotubes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.3 Gene transcription of *GLUT4*

Furthermore, the expression of the *GLUT4* gene was investigated. *GLUT4* is an insulin-responsive glucose transporter that regulates glucose uptake. *NRF-1* controls the *MEF2A* gene and in turn regulates *GLUT4*. The results in figure 4.3 show that the TZD only increased *GLUT4* expression ~5-fold compared to the control. The palmitate only treatment significantly reduced *GLUT4* expression below the control group level. The palmitate + TZD expression of *GLUT4* was slightly higher than that of the palmitate + metformin treatment. The palmitate + metformin treatment in fact produced a lesser yield of the gene than the control. The similar trend of expressions observed with other glucose transport related genes, *MEF2A* and *NRF-1,* was also observed with the

GLUT4 gene in this experiment. This indicates the positive influence of TZD on glucose transport genes.

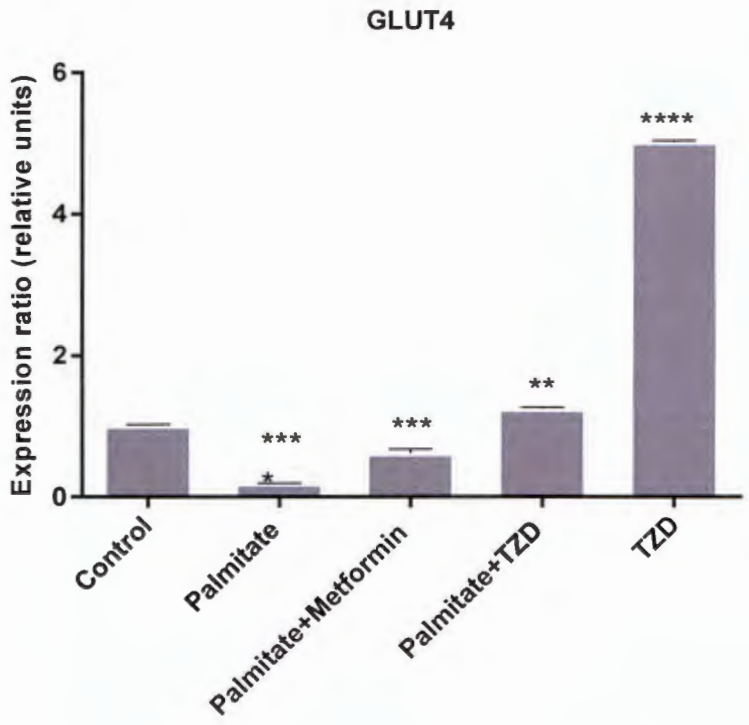


Figure 4.3: *GLUT4* gene expression in response to a variety of treatments in C2C12 myotubes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control proup (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.4 Gene transcription of *pCAF*

Acetylation has long been an established posttranslational modification that results in gene transcription. Various reports have linked increased expression of glucose transport related genes with various histone acetyltransferases (HATs) responsible for their acetylation. In a similar vein, HATs associated with glucose transport gene expression were evaluated. The first HAT studied in this experiment was *pCAF*, which is a HAT associated with *NRF-1* and *MEF2A*. The results shown in figure 4.4 indicate that the gene was upregulated ~4-fold in the TZD treatment group when compared with the control. The palmitate only group, as expected, significantly reduced *pCAF* expression

under induced insulin resistance conditions in C2C12 myotubes. When palmitate was used together with TZD, the expression of *pCAF* was increased by ~3-fold, whereas when palmitate was used with metformin the increase was only moderate.

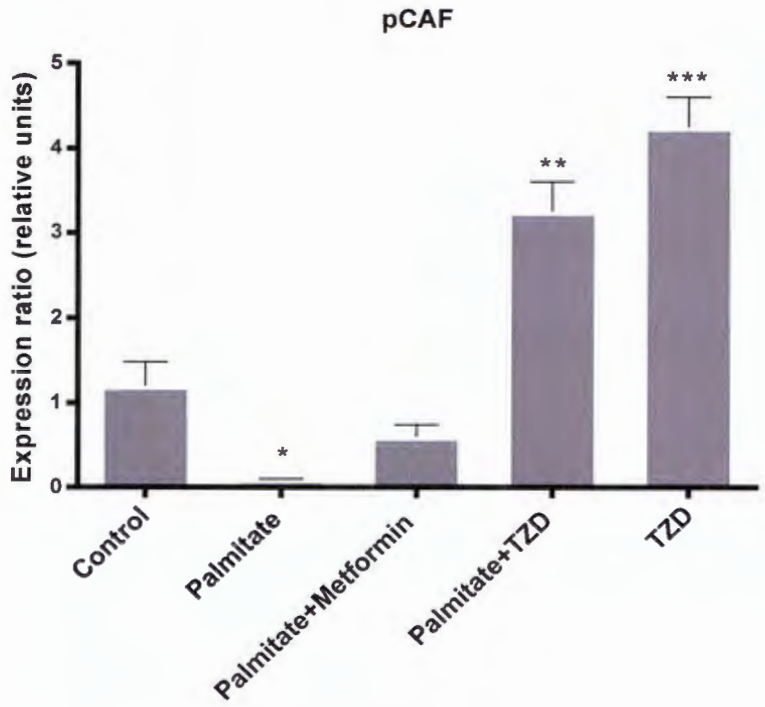


Figure 4.4: *pCAF* gene expression in response to a variety of treatments in C2C12 myocytes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.5 Gene transcription of *p300*

Another important HAT associated with glucose transport gene expression is *p300.* Research has shown that it associates with both *NRF-1* and *MEF2A* transcription factors to induce gene expression. Both the *p300* and *pCAF* associated factors liaise with several transcription factors and act to intensify the expression of their target genes. The results in figure 4.5 illustrate that the TZD only increased the *p300* expression 1.4-fold. Palmitate only also reduced the expression of *p300* significantly when compared to the control. When palmitate was used with either metformin or TZD,

the expression of *p300* was upregulated, although it was not above the control group levels.

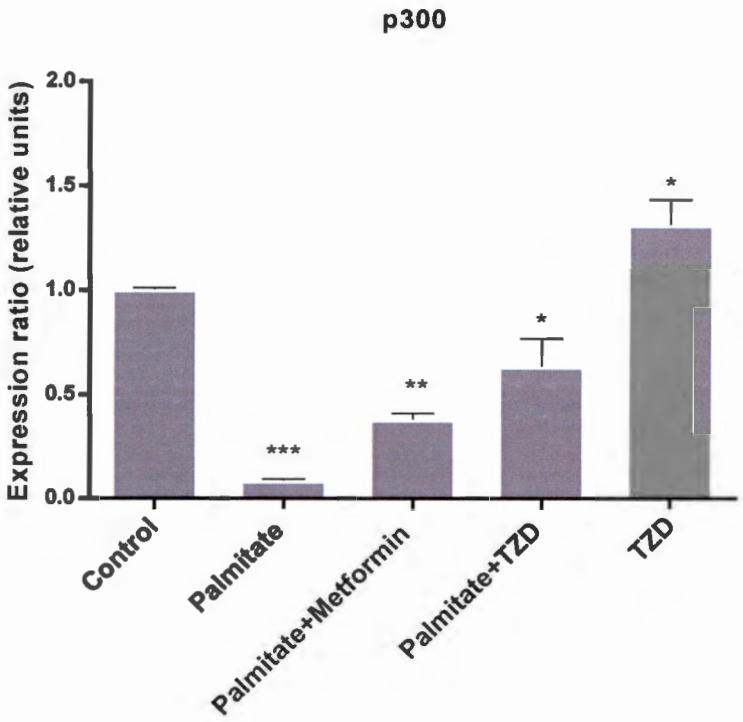


Figure 4.5: *p300* gene expression in response to a variety of treatments in C2C12 myocytes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.6 Gene transcription of *GCN5*

The last HAT to be evaluated was the *GCN5* which mainly acts as a transcriptional activator and acetylates histones genome-wide (global acetylation).

The results shown in figure 4.6 reveal that *GCN5* gene expression increased ~9-fold after treatment with the TZD compared with the control group. The palmitate group was significantly reduced levels below the control group as expected. When palmitate was used together with metformin, the expression of *GCN5* was increased to ~3.8-fold

compared to the control and ~4-fold compared to the treatment with palmitate alone. Although not significant, the gene expression of the palmitate + metformin was slightly higher than that of the control. Consistently, the three HATs studied showed that the TZD significantly increased their expressions in an analogous manner observed with the glucose transport related genes, concomitantly significantly reduced by palmitate.

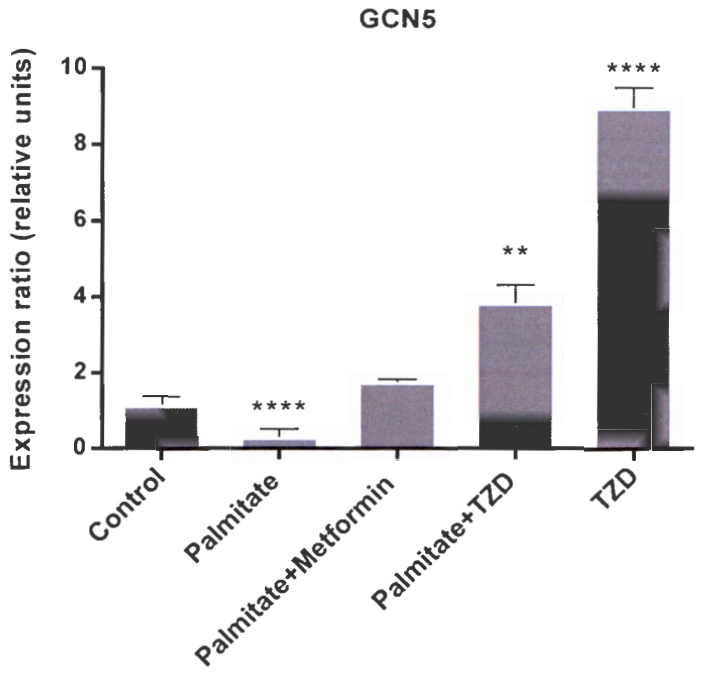


Figure 4.6: *GCN5* gene expression in response to a variety of treatments in C2C12 myocytes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.1.7 Gene transcription of *HDAC4*

HATs function antagonistically with histone deacetylases (HDACs) which are associated with the down regulation of gene expression. *HDAC4* expression condenses chromatin thus masking the *MEF2A* sites and obstructing entrance for transcription regulators. This in turn inhibits *GLUT4* gene expression and subsequently glucose transport.

Results in figure 4.7 reveal that the *HDAC4* gene was downregulated in all the four treatments as compared to the control. It is not surprising that the expressions were generally similar in all treatments since HDACs are associated with the suppression of genes by compacting the chromatin such that transcription factors are unable to access their respective DNA binding domains in the promoters.

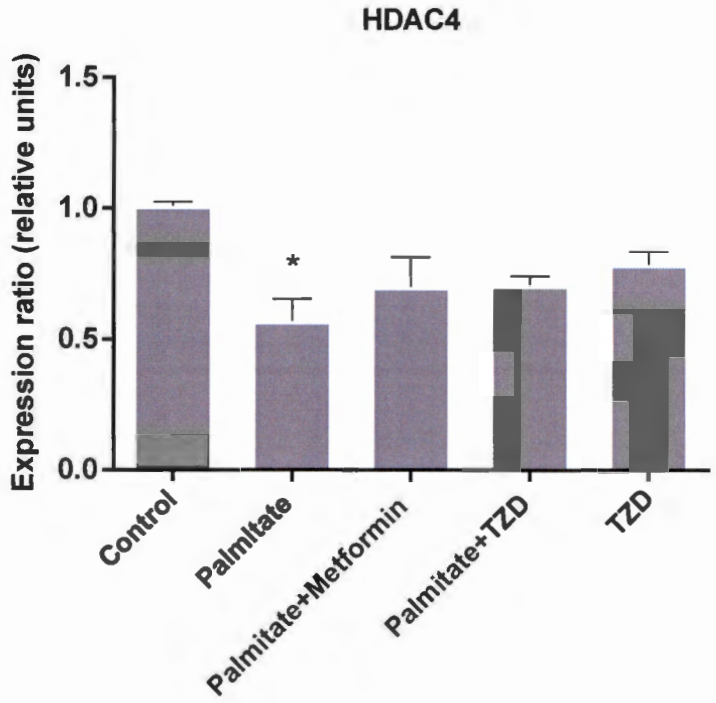


Figure 4.7: *HDAC4* expression in response to a variety of treatments in C2C12 myocytes as assessed by qPCR. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.2 Determination of *MEF2A* binding to the *GLUT4* promoter

Results in figure 4.8 indicate that palmitate, as expected, reduced the level of binding whereas treatments with either metformin/TZD or with TZD only showed some modest increase in *MEF2A* binding to the *GLUT4*. Binding of transcription factors to their respective binding domains precedes gene transcription. This experiment was conducted to study the model through which TZD regulate glucose transport genes.

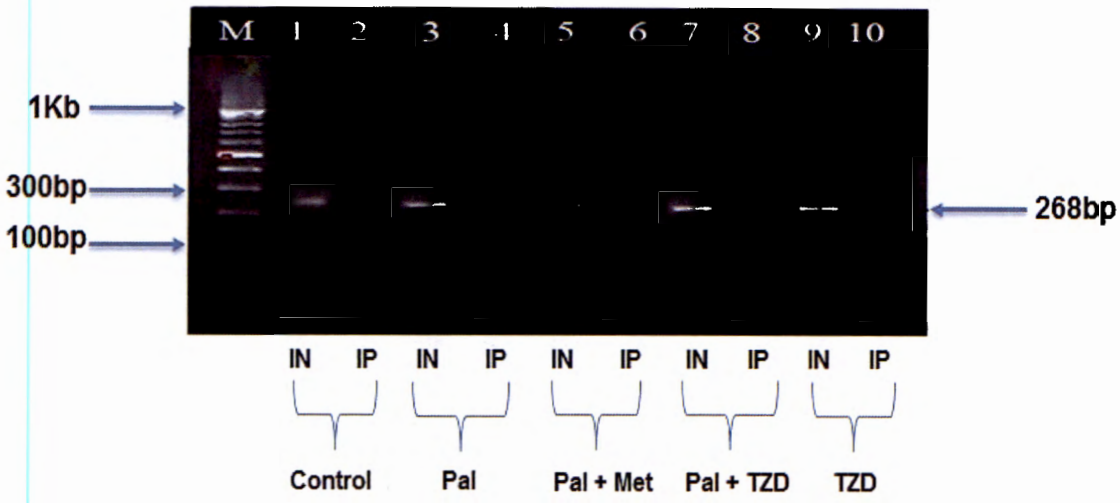


Figure 4.8: Agarose gel showing a ChIP assay performed on C2C12 treated cells. The PCR was performed with *GLUT4-MEF2A* primers (268bp). Lanes 1 and 2 represent the control, lanes 3 and 4 represent the palmitate (Pal), lanes 5 and 6 represent palmitate + metformin (Pal + Met), lanes 7 and 8 represent palmitate + TZD (Pal + TZD), and lanes 9 and 10 represent the TZD treatments respectively. Lane M represents the 1Kb marker. IN represents the input samples while IP represents the immunoprecipitated samples.

4.3 Assessment of TZD on antioxidant properties.

Antioxidants help to balance ROS levels in the body as an imbalance of these may lead to oxidative stress which has been implicated in a wide array of diseases including diabetes (Tangvarasittichai, 2015). Another objective of this project was to determine the influence of the TZD on antioxidant activity using TEAC and FRAP assays.

4.3.1 Trolox equivalent antioxidant capacity (TEAC)/ ABTS radical scavenging activity of the treatments

The results in figure 4.9 indicate that the ABTS radical scavenging activity of palmitate only was reduced significantly to approximately half that of the control. When palmitate

was used with metformin, there was some modest increase compared to palmitate only. However, when palmitate was used with TZD, there was ~2-fold increase in scavenging activity; similarly, with TZD only.

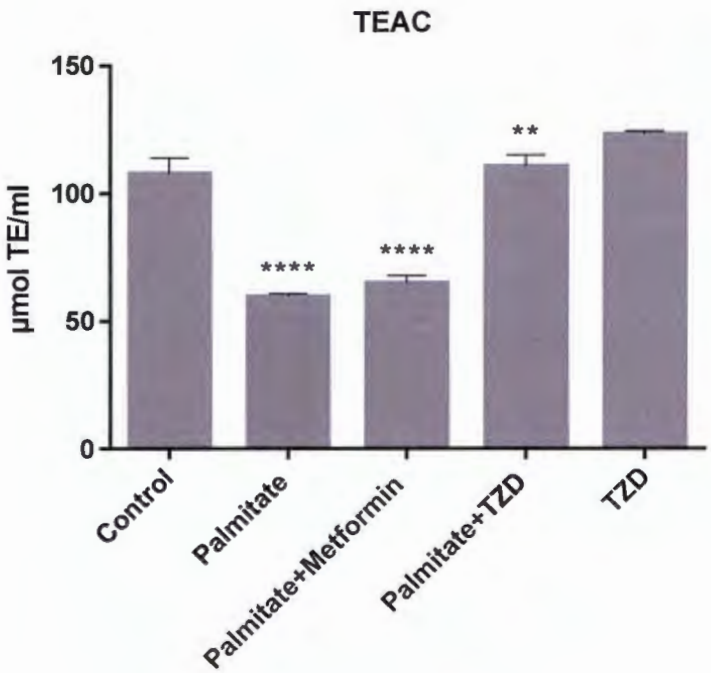


Figure 4.9: ABTS radical scavenging activity of the treated C2C12 myocytes. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.3.2 Ferric reducing antioxidant power (FRAP) of the treatments

The results in figure 4.10 show that the ferric reducing antioxidant power of both the TZD only, and palmitate with TZD treatments were increased by ~1.5-fold compared with the control. On the other hand, palmitate + metformin treatment showed ~1.4-fold increase in FRAP. The FRAP value of the palmitate treatments were almost like the control group.

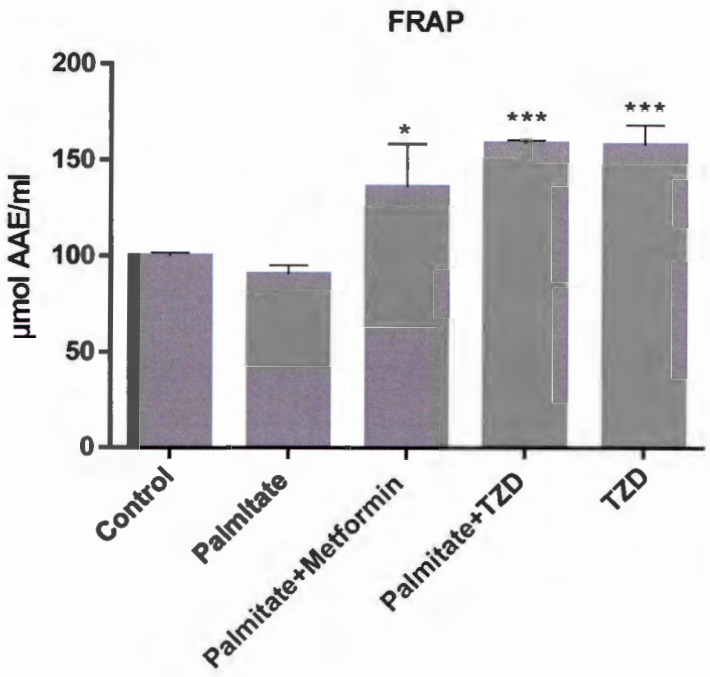


Figure 4.10: Ferric reducing antioxidant power of the treated C2C12 myocytes. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; ** < 0.01; ***<0.001; ****<0.0001).

4.4 Enzymatic assays of the treatments

Enzymatic assays are laboratory procedures used for measuring enzyme activity. All enzymatic assays either measure consumption of substrate or production of product over time (Acker & Auld, 2014). Hence, they are vital for the study of enzyme kinetics and inhibition. In this study, enzymatic assays were performed to measure glucose and ATP production.

4.4.1 Glucose oxidase analysis

Diabetic patients have blood glucose concentrations ranging from 9-40mM (Periasamy *et al.*, 2011). The detection of glucose has been achieved through electrochemical sensing systems (Chen *et al.*, 2014). To provide selectivity towards glucose, most of the electrochemical systems require glucose oxidase (GOx). Glucose oxidase enzyme also

known as notatin is an oxidoreductase that catalyses β-D-glucose to glucono-δ-lactone and H_2O_2 in the presence of oxygen (Roy *et al.*, 2012). A glucose oxidase assay was performed in this study to determine if the observed decrease in *GLUT4* gene expression would result in decreased glucose transport. The experiment was performed using the Glucose Oxidase Activity Assay Kit (Sigma-Aldrich) following the manufacturers protocol (section 3.5).

The results in figure 4.11 show the glucose oxidase activity of the TZD only treatment was almost equal to that of the control. However, when myotubes were treated with palmitate only, the glucose activity was significantly dampened. Addition of either metformin or TZD slightly enhanced the glucose activity compared to the palmitate only group. The glucose oxidase activity in TZD only was increased ~2-fold compared to palmitate only but did not vary with the control.

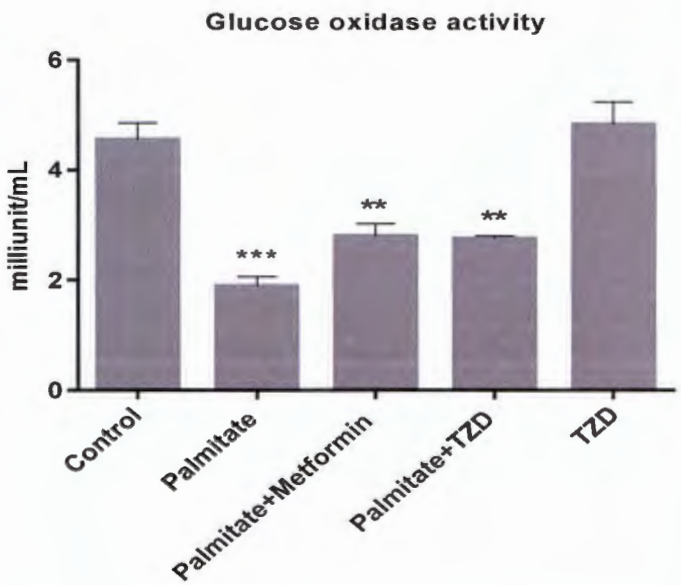


Figure 4.11: Glucose oxidase activity of the treated C2C12 myocytes. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

4.4.2 ATP content analysis

The breakdown of glucose also results in the production of ATP molecules. Adenosine triphosphate (ATP) is a nucleoside that transports chemical energy within cells for metabolism (Peleli & Carlstrom, 2017). ATP flux is generally controlled by ATP demand, a paradigm that is acknowledged by both advocates and challengers of the 'mitochondrial insufficiency' model (Goodpaster, 2013). There is mounting evidence indicating that adenosine signalling pathways have critical roles in modifying the development of type 2 diabetes primarily through interfering with the function of metabolism-regulating organs (Antonioli *et al.*, 2015). An ATP assay was performed in this study to determine the effect of hybrid compound 2d' on the production of ATP using the ATP Detection Kit (Invitrogen).

The results in figure 4.12 show that the ATP content of the palmitate only group was reduced significantly compared to the control. The TZD only and the palmitate with TZD yielded ~1.5-fold increase compared to the control and ~4-fold compared to the palmitate only group. Therefore, the ATP content was reduced in the induced insulin resistant state while it was increased after treatment with the TZD alone.

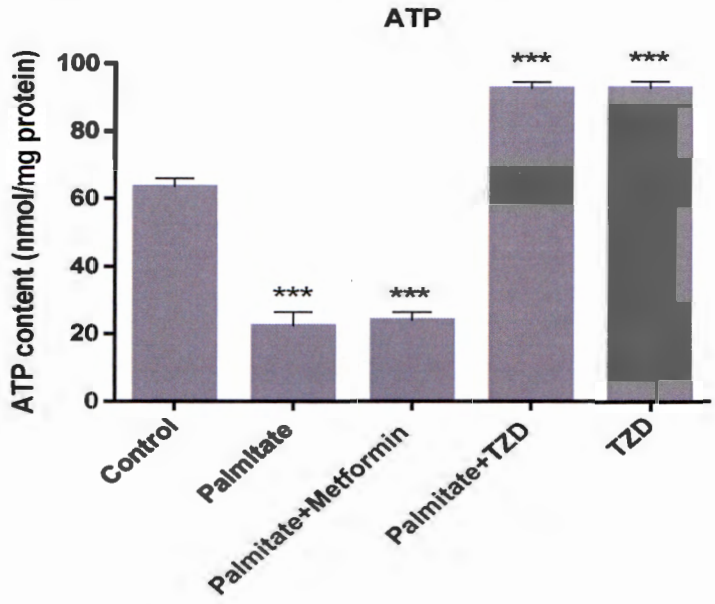


Figure 4.12: Measurements of the ATP content of the treated C2C12 myocytes. The level of significance in statistical analysis is indicated by p-values which are represented by stars to show the degree of significance in the various treatments performed as compared to the control group (*<0.1; **<0.01; ***<0.001; ****<0.0001).

CHAPTER 5

Discussion

The purpose of this study was to determine if the newly synthesized TZD with the added methoxy group could better influence the expression of genes that confer protection against diabetes. The gene expressions studied in this project were those associated with glucose transport (*NRF-1, GLUT4* & *MEF2A*) as well as those involved in the acetylation or deacetylation of these anti-diabetic genes (*pCAF/p300, GCN5* & *HDAC4*). Furthermore, this study had also evaluated the influence of this TZD on antioxidant properties since diabetes is also caused by oxidative stress. The current study was inspired by the fact that type 2 diabetes mellitus is growing to epidemic levels with no cure to date (Hebrok, 2017). Studies like these that seek to find better therapeutic means to treat or better manage diabetes are necessary in finding solutions to this growing epidemic that results in heavy financial burden to our governmental resources.

Genes play various essential roles in the cell and are therefore linked to several diseases. Numerous genes such as *NRF-1*, *MEF2A* and *GLUT4* are known to confer protection against the disease. In this study the TZD with an added methoxy group increased the expression of all these genes in cells, which is a positive indication of its role as an anti-diabetic compound. All these findings are still at preliminary phases, and the ability of the TZD to enhance these genes as well as those involved in the acetylation of anti-diabetic genes is a significant step towards finding new therapeutics for the disease.

Some studies have shown that some genes are down-regulated in diabetes, especially those known to be involved in glucose transport and lipid oxidation. For example, *NRF-1* and its target genes are down-regulated in individuals with type 2 diabetes and also in

those subjects with family history of the disease (Patti *et al.*, 2003). *NRF-1* regulates *MEF2A* which influences the expression of the *GLUT4* gene and subsequently improves glucose transport (Joseph *et al.*, 2017). In this study, palmitate significantly reduced *NRF-1* gene expression while the TZD upregulated the gene together with its target genes. The *NRF-1* target genes (*MEF2A* and *GLUT4*) were also assessed. *MEF2A* is the main transcription factor of the *GLUT4* gene. *GLUT4* protein is now being targeted as a therapeutic means to treat type 2 diabetes since it is the main transporter of glucose from the blood into the cells. While the TZD increased the expressions of *NRF-1* and its target genes (*MEF2A* & *GLUT4*), conversely, palmitate reduced their expressions even below control levels. These results suggest that the TZD is a potential compound that could be turned into a drug for the treatment or management of diabetes.

Since *MEF2A* regulates the *GLUT4* gene, we further assessed the binding of *MEF2A* to the *GLUT4* promoter in response to treatment with TZD. Again, the TZD enhanced the binding extent of *MEF2A* to the *GLUT4* promoter. The results observed in this study using a hybrid compound have similarly been also observed in an exercise model (Joseph *et al.*, 2017). Binding of *MEF2A* to the *GLUT4* promoter increases glucose transport which in turn alleviates the effects of insulin resistance.

It is important to note that most genes are generally expressed because of posttranslational modifications or epigenetic processes such as acetylation. For example, *NRF-1* transactivation is through acetylation which results in increased binding to its target genes and also their expressions (Evans & Scarpulla, 1990). The HATs such as *p300*, *pCAF* and *GCN5* are all associated with *NRF-1* resulting in increasing its binding to its binding domains of the target promoters. In this study the TZD increased

the expression of all these HATs (*p300*, *pCAF* and *GCN5*) whereas palmitate reduced the expression of all these acetylation genes. However, when palmitate was used with the TZD, there was reversal of the observed down regulation of all the three HATs studied. This implies that the TZD was effective in enhancing the expression of *p300*, *pCAF* and *GCN5* which subsequently increased the expression of the *NRF-1* and its target genes thus reducing the impact of palmitate induced insulin resistance. These results are crucial since the TZD reversed most of the down regulation of the genes studied under insulin resistance (known to be the hallmark of type 2 diabetes) and similar patterns where also observed in previous studies.

On the other hand, HDACs (which work antagonistically with HATs) deactivate the process of acetylation consequently hindering gene expression (Yang & Seto, 2007). In fact, insulin resistance is often associated with high levels of class II HDACs such as *HDAC4* (Pirola *et al.*, 2012). In this study, we observed that *HDAC4* gene expression was reduced after treatment with both palmitate alone or with TZD treatments. This means that the acetylation rate was increased, and more genes were able to bind to their suitable binding domains. This also corresponds to the increased expressions of the above mention HATs as they operate in a method contradictory to that of the HDACs. Therefore, the TZD could confer the deacetylation associated with insulin resistance. A similar study conducted by (Kim *et al.*, 2016) reported that HDAC inhibition was found to ameliorate hyperglycaemia through the downregulation of gluconeogenesis in type 2 diabetic rats.

The treated C2C12 myotubes were further tested for their ABTS radical scavenging activity as well as their ferric reducing power through TEAC and FRAP assays. The TEAC and FRAP values were reduced following treatment with palmitate. However, the

TZD managed to salvage the palmitate treated cells slightly higher than the control. An imbalance in ROS can lead to oxidative stress and subsequently diabetes. Since the TZD increased antioxidant activity, it can be said that the drug has the potential to serve as an improved therapeutic treatment for oxidative stress. These results also correlate with those obtained in a recent similar study conducted by Ayeleso *et al.*, in 2017.

Lastly, the influence of the TZD on glucose oxidase activity as well as the production of ATP was evaluated. Enzymatic assays were performed to determine if the hybrid compound would increase the contents of the two important energy molecules involved in cell metabolism, that is glucose and ATP. The palmitate significantly reduced the glucose oxidase activity while the TZD managed to increase it above the control levels. This suggests that the TZD did increase glucose transport and hence counteracted the effect of diabetes. The breakdown of glucose results in the production of ATP molecules. Palmitate again managed to reduce the production of ATP. However, after treatment with the TZD, the ATP was significantly increased. These findings propose that hybrid compound 2d' has the potential to increase ATP production and consequently lower the impact of metabolic dysfunction. Numerous experiments and clinical studies have shown variations in adenosine receptor expression and signalling during metabolic complications, targeting adenosine receptors with appropriate pharmacological agents is not trivial (Peleli & Carlstrom, 2017).

CHAPTER 6

Conclusions and Recommendations

Palmitate significantly reduced the expression of all the genes studied. This confirms that the palmitate induced insulin resistance model was successful as expected. However, the TZD averted the induced insulin resistance in most of the experiments carried out. This study has shown that the TZD has a positive influence on the expression of glucose transport and acetylation genes. Although these results are still at preliminary stages regarding this newly synthesized TZD with an added methoxy group, there are many positive results that were obtained in this study, showing the potential influence of this compound in anti-diabetic genes and in the influence of chromatin posttranslational modification. Although metformin has been used widely, it still does not cure the disease and various side effects have been reported (Al-Majed *et al.*, 2016). The TZD in this study showed better reversal of palmitate induced insulin resistance than metformin.

Like any study, there were some limiting aspects to this research project. Since the hybrid compounds are newly synthesized, the study is still at preliminary stages. In future, we intend to use qPCR to assess the extent of *MEF2A* binding to the *GLUT4* promoter, as opposed to only using gel electrophoresis. This will also be used for other binding assays in future such as *NRF-1* binding to the *MEF2A*. The study could have also been improved by looking at some of the HATs studied to assess their association with either *NRF-1* or *MEF2A*.

The results obtained also emphasize the need for further study not only on the binding of MEF2 site, but also to determine whether this binding was associated with hyper-acetylation. Western blots could also be carried out to assess the expression of proteins associated with these genes studied. Since these experiments were carried out in cell culture models, it would be essential to escalate them in an *in vivo* system such as rats/mice models.

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