The role of thiazolidinedione on adenosine monophosphate-activated protein kinase: Implications on diabetes

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DECLARATION OF ORIGINALITY

I Brian Moono Simapulasi Munansangu (22937803), hereby declare that the work written herein has not been published previously and that as far as I know, no part of this work has been submitted before for higher degree examination in any University.

I declare that any contribution, material and works of other people in this dissertation have been attributed, cited and referenced accordingly.

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ABSTRACT

The incidence and prevalence of type 2 diabetes mellitus (T2D) has nearly quadrupled since 1980 and is increasing at disturbing rate worldwide, particularly in low and middle -income countries. The causes are complex, but the rise is due in part to increase in the number of people who are overweight, increase in obesity, and in a widespread lack of physical activity. Besides environmental factors, the combination of heterogeneous genetic defects also plays an important role in determining susceptibility to T2D.

Skeletal muscle and adipose tissue are the major tissues involved with post-prandial peripheral glucose disposal in response to insulin. Insulin resistance reduces the ability to clear glucose from the circulation resulting in hyperinsulinemia and hyperglycaemia with the development of T2D. On the other hand, the currently used biguanides and the thiazolidinediones (TZDs) activate AMPK pathway, but these drugs have a wide range of unwanted side effects to the users. A new class of up to eight (08) thiosemicarbazone-triazole hybrids (labelled 1a-h) have been developed which have electron-donating groups such as methoxy substituent attached in one of the aromatic rings resulting in superior mechanisms which can alleviate the symptoms of T2D. In this dissertation, we refer to the thiosemicarbazone-triazole hybrid as hybrid compound b´ or TZD throughout this work)

This study focused on assessing if newly synthesized thiosemicarbazone-hybrid (hybrid compound b´) can stimulate the major enzyme in cellular and whole-body energy homeostasis, AMP-activated protein kinase (AMPK). We further assessed the influence on transcription genes involved in T2D such as NRF-1, MEF2A and GLUT-4 as well as its antioxidant capabilities.

Mouse C2C12 myoblasts were differentiated from myocytes to myotubes by serum deprivation. Myocytes were cultured in media containing 90% DMEM, 10% FBS, and 1% antibiotic (containing penicillin, streptomycin and fungizone). Thereafter, media was switched to differentiation media (70% DMEM, 2% FCS, 10% DMSO) and then to media with 0.75 mM of Sodium palmitate and metformin 1 µM, a total volume of 5 µL (10 mg/mL) hybrid compound was used at various intervals i.e. 4h,16h, and 24h respectively. The effect of treatment with compound b´ on cell viability was evaluated by MTT assay and total Messenger ribonucleic acid (mRNA) was extracted using the Purelink RNA mini kit and converted to cDNA using the Superscript Vilo kit. Catalytic AMPKα was silenced through a predesigned PRKAA2 siRNA, while mRNA extracted from the siRNA was processed using Cells to CT™ kit. Gene expression was analysed via qRT-PCR. Western blot was employed for protein analysis while ChIP assay was used to assess

protein-DNA interactions of MEF2A to NRF-1 promoter. Lastly, the FRAP and TEAC assays were used to assess the Ferric Reducing Antioxidant power of the hybrid compound.

The study showed that the newly synthesized thiosemicarbazone-triazole hybrid increased expression levels of glucose transporter genes i.e. GLUT-4, MEF2a, and NRF-1 in including lipid metabolising genes such as PPARα, on palmitate-induced insulin-resistance in C2C12 myotubes. The up-regulation of these genes is a positive effect in that they could help in alleviating the symptoms for type 2 diabetes. The results showed that the hybrid compound was dependent on AMPK pathway; thus AMPK (α1 and α2), as silencing resulted in down-regulation of genes associated with glucose transport. Furthermore, the thiosemicarbazone-triazole hybrid exhibited antioxidant potential activity that could mitigate the effects of free radicals that are usually present in type 2 diabetes.

LIST OF ABBREVIATIONS

AA	Amino acid
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACC	Acetyl-CoA carboxylase
AICAR	Aminoimidazole carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BMI	Body Mass Index
CAMKK	Calcium $^{2+}$/ calmodulin dependent protein kinase kinase
cDNA	Complementary DNA
CPT-1	Carnitine palmitoyl transferase 1
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylene diamine tetra acetic acid
eNOS	Endothelial nitrogen oxide synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
FRAP	Ferric reducing antioxidant power
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-4	Glucose transporter 4
HDL	High-density lipoprotein

IN	Sample (input) used for ChIp assay in immunoprecipitation step
IRS	Insulin receptor substrate
KO	Knockout
LBD	Ligand binding domain
MEF2	Myocyte enhancer factor 2
MET	Metformin
MO25	Mouse protein 25
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NO	Nitric oxide
NRF-1	Nuclear respiratory factor
PA	Palmitate
PAI-1	Plasminogen activator inhibitor-1
PBST	Phosphate buffered saline (PBS) containing 0.1 % tween 20
PI3	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PRKAA2	Protein kinase AMP-activated catalytic subunit alpha 2 catalytic subunit
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
S6K	p70 ribosomal S6 protein kinase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SHP	Small heterodimer partner
siRNA	Small interference ribonucleic acid
STRAD	STE20-related kinase adapter protein
T2D	Type 2 diabetes
TEMED	Tetra methyl ethylene diamine
TG	Triglyceride
TZD	Thiazolidinedione
WHO	World Health Organization
ZMP	5-aminoimidazole-4-carboxamide ribonucleotide monophosphate

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1.0 INTRODUCTION AND STUDY BACKGROUND

1.1 Background

Two main types of diabetes, which are type 1 and 2 diabetes mellitus, are recognised. Type 1 diabetes mellitus, also known as insulin-dependent diabetes mellitus, is due to the inability of the pancreas to secrete insulin (Skyler, 2007). On the other hand, type 2 diabetes (T2D) is associated with chronic insulin resistance especially in muscle, liver and adipocytes and insufficient compensatory β-cells insulin secretion which results in hyperglycaemia (DeFronzo, 2009; Bergman *et al.*, 2002).

The world burden of T2D was estimated to be 366 million in 2011 and approximately half of that number is undiagnosed (Ruta *et al.*, 2013; Vlad and Popa, 2012). The figures are likely to increase to 522 million by 2030 if no appropriate measures are taken (Ruta *et al.*, 2013). Africa has about 19.8 million people afflicted with T2D and the number is expected to rise 41.4 million people by 2035 (Mbanya *et al.*, 2014). South Africa, particularly, has about 2.6 million people with T2D (Mbanya *et al.*, 2014).

Insulin is the primary anabolic hormone that stimulates uptake and storage of glucose in skeletal muscle, liver and adipocytes (Dimitriadis *et al.*, 2011). When energy balance is disturbed due to overeating of high calorie food, and lack of physical exercise, it leads to a high prevalence of type 2 diabetes (Klein *et al.*, 2004; Wing *et al.*, 2001) and a metabolic disorder associated with insulin resistance of peripheral tissues (Hardie, 2004).

Kemp and associates (Kemp *et al.*, 2003) have shown that in mammalian tissues, the AMP-activated protein kinase (AMPK) regulates glucose and lipid metabolism. Additionally, AMPK integrates signalling pathways between peripheral tissues and hypothalamus to control food intake and whole-body energy expenditure (Kemp *et al.*, 2003).

Even though there are five classes of oral agents and a variety of insulin preparations available for the treatment of T2D, a glycaemic control remains suboptimal (Schimmack *et al.*, 2006) in about 50% of patients 6 years after being diagnosed (Group, 1995). This relates, in part, to the observation that neither sulfonylurea nor metformin therapy stops the progressive decline in β-cell function observed in T2D (Turner *et al.*, 1999; Group, 1995). Novel agents thiazolidinediones

(TZDs), pioglitazone and rosiglitazone are insulin sensitising antidiabetic agents that bind with high affinity to the peroxisome proliferator-activated receptor (PPAR-γ) (Olefsky, 2000; He *et al.*, 2015; Yki-Järvinen, 2004). These agents have been shown in animal (Finegood *et al.*, 2001) and human (Buchanan, 2003) studies that they preserve β-cell viability and function, and as a result, delay the progression of T2D. The use of TZDs as monotherapy (Phillips *et al.*, 2001) or in combination with a sulfonylurea, metformin, or insulin (Raskin *et al.*, 2001; Kipnes *et al.*, 2001) has shown improved decline in the levels of glycohaemoglobin (HbA_{1C}) in comparison to a placebo (Lebovitz *et al.*, 2001). However, the effectiveness of these drugs currently used to manage T2D is limited and besides, they present with a lot of side effects (O Moore-Sullivan and Prins, 2002; Boyle *et al.*, 2002). Metformin, for example, is associated with weight loss and lactic acidosis (Kirpichnikov *et al.*, 2002; Al-Jebawi *et al.*, 1998), while rosiglitazone is known to increase the risk of heart attack and stroke by as much as 43% (Chen *et al.*, 2012). There is an urgent need for a novel agent which comprise two or more drug pharmacophores in one molecule with the desire to exert multi-drug action (Morphy *et al.*, 2004; Kinfe *et al.*, 2013) that can better manage the disease effectively and with lesser side effects. In this regard, exploration was done on newly synthesized thiosemicarbazone and triazole hybrids by (Kinfe *et al.*, 2013) and evaluated their antidiabetic activities. This class of drugs has several metal binding sites and have a wide spectrum of biological activities which include antidiabetic (Kinfe *et al.*, 2013), anti-obesity (Oliva *et al.*, 2010), anticancer (Đilović *et al.*, 2008), antiviral (Glisoni *et al.*, 2012), and antimalarial (Kinfe and Belay, 2013) activities. To help address the mentioned side effects of the drugs used to manage diabetes, investigations were done into the activities of the AMP-activated kinase (AMPK) which plays a key role in the regulation of metabolism within the muscle cell and has been implicated as a potential target in type 2 diabetes mellitus and in obesity (Winder and Hardie, 1999; Moller, 2001; Minokoshi *et al.*, 2002). The description of this kinase which demonstrated the activation of AMPK by metformin in both hepatocytes and skeletal muscle is further discussed in detail in Section 2.2.8 (Zhou *et al.*, 2001). Metformin, as already mentioned, is a widely used oral drug to treat type 2 diabetes, it decreases hyperglycaemia and has beneficial effects on circulating lipids and does not affect insulin secretion (Wu *et al.*, 1990; Stumvoll *et al.*, 1995). AMPK activation by metformin was found to be required for the decrease in glucose production and the increase in fatty acid oxidation in hepatocytes and for the increase in glucose uptake in skeletal muscles (Zhou *et al.*, 2001). In this dissertation, we check if AMPK is activated by the newly synthesized thiosemicarbazone and triazole hybrid (which we shall refer to as Compound b' in this study). We further investigate the roles it plays in adjusting the levels of cellular reactive oxygen species.

1.2 Problem statement

There is no known cure for type 2 diabetes and the number of people living with and dying of type 2 diabetes across the world is rapidly increasing. The drugs (Biguanides and thiazolidinediones (TZDs)) widely used to manage the disease are associated with a lot of side effects such as weight gain, oedema, and abnormal lipid profiles. There is a pressing need to develop novel drugs to manage T2D with lesser side effects. There is substantial evidence suggesting that AMPK is dysregulated in skeletal muscle and adipose tissue of humans with or T2D, and that AMPK activation (physiological or pharmacological) can improve insulin sensitivity, glucose uptake, fatty acid oxidation and ameliorate some of the pathologies of T2D. Therefore, this work explored whether the effects of newly synthesized thiosemicarbazone-triazole hybrid was dependent on the AMPK pathway and evaluated if the genes involved in T2D are up-regulated or down-regulated.

1.3 Aim

To establish whether the synthesized thiosemicarbazone-triazole hybrid (compound b´) is dependent on AMPK and if this can ameliorate experimentally-induced insulin-resistance in C2C12 skeletal muscle cells and elevate transcription of genes involved in lipid and glucose transport.

1.4 Objectives

- To evaluate if thiosemicarbazone-triazole hybrid (Compound b´) up-regulates or down-regulates genes associated with glucose transport (NRF-1, GLUT-4, MEF2A) and AMPK using qualitative real-time polymerase (qRT-PCR).
- To assess protein expression level of AMPKα by Western blotting technique.
- To silence AMPK (PRKAA α2) gene using small interfering ribonucleic acids (siRNA) and measure gene expression levels of AMPK, GLUT-4, MEF2A, and NRF-1 after treatment with hybrid compound b´.
- To assess Protein-DNA interactions (MEF2A-NRF) within the chromatin of C2C12 cells using the chromatin immunoprecipitation (ChIP) assay.
- To investigate the influence of thiosemicarbazone-triazole hybrid compound b´ for antioxidant properties via TEAC and FRAP.

CHAPTER 2

2.0 REVIEW OF LITERATURE

2.1 Diabetes mellitus, insulin resistance, obesity, Metabolic syndrome and treatment

This chapter focuses on an in-depth overview of diabetes mellitus, insulin signalling and resistance, and metabolic syndrome. The causes and treatment available for T2D, hybrid compounds from thiosemicarbazone-triazole as treatment for T2D. The AMPK pathway is described in depth and the potential role it has on regulation of cellular antioxidant defence in T2D are discussed.

2.2 Diabetes mellitus

Diabetes mellitus is a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO, 1999).The diagnosis of diabetes usually involves symptoms of diabetes plus either casual plasma glucose concentration ≥200 mg/dl (11.1mmol/l), a fasting plasma glucose ≥126 mg/dl (7.0 mmol/l), or a 2-hour post-load glucose ≥200 mg/dl (11.1mmol/l) during an Oral Glucose Tolerance Test (OGTT) conducted as per WHO recommendations using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water (Association, 2014). More recent methods for diagnosis of diabetes mellitus such as measurement of glycated haemoglobin (HbA_{1c}) have been included by the American diabetes association and adopted by the European association for diabetes and WHO (Fox *et al.*, 2015). Therefore, an individual presenting with these symptoms and coupled with a random blood glucose level above 11.1 mmol/L is categorised as diabetic (Ren and Davidoff, 1997; Seino *et al.*, 2010; Bailey *et al.*, 2016). Additionally, clinical symptoms such as polyuria, polydipsia, body wasting and the less familiar description of urine sweet enough to attract flies and ants have been described as far back as 1500 BC when Hindu scholars initially described the illness (Howlett and Bailey, 2007).

The hallmark of all forms of diabetes mellitus is abnormal insulin physiology, persistent hyperglycaemia, insulin resistance and relative lack of insulin (Farris *et al.*, 2003; Ahmad, 2013). Two traditional paradigms of diabetes are recognised viz type 1 and type 2 diabetes mellitus. Type

1 diabetes mellitus is also referred to as insulin-dependent diabetes mellitus and is due to the inability of the pancreas to secrete insulin (Skyler, 2007). Type 2 diabetes (T2D), also called non-insulin dependent diabetes or adult-onset diabetes, is the most common form of diabetes accounting for approximately 90% of cases worldwide (Nolan *et al.*, 2006; Association, 2018). However, other forms of diabetes occur such as gestational diabetes mellitus (GDM) (diagnosed in the second or third trimester of pregnancy that was not overt diabetes prior to gestation) and drug or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) (Association, 2018).

Recently, researchers proposed the term 'Type -3-diabetes' for Alzheimer's disease (AD) because of the shared molecular and cellular features among Type-1 and Type-2 diabetes and insulin resistance associated with memory deficits and cognitive decline in elderly individuals (Kandimalla *et al.*, 2017; Leszek *et al.*, 2017).

T2D has been identified as a major cause of morbidity and mortality (Roglic *et al.*, 2005; Association, 2013). As already mentioned above, T2D is a multifactorial disease that occurs primarily in adults and is associated with insulin resistance, dyslipidaemia and obesity and is becoming prevalent in obese children (Rosenbloom *et al.*, 2009). An estimated 422 million people are afflicted with T2D according to (WHO, 2016). The figures of people with diabetes are expected to reach 592 million by the year 2035 (Diabetes, 2013). In the past, T2D was considered a disease of industrialised nations; however, current evidence indicates that there are up to 90%–95% diabetes cases in Low and Middle-Income Countries (LMICs) (WHO, 1994; WHO, 2016). This new trend has been associated with the changing demographic profile together with rapid urbanization and changing lifestyles in both rural and urban settings in Africa (Gwatkin *et al.*, 1999).

In sub-Saharan Africa, the number of people afflicted with diabetes was at 19.8 million in 2013 and the figure is projected to rise to 41.5 million by 2035 (Bailey *et al.*, 2016; IDF., 2013). In South Africa particularly, an estimated 2.5 million people are diabetic and half of that is thought to be undiagnosed. In addition, the international diabetes foundation estimates that 5 million South Africans have pre-diabetes conditions. The highest prevalence of diabetes in South Africa is among the Indian population (11-13%) followed by the Coloured community with 8-10%, while among the Black it is estimated at 5-8% and amongst Whites, 4% (IDF., 2013; Erasmus *et al.*, 2012)

2.2.1 Insulin receptor

The insulin receptor (IR) belongs to the family of cell surface receptors possessing intrinsic tyrosine kinase activity, insulin-like growth factor (IGF) receptor and the insulin receptor-related receptor (IRR) (Saltiel and Kahn, 2001; Patti and Kahn, 1998). It is a heterotetrameric protein composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulphide bonds. When insulin binds to the α-subunit, it induces tyrosine autophosphorylation of the IR ß-subunit. The activated IR subsequently phosphorylates its substrates, including the insulin receptor substrate (IRS) proteins, Shc (Src homology collagen) and APS (adaptor protein with a PH and SH2 domain) (Saltiel and Kahn, 2001; Patti and Kahn, 1998). Biological dysfunction of the IR has been implicated in pathological conditions such as insulin resistance, obesity, and hyperinsulinemia (Moller *et al.*, 1996; White, 2002).

Lower organisms such as *Drosophila*, *Caenorhabditis elegans* and metazoan marine sponges have been shown to exhibit homologues of the Insulin/IGF-I receptor. These lower organisms use some of the downstream signals vital to the regulation of mammalian cells, including phosphatidylinositol-3-OH kinase (PI(3)K), Akt and Forkhead transcription factors (Skorokhod *et al.*, 1999). The Forkhead box protein (FOXO1) has been shown to downregulate IR gene expression in the presence of insulin (Puig and Tjian, 2005).

2.2.2 Insulin-receptor substrates

There are at least nine intracellular substrates of the insulin/IGF-I receptor kinases which have been identified. Four (4) of these belong to the family of insulin-receptor substrate (IRS) proteins (White, 2002). According to (Pessin and Saltiel, 2000), the other substrates include Gab-1,$p60^{dok}$, Cbl, APS and isoforms of Shc. When phosphorylated tyrosines in these substrates function as receptor sites for proteins that contain a distinct subset of signalling proteins housing SH2 (Src-homology-2) domains (Saltiel and Kahn, 2001). The IRS proteins are highly homologous and serve different functions in insulin/IGF-1 signalling. IRS-1 knockout (KO) mice show pre- and post-natal growth retardation, as well as impaired insulin action in peripheral tissues, especially in muscles and impaired glucose tolerance (Araki *et al.*, 1994; Tamemoto *et al.*, 1994).

IRS-2 (KO) mice also display insulin resistance in both peripheral tissues and liver, but have retarded growth in selective tissues such as certain neurons, islets and retina (Gutierrez *et al.*, 1998; Kido *et al.*, 2000). These multifactorial defects combined with decreased β-cells culminate to the development of type 2 diabetes (Withers *et al.*, 1998). By contrast, at cellular level, IRS-1 KO

exhibits reduced IGF-I stimulated DNA synthesis (Araki *et al.*, 1994; Tamemoto *et al.*, 1994) and preadipocytes show defects in differentiation (Withers *et al.*, 1998), whereas IRS-2 KO preadipocytes differentiate normally, but have impaired insulin-stimulated glucose transport (Yu-Hua *et al.*, 2005; Miki *et al.*, 2001).

IRS-3 and IRS-4 expression are more restricted in tissue distribution pattern, and in rodents they most abundant in adipocytes, liver and lung (Sciacchitano and Taylor, 1997). In humans, the IRS-3 is known to be a pseudogene, hence no protein is produced at all (Björnholm *et al.*, 2002). The roles of IRS-3 and 4 are not fully known in cultured cells, although evidence indicates that these substrates may act as negative regulators of IRS-1 and -2 (Tsuruzoe *et al.*, 2001). IRS-5, (also known as DOK4) and IRS-6 (DOK5) are expressed limitedly in tissues (Cai *et al.*, 2003) and are subsequently poor IR substrates (Versteyhe *et al.*, 2010).

2.2.3 Insulin signalling

Cellular insulin signalling exerts its influence via two key pathways. The first pathway involves insulin receptor substrate 1 (IRS-1), which acts upstream of the phosphatidylinositol 3-kinase (PI3K)-protein. Kinase B (also known as Akt) signals a transduction pathway to elicit predominantly metabolic responses (Jia *et al.*, 2016). The PI3K are heterodimers consisting of a regulatory and catalytic subunit existing in several isoforms (Vadas *et al.*, 2011).The involvement and activation of the PI3K depend on the binding of the two SH2 domains to tyrosine-phosphorylated IRS proteins (Myers *et al.*, 1992; Shaw, 2011). Once this activation occurs, it phosphorylates phosphatidylinositol 4,5-bisphosphate to generate the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate, which initiates a kinase cascade and further activates the Akt signalling pathway. The activation of AKT increases the translocation of GLUT4 from the intracellular vesicles to the plasma membrane where it facilitates glucose uptake in the cell (Samuel *et al.*, 2010; Khan and Pessin, 2002). The second pathway involves signal transduction via mitogen-activated protein kinase (MAPK) (Witteles and Fowler, 2008). This kinase contributes to cell growth and protein synthesis (Jia *et al.*, 2015).

2.2.4 Insulin resistance as a primary factor of T2D

Insulin resistance can be defined as an impaired biological response to either exogenous or endogenous insulin (Association, 1998). It is often present when the circulating levels of either free fatty acids (FFAs) or triglycerides (TGs) are abnormally high (Despres *et al.*, 1995). As far back as 1963, Randle and colleagues proposed a connection between muscle insulin resistance and

elevated FFA concentrations which is a negative effect of increased tissue lipid content. In the fasted state, blood glucose level is low and FFA levels are elevated due to their release from adipose tissue. On the other hand, in the fed state, blood glucose and insulin levels rise and FFA levels fall due to the suppression of lipolysis by insulin (Nolan *et al.*, 2015; Schenk *et al.*, 2008) Many studies have indicated that circulating FFA may directly contribute to the underlying pathophysiology of T2D, in particular, the development of insulin resistance both in the periphery and liver (Boden, 1997; Shulman, 2000). Some forms of insulin resistance may involve the insulin receptor itself. Alterations in insulin receptor expression, binding, phosphorylation state, and/or kinase activity could account for many insulin- resistance phenotypes such as type A syndrome, leprechaunism, Rabson-Mendenhall syndrome, and lipoatrophic (Taylor and Arioglu, 1998; Krook and O'Rahilly, 1996). The mechanism by which insulin resistance develops is poorly understood. However, several factors for the development of insulin resistance are associated with a vast interplay of genetics, ethnicity, age, overweight, unhealthy diet, lack of physical activity and metabolic factors (Organization and Unit, 2014).

2.2.5 Metabolic syndrome (Met S)

The ever-rising incidence of obesity has contributed to the increased global prevalence of metabolic syndrome and associated complications (Janus, 2004). There is currently no universal definition for metabolic syndrome, although several definitions have been proposed to identify persons at risk for the development of T2D. At present, the mostly widely accepted definition encompasses the core characteristic of metabolic syndrome which are: obesity, insulin resistance, dyslipidaemia and hypertension.(Organization, 1999; Balkau and Charles, 1999). Therefore, metabolic syndrome can be described as a complex condition with multiple metabolic conditions such as insulin resistance, impaired glucose tolerance and lipid metabolism, central obesity, hypertriglyceridemia, low HDL, hypertension and hyperglycaemia (Isomaa *et al.*, 2001; Organization, 1999; Janus, 2004).

2.2.6 Treatment of T2D

The first line in the management of T2D is the use of non-pharmacological methods which include, weight control through diet modification and regular physical exercise coupled with patient education. When lifestyle modification fails to achieve or sustain adequate glycaemic control, insulin or oral anti-diabetic agents are typically used to manage the disease (Viollet *et al.*, 2009b). Oral agents are most commonly used and these include metformin which is an insulin- sensitising drug via inhibition of hepatic glucose production, thiazolidinediones (TZDs) (insulin sensitizers

via Peroxisome proliferator-activated receptor gamma (PPARγ) activation), α-glucosidase inhibitors (inhibition of gut glucose absorption), and sulphonylureas (β-cell secretagogues) (Viollet *et al.*, 2009b). Other drugs approved for treatment of T2D are dipeptidyl peptidase 4 (DPP4) inhibitors, sodium-glucose co-transporter 2 (SGLT2), glinides, bile-acid-binding resins and dopamine-receptor agonists (Kahn *et al.*, 2014). Several new drugs with glucose-lowering efficacy offering certain advantages have recently become available, such as injectable glucagon-like peptide-1 (GLP-1) receptor agonists, rapid-acting and short-acting insulin injectables, intermediate-acting insulin and long-acting insulin injectables (Kahn *et al.*, 2014; Gallwitz, 2011). Typically, metformin is usually prescribed first as already indicated, but the choice of therapy usually depends on a number of factors including age of the patient and taking into account contraindications (Zhang *et al.*, 2012). Cognisance should be taken that these class of oral agents differ in mechanism and duration of action, and the degree to which they reduce blood glucose and their side-effect profile (Viollet *et al.*, 2009b).

(LeBrasseur *et al.*, 2006; Zhang *et al.*, 2012) have shown that AMPK, a phylogenetically conserved (in all mammalian cells) serine/threonine protein kinase, is one of the probable targets of major antidiabetic drugs, metformin and TZDs, and of insulin-sensitizing adipokines (LeBrasseur *et al.*, 2006). In the same vain, in this work, more insight is sought into the effect of newly synthesized thiosemicarbazone-triazole hybrids on AMPK and ultimate result on type 2 diabetes.

2.2.7 Hybrid compounds from thiosemicarbazone-triazole as treatment for T2D

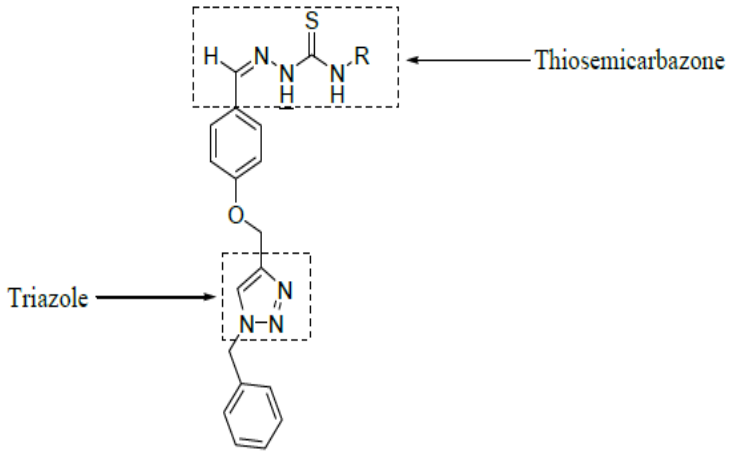


Figure 2-1: The general chemical structure of the thiosemicarbazone-triazole hybrids. R-represents the various alkyl and aryl group. Structure adopted from (Kinfe and Belay, 2013).

Thiosemicarbazones display various biological activities such as anti-obesity and antiviral, hence have therefore considerable pharmaceutical interest (Hu *et al.*, 2006). Thiosemicarbazones belong to a vast group of thiourea derivatives, whose biological activities are a function of parent aldehyde or ketone moiety (Du *et al.*, 2002; Jawed Ahsan, 2013; Lovejoy and Richardson, 2002). A considerable number of thiosemicarbazones derivatives have been shown to have a wide spectrum of biological activities which include antidiabetic (Kadowaki *et al.*, 2013), anticancer, antibacterial (Lovejoy and Richardson, 2002), antimalarial activities, antifungal activities (Usman *et al.*, 2002), and antiviral activity (Glisoni *et al.*, 2012). The other part of the combination of this hybrid is the triazoles, which can interact with biological targets through hydrogen bonding and dipole-dipole interactions (Kolb and Sharpless, 2003). Triazoles are heterocyclic compounds featuring a five-member ring of two carbon atoms and three nitrogen atoms as part of the aromatic five-member ring (Figure 2-1) with molecular formula $C_3H_3N_3$ (Wakale *et al.*, 2013). In this regard, this report is based on the antidiabetic effects of newly synthesized thiosemicarbazone-triazole hybrid studied by knife and colleagues (Kinfe *et al.*, 2013).

Table 2-1 List of newly synthesized thiosemicarbazone-triazole hybrids.

Number	RNH_2	Compound	Yield (%)
1	$PhCH_2NH_2$	1a	72
2	$CH_3CH_2CH_2CH_2CH_2NH_2$	1b	80
3	$PHCH_2CH_2NH_2$	1c	74
4	$(CH_3)_2NCH_2CH_2NH_2$	1d	78
5	$HOCH_2CH_2CH_2CH_2CH_2CH_2NH_2$	1e	66
6	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2NH_2$	1f	85
7	$CH_3CH(CH_3)CH_2NH_2$	1g	70
8	$HOCH_2CH_2NH_2$	1h	62

The hybrids 1a-h were synthesized according to the reported protocol as outlined in the mechanism by knife and colleagues (Kinfe *et al.*, 2013). Production of these hybrids was initiated with the alkylation of commercially available 4-hydroxybenzaldehyde with propargyl bromide in the presence of K_2CO_3 to give compound 4 which integrates an alkynyl group needed for click chemistry. Compound 4 underwent click chemistry with freshly prepared benzyl azide to provide1,4-disubstituted triazole 5. Methylhydrazinecarbodithioate 7, which was prepared in a one-pot synthesis from the condensation of hydrazine monohydrate, CS_2 and methyl iodide, reacted with triazole compound 5 under Schiff's base condensation reaction conditions to give compound 8. The latter then underwent nucleophilic substitution reactions with a series of amines to provide an array of hybrid compounds 1a-h, as illustrated below in (Figure 2-2). After successful synthesis of these hybrids, 2a-h were synthesized such that electron-donating groups such as methoxy substituent were attached in one of the aromatic rings under similar conditions as hybrid 1a-h were synthesized.

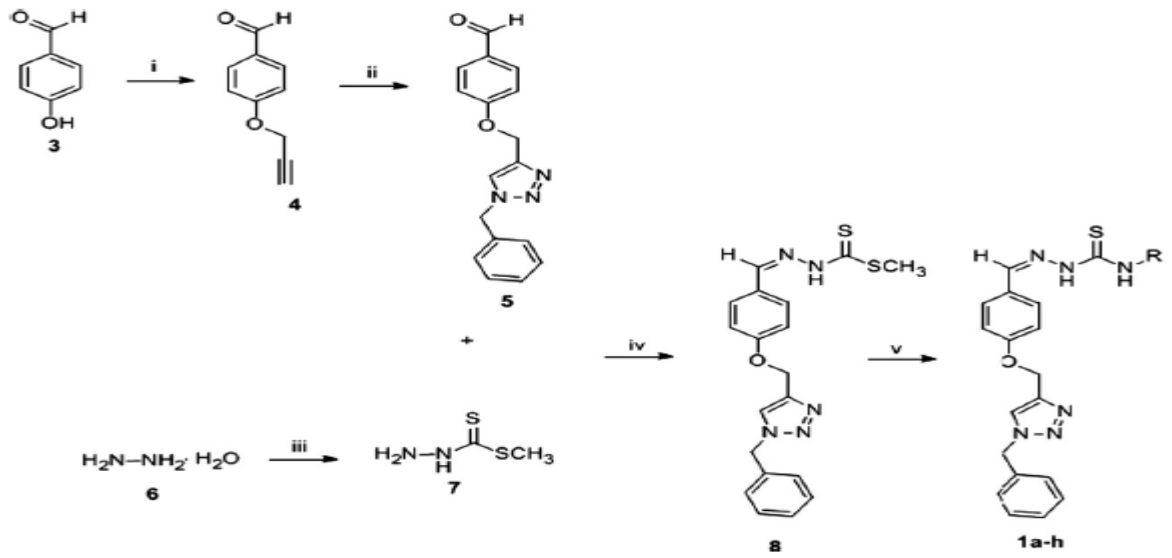


Figure 2-2: Reagents and conditions. (i) K_2CO_3, propargyl bromide, acetone, reflux, 2.5h, 92%; (ii) BnN_3, $CuSO_4.5H_2O$, sodium ascorbate, DMF:H_2O (4:1), 60°C, 3h, 80%; (iii) CS_2, KOH, CH_3I, H_2O:isopropanol (1:1), r.t, 4h, 90%: (iv) 5,7 MeOH, reflux, overnight, 89%; (v) RNH_2 MeOH, reflux, 24h, 62-85%. Taken from Knife (Kinfe *et al.*, 2013).

2.2.8 AMP-activated protein kinase (AMPK)

2.2.8.1 AMPK isoforms and subunits

AMPK is a heterotrimeric protein present in all tissues and is composed of three different subunits namely, the catalytic α, regulatory β and γ (O'Neill *et al.*, 2013). It plays a central role in the regulation of cellular and whole-body energy homeostasis (Viollet *et al.*, 2009b). It has been described as a "master switch'' that coordinates or mediates the cellular adaptations to nutritional and environmental fluctuations consuming intracellular ATP levels, such as heat shock, starvation, hypoxia or prolonged exercise (Viollet *et al.*, 2009b; Hardie and Carling, 1997; Kemp *et al.*, 1999). Generally, these factors activate AMPK and trigger catabolic pathways that produce ATP while turning off anabolic pathways that consume ATP to maintain cellular energy levels. Homologues of these subunits have been identified in mammals, drosophila, worm, yeast, plants and primitive protozoon, with a high degree of conservation (Halford and Hardie, 1998; Hardie *et al.*, 2003). In humans, the heterotrimeric complexes form various combinations i.e. α subunit (α1 or α2), with β (β1 or β2) and γ (γ1 and γ2 or γ3) regulatory subunits encoded by separate genes to produce 12 heterotrimeric combinations (Figure 2-3). All these subunits have different tissue distribution and level of expression (Viollet *et al.*, 2009b). The α1 subunit is abundantly expressed in kidney, lung and adipose tissue, whereas the α2 catalytic is highly expressed in the heart and skeletal muscles (Stapleton *et al.*, 1996; Woods *et al.*, 2000). The regulatory β1 subunit is usually expressed in the liver and β2 in the skeletal muscle (Viollet *et al.*, 2009b). On the other hand, the regulatory γ1 and

γ2 subunits have a broad tissue distribution than the γ3 subunit which is usually found in the glycolytic muscle (Viollet *et al.*, 2009b).

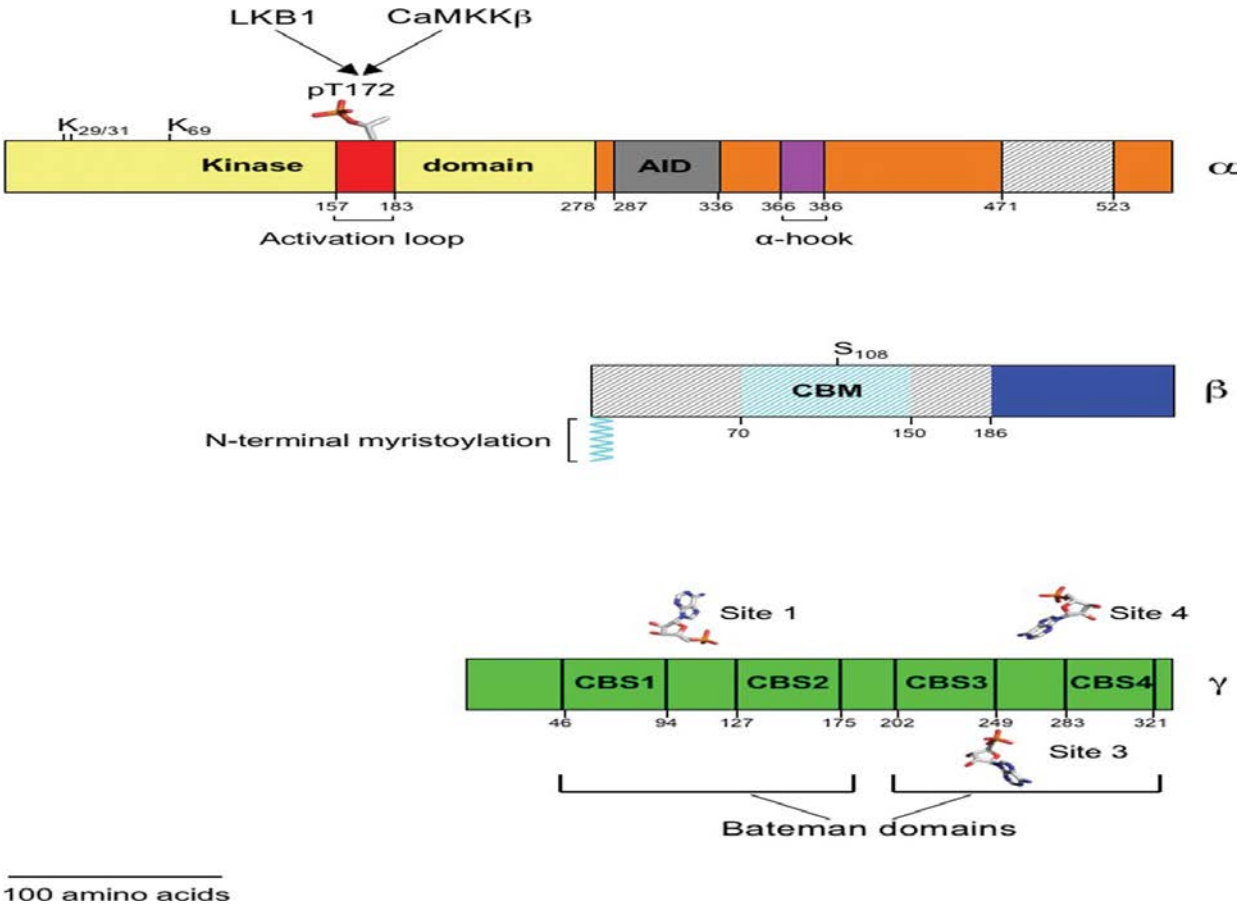


Figure 2-3 Schematic representation of AMPK subunits, highlighting key amino acid residues and regions implicated in the regulation of AMPK activity, is shown. The major upstream kinases phosphorylating Thr^{172} are LKB1 and CaMKKβ. AMP is shown bound to each of the three nucleotide-binding sites in the γ subunit. The numbering is taken from the rat α1, β1 and γ1 isoforms. Adapted from (Carling *et al.*, 2012).

2.2.8.2 The α-Subunit

The alpha subunit is the main functional component of the AMPK complex and it houses the catalytic α subunit (AMPKα) – a protein with a molecular weight of 63kDa encoded by the PRKAA1 gene (Mitchelhill *et al.*, 1994; Davies *et al.*, 1994). AMPKα includes the following structural elements: a serine/threonine kinase domain, autoinhibitory domain (AID), and a sequence responsible for binding of the β subunit (Crute *et al.*, 1998). The vital site for AMPK activation is located on the activation loop (Birnbaum, 2005) of the kinase domain (KD) as threonine 172 (Thr172) and is conserved among kinases (Zorman, 2013; Birnbaum, 2005). It is located in the N-terminus of the protein (Crute *et al.*, 1998). The C terminal region of AMPKα forms a globular region which the C-terminal region of the β subunit which interacts with the α and γ subunits, acting as a scaffold for the formation of the heterotrimeric αβγ complex (Woods *et*

al., 1996; Xiao *et al.*, 2011). The α1 subunit facilitates non-nuclear localization, whereas the α2 subunit localizes both in the nucleus and in the cytoplasm (Salt *et al.*, 1998). However, the localization of AMPK is not strictly determined by the structure, and under different conditions, the distribution of the kinase complex between the nucleus and cytoplasm can vary (Kazgan *et al.*, 2010).

2.2.8.3 The β-Subunit

AMPK-β is a protein of 270 aa (38 kDa) encoded by the PRKAB1 gene, and AMPK-β2 is a protein of 271 aa (34 kDa) encoded by the PRKAB2 gene (Sanz, 2008). The AMPK-β subunits function as a scaffolding structure to form the α and γ subunits, via binding to their conserved Kinase interacting sequence (KIS) and association with Snf1 complex (ASC) domains (Sanz, 2008). It also houses another specific region called the glycogen-binding domain (GBD) (Hudson *et al.*, 2003). The presence of the GBD allows the AMPK complex to associate with glycogen and oligosaccharides with different affinity depending on the isoform (Novikova *et al.*, 2015; Bieri *et al.*, 2012). AMPK-β subunits are phosphorylated at multiple sites, Ser24/25, Ser108 and Ser182 (AMPK-β2 lacks Ser24/25 site) (Sanz, 2008). In addition, both β-subunits or isoforms are myristoylated at the N-terminus on Gly2 (Mitchelhill *et al.*, 1997; Novikova *et al.*, 2015). Literature indicates that N-myristoylation of proteins mediates the membrane binding and membrane targeting, and in the case of AMPK results in a more homogeneous distribution of the AMPK-β subunits inside the cell and in an increase in AMPK activity (Novikova *et al.*, 2015; Warden *et al.*, 2001; Sanz, 2008).

2.2.8.4 The γ Subunit.

This subunit contains 3 AMPK-γ subunits namely γ1, γ2 and γ3. AMPK-γ1 is a protein of 331 aa (37 kDa), AMPK-γ2 is a protein of 569 aa (63 kDa) and AMPK-γ3 is a protein of 492 aa (55 kDa), encoded by the PRKAG1, PRKAG2 and PRKAG3 genes, respectively (Sanz, 2008). All these subunits differ in the length of the N-terminal sequence domain of the proteins (CHEUNG et al., 2000; Sanz, 2008). The γ-subunits contain four evolutionarily conserved tandem repeats of a structure module called cystathionine-β-synthase (CBS) (Bateman, 1997). The primary function of CBS is to control the intracellular metabolites, and particularly to bind the adenine nucleotide regarding AMPK (Novikova et al., 2015; Scott et al., 2004). The γ-subunit makes the AMPK complex function as a sensor of AMP level within the cell (Moffat & Ellen Harper, 2010; Novikova et al., 2015).

2.2.8.5 AMPK ACTIVATION

The fundamental purpose of AMPK is to maintain energy homeostasis by switching off ATP-consuming pathways and switching on pathways that regenerate ATP (Hadie and Carling, 1997; Kemp *et al.*, 1999; Schimmack *et al.*, 2006). Cellular stresses (Hardie *et al.*, 2012b) such as those described in this section increase AMP/ATP and creatine (Cr) phosphocreatine (PCr) ratios. This, in turn, activates AMPK by two mechanisms: i) allosteric activation of AMPK and ii) binding of AMPK making it a worse substrate for phosphatases (Sanz, 2008; Hardie *et al.*, 1998; Gimeno-Alcañiz and Sanz, 2003; Halford and Hardie, 1998; Hardie *et al.*, 1999). The combination of allosteric and phosphorylation effects causes a >1000-fold increase in AMPK activity (Suter *et al.*, 2006). Phosphorylation, particularly of the α subunit at site Thr172 within the 'activation loop 'of the kinase domain, is the main mechanism responsible for the increase in enzyme activity (Hadie and Carling, 1997; Kemp *et al.*, 1999; Schimmack *et al.*, 2006; Stein *et al.*, 2000). The phosphorylation is achieved by an upstream AMPK kinase (Sanz, 2008) and inhibition of dephosphorylation of Thr172 by protein phosphatases (Viollet *et al.*, 2009b; Hurley *et al.*, 2005). Two protein kinase have been identified to be capable of phosphorylating Thr172 in vivo, the tumour suppressor LKB1 (Hawley *et al.*, 2003; Viollet *et al.*, 2009b) and $Ca2^{+}$/calmodulin-dependent kinase kinase (CaMKK) (Hawley *et al.*, 2005), especially the β isoform (CaMKKβ). In 2006 (Sanders *et al.*, 2007) and colleagues identified a genetic selection for the mammalian Snf1-acting kinase in yeast and identified TAK1 (transforming growth factor-β-activated kinase), a member of the mitogen-activated protein kinase kinase family (MAPKKK), as an additional AMPK upstream kinase (Sanz, 2008).

2.2.8.6 AMPKK: LKB1

LKB1 is a Ser/Thr kinase and a tumour suppressor identified with an inherited form of cancer called Peutz-Jeghers syndrome (Alessi *et al.*, 2006). As indicated above, it is an upstream kinase (AMPKK) that can phosphorylate Thr-172 in vivo (Shaw *et al.*, 2004) as well as in vitro (Hong *et al.*, 2003). LKB1 requires two accessory protein subunits called Sterile-20-related adaptor (STRAD) and Mouse protein 25 (MO25) to form a heterotrimeric complex (Hawley *et al.*, 2003). The pseudo kinase domain STRAD activates LKB1 and is responsible for exporting the complex from the nucleus to the cytoplasm (Hawley *et al.*, 2003), while MO25 acts as a scaffold of the protein. It also stimulates the heterotrimer and upregulates the kinase activity of LKB1 by more than 10-fold (Boudeau *et al.*, 2003). Mutations in LKB1 identified from Peutz-jeghers sufferers fail to interact with STRAD-MO25, thus leading to the pathology (Boudeau *et al.*, 2004). LKB1

immunoprecipitation with anti-LKB1 antibodies has been shown to reduce the AMPK activity while recombinant complexes of LKB1-STRAD-MO25 activate AMPK via phosphorylation of AMPK-α at Thr-172 (Sanz, 2008; Hawley *et al.*, 2003; Woods *et al.*, 2003a). Research in LKB1-deficient mice has shown that LKB1 is the main AMPK kinase in muscle and liver (Sakamoto *et al.*, 2005; Koh *et al.*, 2006). LKB1 KO mice display impaired AMPK α2 activation after administration of AMP-mimetric such as AICAR and phenformin (Sakamoto *et al.*, 2005; Koh *et al.*, 2006; Shaw *et al.*, 2004; Cantó and Auwerx, 2010). In the liver, deletion of the LKB1 reduces phosphorylation and activity of AMPK which leads to increased hyperglycaemia due to elevated hepatic gluconeogenic and lipogenic gene expression (Zhou *et al.*, 2001; Shaw *et al.*, 2005).

2.2.8.7 CaMKK

LKB1 is related to activation of AMPK caused by a change in the AMP/ATP ratio. Therefore, when LKB1-knockout cells (Suzuki *et al.*, 2004) showed that despite decrease in AMPK phosphorylation complete suppression of its activity did not occur, it became clear that an alternative kinase existed for AMPK, and the simultaneous work of David Carling and Grahame Hardie found this kinase: $Ca2^{+}$/calmodulin dependent kinase kinases (CAMKKs) (Woods *et al.*, 2005; Hawley *et al.*, 2003; Novikova *et al.*, 2015; Altarejos *et al.*, 2005). It is usually found in the brain (Hardie and Sakamoto, 2006) and other tissues like muscles also express CAMKKα but not clearly CAMKKβ (Rose *et al.*, 2006; McGee *et al.*, 2008a). The activation of this kinase is dependent on the increase in intracellular $Ca2^{+}$ level (Sanders *et al.*, 2007; Witczak *et al.*, 2007). The work of (Witczak *et al.*, 2007) indicated that over expression of CAMKKα and CAMKKβ in muscle was sufficient to increase AMPK phosphorylation (Cantó and Auwerx, 2010), while muscle overload is known to increase AMPK activity in LKB1 knock-out mice (McGee *et al.*, 2008a; Cantó and Auwerx, 2010) and inhibited by STO-609, a specific inhibitor of CaMKK, and by specific siRNAs corresponding to CaMKKβ (Woods *et al.*, 2005; Hurley *et al.*, 2005; Suzuki *et al.*, 2004).

2.2.8.8 TAK1

Transforming growth-β-activated kinase 1 (TAK1) is the third kinase which has been identified to directly phosphorylate AMPK in various tissues including muscle and heart (Momcilovic *et al.*, 2006; Xie *et al.*, 2006). TAK1 phosphorylates AMPK-α at Thr-172 and expression of its binding conjugate TAB1 in HeLa cells or treatment of these cells stimulated phosphorylation at Thr-172 in AMPK. The physiological conditions under which TAK1 modulates AMPK activity are yet to be fully understood (Momcilovic *et al.*, 2006).

2.2.8.9 AMPK activation by oxidative stress and genotoxic treatments

Studies indicate that some types of cellular stress can activate AMPK by a mechanism that does not involve the AMP/ATP or $Ca2^{+}$ levels (Toyoda *et al.*, 2004). In mouse skeletal muscle, AMPK is activated by oxidative stress and enhances glucose transport (Toyoda *et al.*, 2004). Similarly, in cell culture conditions, hypoxia-induced AMPK activation was reported to be dependent on mitochondrial reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) without tangible changes in AMP/ATP ratio (Emerling *et al.*, 2009; Wang *et al.*, 2012). Suggestions have been made by Zmijewski and colleagues (Zmijewski *et al.*, 2010) that H_2O_2 can activate AMPK using a third mechanism that involves a cytoplasmic form of phosphoinositide 3- kinase-like kinase (PIKK) ataxia telangiectasia mutated (ATM) (Alexander *et al.*, 2010; Wang *et al.*, 2012). Genotoxic agents, such as etoposide, doxorubicin and ionizing radiation are DNA-damaging medication and have been identified as other factors that activate AMPK in the nucleus (Sanli *et al.*, 2010). The mechanism in which they operate requires ataxia telangiectasia mutated (ATM) but, surprisingly, they do not require LKB1 because the pathway is functional in LKB1-null cells (Fu *et al.*, 2008; Sanli *et al.*, 2010). However, detailed mechanism of this effect remains unclear (Hardie *et al.*, 2012a).

2.2.8.10 AMPK regulation of gene and protein expression via transcription

Short-term effects of AMPK activation can be explained by direct phosphorylation and regulation of metabolic enzymes in the cytoplasm; however, long-term activation of the kinase has an impact on the pattern of gene expression in a variety of circumstances. For example, in the pancreatic β-cell line, activation of AMPK by a shift to low glucose, or by AICAR treatment, causes a reduction in the expression of metabolic genes including GLUT2, aldolase B and liver-type pyruvate kinase (L-PK)(da Silva Xavier *et al.*, 2000; JOHNSON *et al.*, 1998). A Single subcutaneous AICAR injection has been shown to increase uncoupling protein-3 (UCP-3)(Zhou *et al.*, 2000) and hexokinase (HK)-II (Stoppani *et al.*, 2002) mRNA, and UCP-3 protein expression in as little as 30 minutes (Zhou *et al.*, 2000). On the other hand, longer upregulation of GLUT 4 mRNA 13 hours after a subcutaneous AICAR injection has also been shown in white and red quadriceps (Zheng *et al.*, 2001). AMPK has also been shown to regulate the expression of GLUT4 and subsequent control of skeletal muscle carbohydrate metabolism which increases the maximal rate of glucose transport into muscles (Zheng *et al.*, 2001). In addition to acute regulation of metabolic enzymes, AMPK has also been shown to modulate a variety of transcriptional regulators involved in the

control of metabolism and mitochondrial changes (Vo and Goodman, 2001). These include PPAR*γ*, PGC-1*α* (PPAR*γ* co-activator 1*α*), NRF1/NRF-2 (nuclear respiratory factors-1 and -2), Tfam (mitochondrial transcription factor A) (Garcia-Roves *et al.*, 2008) (McGee *et al.*, 2008a) and FoxO1 (forkhead box O1) (Jørgensen *et al.*, 2005). As indicated in Figure 2-4, PGC-1α serves as a transcriptional co-activator for DNA-binding transcription factors in conjunction with PPARs which control metabolic gene expression (McGee *et al.*, 2008a; Handschin and Spiegelman, 2006). Furthermore, PGC-1α partially works through co-activation with NRF-1/NRF2, which are vital for the expression of nuclear and mitochondrial-encoded enzymes (McGee *et al.*, 2008a; Handschin and Spiegelman, 2006; Bungard *et al.*, 2010). AMPK and CaMK, have been shown to activate GLUT4 expression by targeting its transcription factors namely the GLUT4 enhancer factor (GEF), myocyte enhancer factor (MEF)-2, and myogenic determination 1 (MyoD) (McGee *et al.*, 2008b). Chronic activation of muscle AMPK has been linked to increased NRF-1 binding activity and increased cytochrome c content and muscle mitochondrial density (Bergeron *et al.*, 2001b).

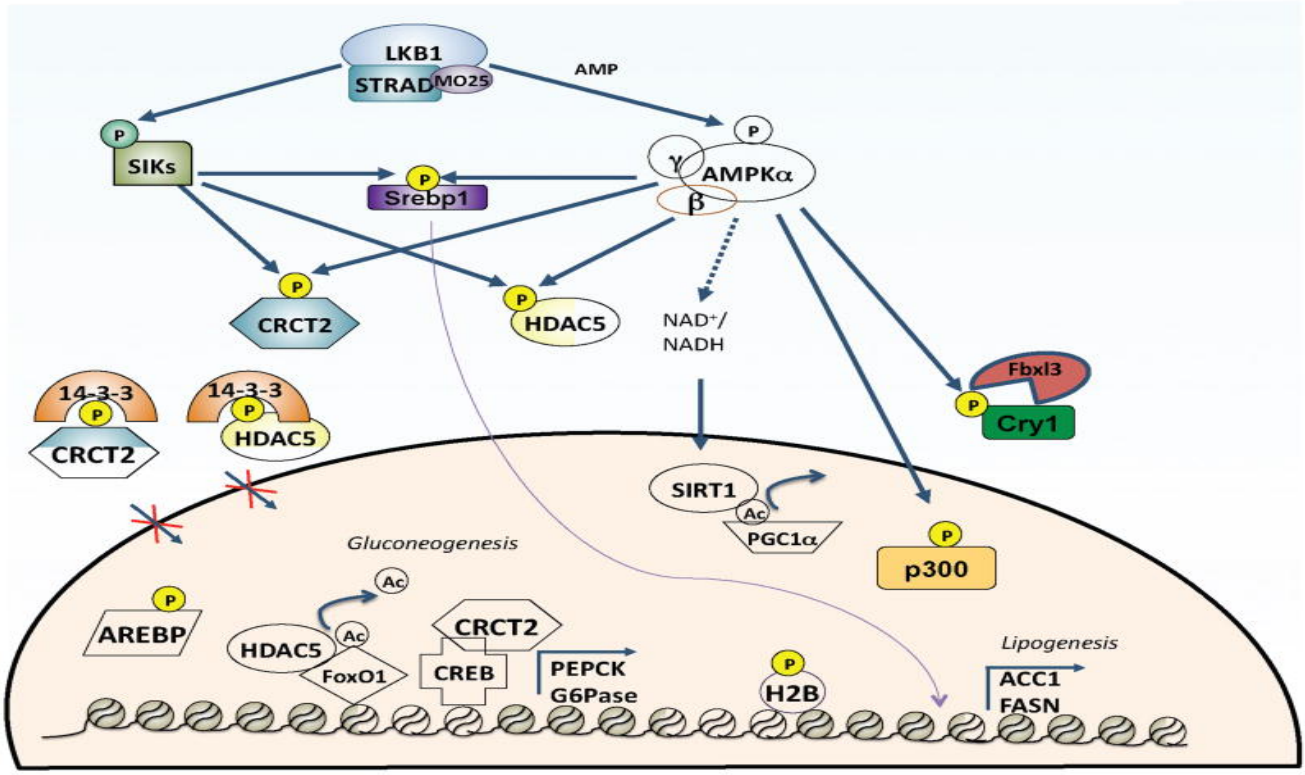


Figure 2-4 Illustration of how AMPK regulates several physiological processes through phosphorylation of transcription factors and co-activators. It shares substrates with its AMPK family related kinases to negatively regulate gluconeogenesis in the liver by phosphorylation and inhibition of the CRCT2 and Class IIa HDACs. These phosphorylation events induce binding to 14-3-3 scaffold proteins and sequestration of these transcription regulators into the cytoplasm. AMPK also regulates transcription factors via inducing their degradation (Cry1), preventing their proteolytic activation and translocation to the nucleus (Srebp1), and by disrupting protein-protein (p300) or protein-DNA interactions (Arebp, HNF4a). AMPK has also been shown to directly control phosphorylation of Histone 2B on Serine 36 as

well as indirectly controlling SIRT1 activity via increasing NAD+ levels. Image and caption adapted from (Mihaylova and Shaw, 2011).

2.2.8.11 Effects of small interference ribonucleic acids (siRNAs) on AMPK

The complex roles of AMPK isoforms in insulin-sensitive tissues have been studied in transgenic mice using small interfering ribonucleic acids (siRNAs), which are double stranded (dsRNA) molecules that can regulate gene expression and are discussed further in section 3.6. Double knockout of the AMPKα1 and AMPKα2 isoforms, simultaneously, results in lethality at embryonic day number ten (Viollet et al., 2009a). PRKAα2-knockout mice were glucose intolerant and insulin resistant and showed impaired glucose uptake on stimulation with the AMPK activator AICAR (Schultze et al., 2012; Viollet et al., 2009a). In addition, deletion of Prkaa2 specifically in β-cells resulted in defective glucose-stimulated insulin secretion (Schultze et al., 2012). Hepatocyte-specific deletion of Prkaa2 in the liver revealed that AMPK inhibits gluconeogenesis and release of glucose in the liver (Andreelli et al., 2006). PRKAβ2-knockout mice have been shown to have reduced maximal and endurance exercise capacities and were more susceptible to diet-induced weight gain and glucose intolerance. PRKAγ3-knockout mice were shown to have impaired AICAR-stimulated glucose uptake (Steinberg et al., 2010). Furthermore, studies have indicated that polymorphisms in LKB1 and subunits of AMPK (α1, α2 and β2) targets myocyte enhancer factor 2A (MEF2A) and MEF2D were found to be associated with reduced response to metformin treatment (López-Bermejo *et al.*, 2010; Jablonski *et al.*, 2010).

2.2.8.12 Pharmacological activation of AMPK

As already stated in activation of AMPK (Section 2.2.8.5) in intact mammalian cells, AMPK is activated by various metabolic stresses, drugs and xenobiotic through the mechanisms described in Section 2.2.8.5, which involve increases in cellular AMP, ADP or $Ca2^{+}$ (Hardie *et al.*, 2012a). However, recent work suggests that other stimuli activate AMPK via mechanisms that do not involve changes in the levels of AMP, ADP and $Ca2^{+,}$ which can, therefore, be termed 'noncanonical' mechanisms (Hardie *et al.*, 2012a) and are discussed in Section 2.2.8.9.

***Biguanides*:** Metformin, which belongs to the biguanide family of drugs is an insulin-sensitizing agent with potent antihyperglycemic attributed to suppressing hepatic glucose production (Kirpichnikov *et al.*, 2002; Rena *et al.*, 2013). It is the most prescribed drug to more than 100 million patients with T2D worldwide and is derived from a product galegine extracted from

Galega officinalis (Hardie *et al.*, 2012a; Zhou *et al.*, 2001).The mechanism of action of metformin has been linked to its inhibition of ubiquinone oxidoreductase (complex I) of the respiratory chain, which ultimately increases the AMP/ATP ratio (Hardie *et al.*, 2006; McGee *et al.*, 2008a). Inhibition of cellular respiration reduces gluconeogenesis (Dominguez *et al.*, 1996) and is considered to induce expression of glucose transporters and subsequent glucose consumption (Ebert *et al.*, 1995). In 2001 (Zhou *et al.*, 2001) reported that metformin activated AMPK in hepatocytes and linked reduction in ACC activity and inducing fatty acid oxidation to the effects of metformin action on AMPK (Zhang *et al.*, 2009). SREBP-1, a key lipogenic transcription factor, was reduced at both mRNA and protein levels in cells following metformin treatment (Zhang *et al.*, 2009). Metformin is associated with initial gastrointestinal side effects, and caution is advised in patients at risk for lactic acidosis such as in advanced renal insufficiency and alcoholism (Inzucchi *et al.*, 2012).

Thiazolidinediones (TZDs) rosiglitazone (RSG) and pioglitazone: these drugs are also insulin-sensitizing, and they are selective ligands of the nuclear transcription factor peroxisome-proliferator-activated receptor γ (PPARγ) (Lehmann *et al.*, 1995). PPARγ is expressed abundantly in adipose tissue, but also in pancreatic beta cells, endothelial cells and to a lesser extent in immune and stem cells (Lehmann *et al.*, 1995; Stafford and Elasy, 2007; Michalik *et al.*, 2006). TZDs are also one of the first drugs administered to tackle the problem of insulin resistance in patients with T2D. The mechanism of action is thought to begin after TZDs bind to PPARγ, resulting in a formation of a heterodimer complex with the retinoid receptor (RXR) (Cariou *et al.*, 2012). This is followed by binding to specific DNA sequences termed peroxisome proliferator response elements (PPREs), found on the promoters of PPARγ target genes which regulate gene transcription involving co-repressors and co-activators such as PGC-1 (Phielix *et al.*, 2011; Cariou *et al.*, 2012). PPARγ activation induces the altering of adipose tissue, including differentiation of preadipocytes into metabolically active adipocytes, and subsequently regional fat distribution towards lipid storage in subcutaneous rather than visceral adipocytes (Belfort *et al.*, 2006; Ratziu *et al.*, 2010; Phielix *et al.*, 2011). Lipogenic genes such as fatty binding protein (aP2), acyl-CoA synthase and SERBP-1 are involved (Phielix *et al.*, 2011), resulting in reduced level of circulating plasma FFA and their flux from visceral fat into the liver, and in turn decrease in muscle, hepatic fat distribution, and insulin resistance (Marchesini *et al.*, 2001) and hepatic gluconeogenesis (Gastaldelli *et al.*, 2006; Belfort *et al.*, 2006). TZDs, via PPAR and adipocytes, also repress the expression of inflammatory-response genes in a mechanism termed ligand-dependent transrepression (Straus and Glass, 2007; Sears *et al.*, 2009) and stimulate the release of adiponectin (Bajaj *et al.*, 2007) which activates AMPK in muscle and liver (Kubota *et al.*, 2007), promoting

fat oxidation and glucose uptake and insulin sensitivity (Kadowaki and Yamauchi, 2005). The main superiority over other antidiabetic drugs is that do not require dose adjustment in patients with renal impairment (Tuttle *et al.*, 2014; Shah and Mudaliar, 2010) and may have renal protective effects, another reason is that they do not increase the risk of hypoglycaemia and may be more durable in their effectiveness than sulfonylureas and metformin (Kahn *et al.*, 2006; Inzucchi *et al.*, 2012). Recognised side effects of TZDs include weight gain, fluid retention leading to oedema in predisposed individuals and increased risk of bone fractures (Dormandy *et al.*, 2005). The major side effects of this class of drugs is the risk for myocardial infarction (MI) and heart failure (Nissen and Wolski, 2007; Wang *et al.*, 2017). Pioglitazone has been reported to have modest benefit on cardiovascular disease while rosiglitazone is no longer widely available owing to concerns of increased myocardial infarction risk (Inzucchi *et al.*, 2012).

AICAR (5-amino-4-imidazole carboxamide riboside): it is the first compound reported for activating AMPK in intact cells and vivo (Corton *et al.*, 1995; Sullivan *et al.*, 1994). Structurally it resembles adenosine (Gadalla *et al.*, 2004) and once it has entered the cells it is converted by adenosine kinase to the monophosphorylated derivative ZMP, which binds to the CBS domains on AMPKs γ-subunit and mimics the effect of AMP both on allosteric activation of kinase and on inhibition of dephosphorylation (Corton *et al.*, 1995). Administration of AICAR to ob/ob mice (Song *et al.*, 2002), fa/fa rats (Bergeron *et al.*, 2001a; Buhl *et al.*, 2001) and high-fat-fed rats (Buhl *et al.*, 2001) was shown to prevent/reverse some aspects of metabolic syndrome (Coughlan *et al.*, 2014). The positive effects that were observed included, improvements in glucose tolerance, reduced plasma triglyceride and free fatty acid levels, elevated whole-body glucose disposal, decreased hepatic glucose output and even a tendency towards a reduction in abdominal fat (Song *et al.*, 2002; Bergeron *et al.*, 2001a; Fogarty and Hardie, 2010a). Treatment of AICAR in normal mice induced the expression of genes involved in oxidative metabolism and enhanced running endurance like the endurance experienced by exercise training (Narkar *et al.*, 2008), indicating a positive benefit of AICAR in the absence of metabolic defects (Fogarty and Hardie, 2010a). Despite these positive effects of AICAR in animal models (Fogarty and Hardie, 2010a), it was discovered that AICAR has AMPK independent effects due to interaction of ZMP with other AMP-regulated enzymes and that ZMP mimics the effect of AMP to inhibit the gluconeogenic enzyme fructose-1,6-bisphosphatase (Longnus *et al.*, 2003) (Coughlan *et al.*, 2014) and to stimulate the muscle isoform of glycogen phosphorylase (Vincent et al., 1991). Unreliable effects of AICAR such as poor bioavailability and short half-life make it an unlikely candidate for use as a treatment drug for T2D (Fogarty and Hardie, 2010a). However, other data have shown that AICAR can be used to treat humans with acute lymphoblastic leukaemia (Sengupta *et al.*, 2007;

Leclerc *et al.*, 2010) and cardiac ischemic injury (Leclerc *et al.*, 2010; Mullane, 1993; Mangano, 1997).

2.2.8.13 Antioxidants

Oxidative stress is defined as a pathological state in which there is an imbalance between the levels of pro-oxidants and antioxidants, resulting in macromolecular damage and disruption of redox signalling and controls (Wang *et al.*, 2012; Sies, 2007). Free radicals owe their destructive nature to one or more unpaired electron which can only be stabilized by pairing with a biological macromolecule such as lipids, proteins and DNA (Goswami and Chatterjee, 2014). Antioxidants help to scavenge free radicals in the body (Goswami and Chatterjee, 2014) and operate through initiation and generation of processes that terminate the chain reaction and slow down the oxidation process (Ayeleso *et al.*, 2017; Khalighi-Sigaroodi *et al.*, 2012). The increased production of reactive oxygen species (ROS) and the inability to detoxify ROS play a causative role in tissue injury in many disease conditions such metabolic syndromes, cardiovascular diseases, cancer and ageing (Yun *et al.*, 2014; Valko *et al.*, 2007). In T2D, oxidative stress is increased because of multiple factors, dominant among these factors is glucose autoxidation leading to the production of free radicals (Rahimi *et al.*, 2005) Another vital factor is the interaction of advanced glycation end products (AGEs) with specific cellular receptors called AGE receptors (RAGE) which are mostly formed under hyperglycaemic conditions (Rahimi *et al.*, 2005). Glycation of protein alters protein and cellular function and binding of AGEs to their receptors can lead modifications in cell signalling and increased production of free radicals (Penckofer *et al.*, 2002). As already outlined in section 2.2.8.9, AMPK can be activated by oxidative stress through the addition of oxygen species such as NO or H_2O_2 to the cell medium (Auciello *et al.*, 2014; Cardaci *et al.*, 2012). Emerging evidence suggests that AMPK also plays an important role in the regulation of cellular antioxidant defence (Wang *et al.*, 2012). Activation of AMPK by AICAR has been reported to inhibit palmitate-induced ROS levels by increasing the expression of thioredoxin (Trx) in human aortic endothelial cells (Li *et al.*, 2009). The thiosemicarbazone-triazole hybrid used in this study proved to have very high antioxidant properties as outlined by the study conducted by Ayeleso and colleagues (Ayeleso *et al.*, 2017). The compound showed high 1-diphenyl-2-picryl- hydrazyl (DPPH) radical scavenging ability and trolox equivalent antioxidant capacity (TEAC) values. Other antioxidant assays carried out such as ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) were also highly expressed indicating that the hybrid could be a potential candidate for antidiabetic and antioxidant effects.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials and methods

All the reagents, materials and instruments/equipment used in this project were of the highest quality. A comprehensive and detailed description of each has been outlined in Appendix 1 and Appendix 2 respectively.

3.2 Justification for use of *in-vitro* cell culture models.

C2C12 immortalized muscle cell lines are derived from mouse myoblasts that readily proliferate in high serum conditions, and differentiates easily (Chan and Lee, 2008). This makes them a perfect model for experiments involving the assessment of physiological and pathological processes related to insulin resistance and type 2 diabetes (Berguiga *et al.*, 2016; Diel *et al.*, 2008). Kumar and Singh (Kumar and Singh, 2010) have pointed out that C2C12 cells differentiate rapidly to form myotubules expressing muscle proteins and (Diel *et al.*, 2008), adds on to this finding as they had shown that these cells have been used *in-vitro* models to study myogenesis and cell differentiation, glucose metabolism and insulin signalling. Using cell lines is a better way of studying cell metabolism. In addition, cell culture experiments are more convenient than *in vivo* experiments in studying pathways that come about from a specific stimulus since they are carried out in an isolated system which excludes the control of the hormones, brain and other physiological processes. When a mammal is under stress conditions such as exercise, many physiological and biochemical responses which occur at the same time make it very difficult to isolate the mechanism involved for the changes observed (Mukwevho, 2010).

3.2.1 Cell lines

C2C12 myoblast (ATCC CRL 1722, Maryland, USA), were purchased from Highveld biologicals in South Africa and another batch was a kind donation by Prof Roan Louw of North-West University School of Chemical and Physical Sciences, Potchefstroom, South Africa.

3.2.2 Thawing cells

Cryopreserved cells were stored in freezing media (70% DMEM, 20% FCS & 10% DMSO) and were thawed by placing the cells in 37°C water. Immediately after thawing, the cells were

transferred to a 1.5 mL tube and centrifuged (ThermoFisher™SL8 and 8R, USA) 2000 g x 3 min. The supernatant was gently removed by aspiration and the pellet was resuspended in 1mL growth media and mixed by pipetting up and down.

3.2.3 Counting of cells

A sample of cell suspension was used, 10 µL of the cell suspension was pipetted into the counting chambers of the hemocytometer.

3.2.4 Subculture

Cells were seeded in a 10 cm petri dish, a total of 10 mL of pre-warmed growth media (90% DMEM, 10% FBS, 1% Antibiotic containing Penicillin, streptomycin and fungizone) was added to the cells. After that, the cells were incubated at 37°C in 5% CO_2 and humidified air for two days until the cells were 70-80% confluent. Media was changed on the second day.

3.2.5 Passage (splitting)

When the cells were 70-80% confluent (starting to aggregate), after 1–2 days, growth media was removed by aspiration and the cells were washed with pre-warmed (37°C) PBS. Trypsinization was done by adding 1 mL of trypsin/EDTA to each culture plate and incubated at 37°C in 5% CO_2 and humidified air for 5-10 min to allow the cells to dislodge from the bottom of the plate. The EVOS microscope (Life technologies, USA) was used to confirm if the cells have dislodged. Trypsin action was halted by adding 2 mL of growth media. Cells were then mixed by pipetting to ensure a single cell suspension and minimise clumping. Cells were then centrifuged at 2000 g for 3 min, and the supernatant was removed by aspiration. Depending on the size of the pellet, an appropriate amount of medium was added and mixed with a pipette. The cells were then seeded as described in Section 3.6 (subculture).

3.2.6 Differentiation and myotube formation of C2C12 cells

To induce differentiation of myoblasts into myotubes, the 70%-80% confluent cells were cultured in DMEM containing 2% horse serum (HS), and 1% penicillin/streptomycin/fungizone (Figure 3-1). Horse serum is a poor growth factor since it lacks the necessary serum factors that aid in cell proliferation and population doubling, which are characteristic of FBS, thus cell proliferation is halted and this results in the myoblasts to undergo myocytic differentiation and begin to form multinucleate myotubes (Franke *et al.*, 2014). Cells were incubated at 37ºC in 5% CO_2 and

humidified air for two days until myotubules were observed by transmission microscope (EVOS FL Microscope, Life Technologies. USA).

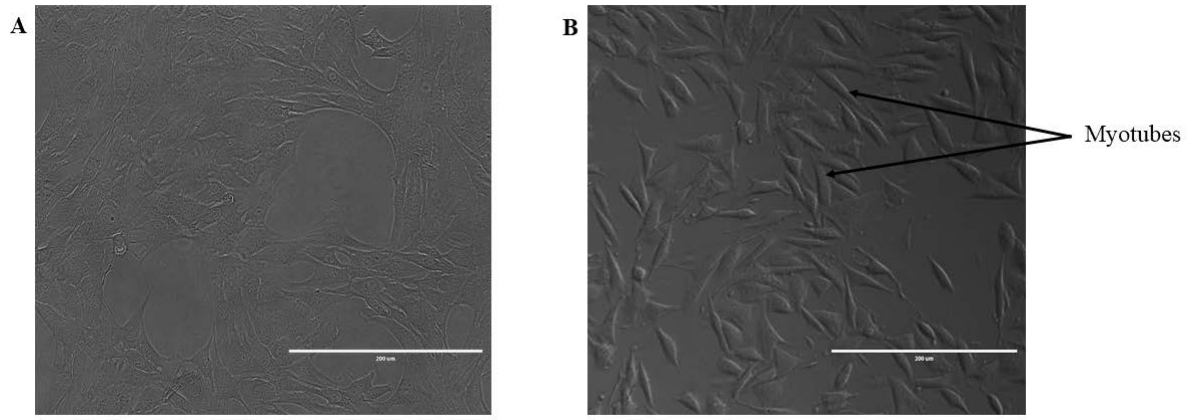


Figure 3-1: Morphology and intensity of Myotube formation in C2C12 cells. A phase contrast photomicrograph of the70-80% confluent C2C12 cells (A). Myotube formation as indicated by the black arrow in photomicrograph (B) after addition of 2% HS. Scale measurement/magnification at 200µm.

3.2.7 Freezing C2C12 cells

To ensure a steady supply of early passage cells and to ensure that the same population of cells was used across all experiments and to keep the seeding densities constant, C2C12 cells were expanded in large quantities in the beginning stage of the project. Cells were sub-cultured at about 70% confluency. Cells were then frozen in freezing medium containing 70% DMEM, 20% FBS and 10% DMSO (Sigma-Aldrich, France).

3.2.8 Experimental design

The differentiated myotubes were treated as illustrated in Figure 3-2, the experiment was done in triplicate. To assess the effect of Compound b´ in various treatment groups, 5 µL of compound b' or metformin 5 µL with 0.75 mM palmitate was added to each culture plate for various length of time. i.e. Group A was untreated (control), group B was treated with palmitate 0.75 mM for 16 hours, and group C was treated with TZD for 24 hours, subsequently group D was treated for 16 hours with palmitate to induce insulin resistance and then followed by 24 hours with hybrid compound b´ and group E was treated with palmitate for 16 hours followed by metformin 5µL for 4 hours. The medium was discarded and replaced with fresh DMEM without fetal bovine serum (FBS) for 6hours before cells were harvested for qRT-PCR.

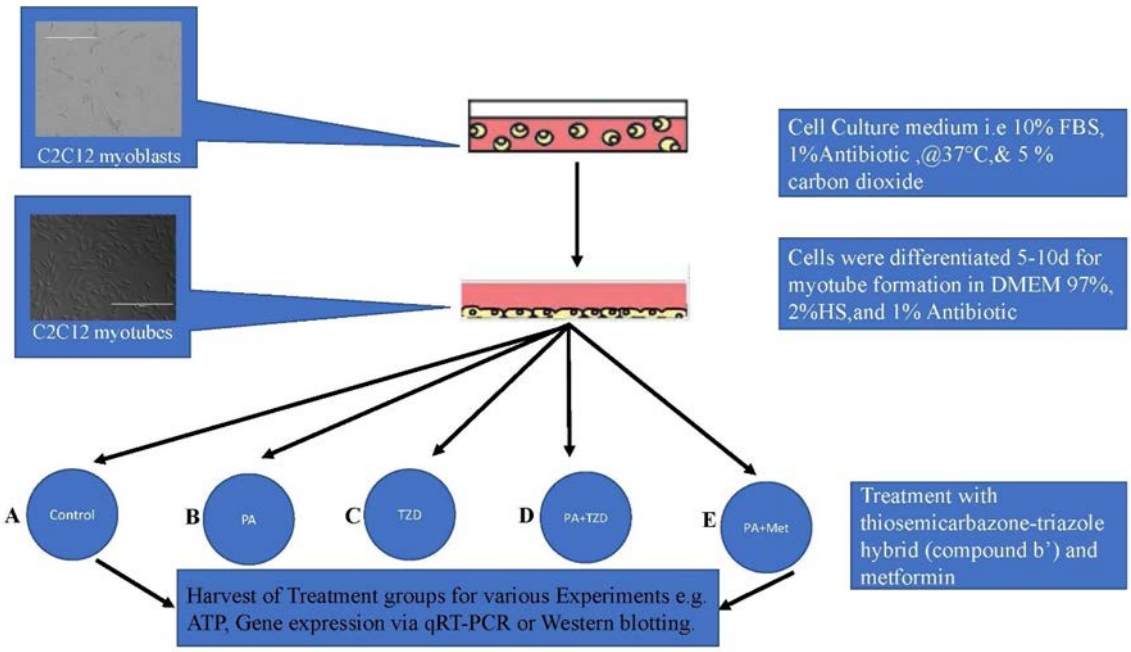


Figure 3-2: Schematic representation of the general outline of how the experiments were conducted

3.3 Determination of cytotoxicity using the MTT assay

The MTT Cell Proliferation assay was used to measure cell proliferation rate. The assay involves the conversion of the water-soluble MTT (yellow colour) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble (blue/purple colour) formazan (Liu *et al.*, 1997; Vistica *et al.*, 1991). The formazan was solubilized using dimethyl sulfoxide (DMSO), and the concentration determined using a spectrophotometer (Multiskan™ GO, USA) at 540 nm. To assess cytotoxicity and determine an optimal working concentration of the new thiosemicarbazone-triazole hybrid compound b´ at four different concentrations i.e. 0.05 g/L, 0.08 g/L, 0.14 g/ L, and 0.35 g/L, the assay was performed according to manufacturer's instructions. Briefly, 100 µL of cells was transferred to a 96 well plate, followed by addition of 10 µL of the MTT reagent and incubated at 37ºC for 4 hours. Afterwards, 50 µL DMSO was added to each well and incubated at 37ºC for 10 min. Lastly, each sample was mixed, and absorbance was read at 540 nm.The percentage growth inhibition was calculated using following formula, % cell inhibition = 100[(At -Ab)/(Ac-Ab)] × 100 where, At = absorbance value of test compound, Ab = Absorbance value of blank and Ac = Absorbance value of control (Nemati *et al.*, 2013).

3.3.1 Treatment with hybrid compound b´

The cells were treated with 5 µL of compound b´; dose-response studies were done using hybrid 1g (Table 2.1) as a model and it was found that 10 mg/mL was optimal. Since the hybrids did not have significant differences in molar mass, 10 mg/1mL was used in this study. Compound b´ and 100 nM metformin (SIGMA Aldrich, USA) were added to the differentiated cells together with 10 mL of DMEM and control was not treated. After 4 days treatment, the medium was changed with non-supplemented DMEM medium. After a 6 hours incubation, total RNA was extracted from the cells.

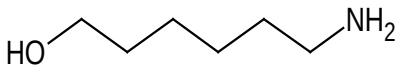


Figure 3-3: Synthesized thiosemicarbazone–triazole hybrid (compound b´)

3.3.2 Palmitate-induced insulin resistance in C2C12 muscle cells

To induce insulin resistance, culture media containing Palmitate was prepared using a method described by (Mazibuko S.E. *et al.*, 2013; Dimopoulos *et al.*, 2006) with slight modification. Palmitate 75 mM was dissolved in 96% ethanol at 95ºC water bath, it was filter sterilised and

diluted 1:100 in DMEM containing 2% BSA to yield a final palmitate concentration of 0.75 mM. The palmitate–DMEM mixture was incubated at 37ºC for 60 min to allow palmitate to conjugate with 2% BSA.

3.3.3 Glucose oxidase test (GOx)

The test was measured using the Glucose Oxidase kit (Sigma Aldrich, USA). A 10 mM H_2O_2 standard solution for colorimetric detection was prepared by diluting 10 µL of 0.88 M H_2O_2 with 879 µL of water. The 10 mM concentration was further diluted to 0.5 mM by diluting 50 µL of the 10 mM standard solution with 950 µL of GOx Assay Buffer. This was followed by adding 0,2,4,6,10 µL of the 0.5 mM standard solution into a 96 well plate, generating 0 (blank), 1,2,3,4 and 5 nmole/well standards. Lastly, GOx Assay Buffer was added to each well to bring the total volume to 50 µL.

H_2O_2 standards for fluorometric detection were prepared also using a 0.5 mM standard solution. 10 µL of 0.5 mM standard solution was diluted with 90 µL of the GOx Assay Buffer to make a 50 µM standard solution. Then 0,2,4,6,8,10 µL of the prepared 50 µm standard solution were added into 96 well plate generating (blank) 0, 0.1, 0.2,0.3,0.4 and 0.5 nmole/well standards. GOx Assay Buffer was added to each well to bring the total volume to 50 µL.

Cell samples were rapidly homogenized with 100 µL of GOx Assay buffer and centrifuged at 15000 x g for 10 min to remove insoluble materials. The reaction mixture was prepared by adding 36 µL of GOx Assay Buffer, 2 µL GOx developer, 2 µL Fluorescent peroxidase substrate, and 10 µL of GOx substrate bringing the total volume to 50 µL. A blank sample was prepared by adding 46 µL GOx assay buffer,2 µL GOx developer, and 2 µL of fluorescent peroxidase substrate without adding GOx substrate. This was followed by adding 50 µL of reaction mix to each well and mixed by pipetting.

After 5 min, the initial measurement ($T_{initial}$) was taken. For colorimetric assays, the absorbance at 570 nm was measured. For fluorometric assays, fluorescence intensity ($FLU_{initial}$, λ_{ex} = 535/λem = 585 nm) was measured. The plates were incubated at 37°C while being protected from light and measurements were taken every 3 min. Measurements were taken continuously until the value of the most active samples was greater than the value of the highest standard.

3.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

3.4.1 RNA extraction (isolation)

Principle: Starting with high-quality, pure, and intact total RNA is critical to many experiments, including RT-PCR, qRT-PCR, array analysis, northern blots, nuclease protection assays, cDNA library constructions and RNA sequencing. However, the single-stranded nature of ribonucleic acid (RNA) is a highly degradable and unstable molecule with a very short half-life due to the presence of RNases (found on skin, blood and bacteria in environment) once it has been isolated from the cell or tissue (Tan and Yiap, 2009; Brooks, 1998). RNA extraction relies on good laboratory technique and RNase-free technique (Doyle *et al.*, 1996). Most common isolation methods make use of RNase inhibitory agents and can be divided into two classes: utilization of 4M guanidinium thiocyanate and utilization of phenol and SDS (Doyle *et al.*, 1996). The commercial RNA isolation kit used in this study relies on guanidine chaotropic salt.

Technique: Total RNA was harvested from the cells using the Purelink RNA mini kit (Ambion®, Life technologies, USA), following manufacturers protocol. The growth medium was aspirated using a pipetted and 1mL of 1x PBS was added followed by scraping to detach cells. The cells were then transferred to a RNase-free tube and centrifuged at 2000 x g for 5 min at 4ºC to pellet. Cells were then lysed by adding 600 µL of lysis buffer containing 2-Mercaptoethanol. The cells were homogenised using homogeniser (Stuart SHM1 homogeniser, UK) at 12000 x g for 2 min. After this, 70% ethanol was added to each cell homogenate and vortexed to mix thoroughly and to disperse any visible precipitate that may have formed by adding ethanol. Then, 700 µL of the sample was transferred to a spin cartridge with collection tube and centrifuged at 1200 x g for 15 secs at room temperature. The flow-through was discarded and the spin cartridge reinserted in the same collection tube. This was repeated until all the sample had been processed. Then, 700 µL of Wash buffer 1 was added and the spin cartridge was centrifuged at 12000 x g for 15 sec. The same procedure was done with 500 µL of Wash buffer II and again centrifuged at 1200 x g for 15 sec. RNA bound to the membrane was centrifuged at 12000 x g for 1-2 min and the collection tube was discarded, and the spin cartridge was inserted into a recovery tube. 30-100 µL of RNase-free water was added to the centre of the spin cartridge and incubated for 1 min at room temperature. This was followed by centrifugation at ≥12000 x g for 2 min at room temperature to elute the RNA.

3.4.2 cDNA synthesis

Reverse transcription of RNA to complimentary DNA (cDNA) was performed using the SuperScript™ VILO MasterMix (Invitrogen, USA) adhering to manufacturers product

information sheet/protocol. The following components were combined in a sterile PCR tube or plate well on ice. To each sample 4 µL, of SuperScript™ MasterMix, RNA (up to 2.5 µg), DEPC-treated water (up to 10 µL) were added. This was followed by gentle mixing and incubating at 25°C for 10 min, and another incubation period at 42ºC for 60 min using the (Bio-Rad T100™ Thermal Cycler, (USA). The whole reaction was terminated at 85°C for 5 min. Synthesized cDNA was used in qPCR or stored at -20°C.

3.4.3 qRT-PCR Procedure for assessing gene expression

Amplification of the cDNA generated from Section 3.4.2 was achieved by using the StepOnePlus real-time PCR machine™ (Applied Biosystems, USA). qRT-PCR was then conducted with either PowerUp™ SYBER® Green Master Mix (Applied Biosystems, USA) or TaqMan-probed primers (Applied Biosystems, USA) in MicroAmp Optical 96-well Plates (Applied Biosystems, USA). The primers used for the SYBR green protocols are listed in Table.3-1 and were diluted to 10 µM per reaction. The cycling conditions used are shown in Table.3-3. The components were mixed thoroughly and briefly centrifuged to spin down the contents and eliminate air bubbles. The contents were then transferred to each well of an optical plate or PCR MicroAmp tubes 0.1 mL (Thermofisher Scientific, USA).

Table 3-1: List of Primers used.

Gene(species)	Primer sequence and direction
NRF-1 (Rat)	Forward: 5´-TTA CTC TGC TGT GGC TGA TGG-3´ Reverse: 5´-CCT CTG ATG CTT GCG TCG TCT-3´
GAPDH(Rats)	Forward: 5'- GAA CAT CAT CCC TGC ATC C-3' Reverse: 5'- CCT GCT TCA CCA CCT TCT T-3'
Actin (Rats)	Forward: 5'- GAC GAG GCC CAG AGC AAG AGA-3' Reverse: 5'- GGG TGT TGA AGG TCT CAA ACA-3'
GLUT-4(Mouse)	Forward: 5´-AAG ATG GCC ACG GAG AGA G-3´ Reverse: 5´-GTG GGT TGT GGC AGT GAG TC-3´
PPAR-α(Mouse)	Forward: 5´-CCT GAA CAT CGA GTG TCG AAT AT-3´ Reverse: 5´-GTT CTT CTT CTG ATT CTT GCA GCT-3´
LKB(Mouse)	Forward: 5´-CTC CGA GGG ATG TTG GAG TA-3´ Reverse: 5´-GCT TGG TGG GAT AGG TAC GA-3´
AMPK-α2(Mouse)	Forward: 5´-TGC GTG TAC GAA GGA AGA ATC C-3´ Reverse: 5´-TGT GAC TTC CAG GTC TTG GAG TT-3´
MEF2A (Rats)	Forward: 5´-CCG CCT CAG AAC TTC TCA ATG-3´ Reverse: 5´-TTG GAG AGG CCC TTG AGT TTA C-3´

Table 3-2: The reaction mixture components for qRT-PCR.

Component	Reaction Volume
	10µl/well
PowerUp™ SYBER® Green Master Mix(2X)	5 µL
Forward	1 µL
Reverse	1 µL
cDNA template + RNase-free water	3 µL
Total reaction volume	10 µL

Table 3-3: Thermal cycling conditions for the RT-qPCR.

Step	Temperature	Duration	Cycles
Uracil-DNA Glycosylase. (UDG) Activation	50ºC	2 min	Hold
AmpliTaq Fast DNA Polymerase, UP Activation	95ºC	2 min	Hold
Denature	95ºC	15 sec	40
Anneal/Extend	60ºC	1 min	

3.4.4 Calculating the ΔC_T value using a reference gene

The method that was employed to calculate the expression of each gene of interest is a variation of the Livak method. It uses a reference gene (such as GAPDH, or β-actin) to measure the relative gene expression where an untreated or baseline sample is chosen as the calibrator, and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator (Schmittgen and Livak, 2008) The resulting expression values obtained are divided by the expression value of a chosen calibrator as shown below in the mathematical equation: Ratio (reference/target) = $2^{C_T(\text{reference}) - C_T(\text{target})}$

3.5 Western Blotting

3.5.1 Protein extraction

Cells were cultured and allowed to differentiate, and then treated according to the procedure described in Section 3.2.6. Carefully, the culture medium was aspirated using a 10 mL pipette and the plate washed with 1X chilled PBS. The PBS was discarded and 1.5 mL of RIPA buffer (1 M Tris-HCL pH 7.4. 2.5 M NaCl, 0.5 mM EDTA, 10% SDS) was added to each plate for 5 min while swirling the plate occasionally on ice. Cells were then scrapped and transferred to a

microcentrifuge tube and homogenized at intervals whilst on ice, followed by centrifugation at 14,000 X g for 15 min at 4ºC. The supernatant was aspirated and either stored at -80ºC or proceeded to perform Bradford assay.

3.5.2 The protein determination assay

This assay was employed to determine the protein concentration of the unknown samples. A standard curve was generated consisting of 0.1-0.5 mg/ml of bovine serum albumin (stock solution 5 mg/mL) and sample concentrations determined from the liner part of the graph (Figure 3-3). The colour intensity (dark blue) was directly proportional to the amount of protein binding to the dye (Coomassie G250) (Bradford, 1976). The Multiskan™ Go spectrophotometer (ThermoFisher Scientific, USA) to measure the absorbance at 595 nm.

A Bradford Protein Assay Dye Reagent Concentrate (5X) (Bio-Rad, USA) was prepared. The 5X Bradford reagent was diluted to a 1:4 ratio taking in consideration the number of unknown samples. To determine the protein concentration, 10 µL of each protein standard was pipetted in triplicate and the same volume was pipetted from the unknown samples into 96 well plate, 200 µL of the prepared dye was added to the wells and incubated for 10 min at room temperature.

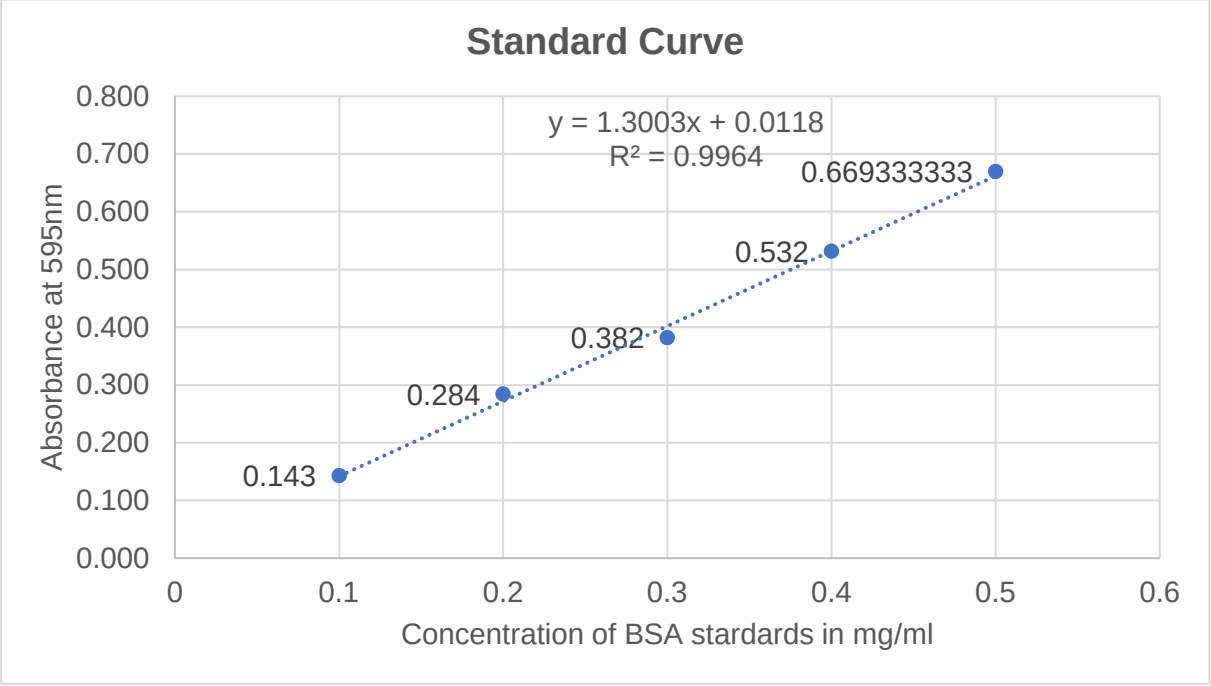


Figure 3-4: Standard curve for bovine serum albumin (BSA).

3.5.3 SDS-PAGE gel electrophoresis

Proteins were resolved on sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad mini-protean II system, (USA) and about 20-40 µg protein per lane was used. The resolving gel consisted of approximately 12.5% acrylamide (percentage can

vary depending on the molecular mass of the protein under study), lower Tris buffer (pH 8.8), 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. The stacking gel consisted of 4% acrylamide, 125mM Upper Tris (pH 6.8), 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED.

Protein samples were mixed with 4 x Laemmli sample Buffer (Bio-Rad, USA) and 10% (v/v) β-mercaptoethanol and heated at 95°C for 5 min to denature the proteins in SDS sample buffer with β-mercaptoethanol as a reducing agent resulting in a standard charge. Samples were loaded into the wells of the stacking gel. Five microlitres (5 µL) of Precision Plus Protein Standards Kaleidoscope™ (Bio-Rad, USA) and Broad range pre-stained molecular weight marker was used to determine the size of the protein of interest. Then 10 µL of the sample was added to a minimum of one well. Gels were immersed in electrode buffer and proteins were resolved using a potential difference of 80V through the gel. Once the protein samples had passed through the stacking gel, a constant voltage of 100V - 150V was applied (generally 120V).

3.5.4 Immunoblotting (protein transfer)

After SDS-PAGE gel electrophoresis, proteins were transferred using the Trans-blot turbo blotting system (Bio-Rad, USA) and trans-blot turbo transfer pack (mini format 0.2 µM nitrocellulose). This was placed in a transfer cassette and transfer of proteins was carried out at 25V, 1.0A, for 30 minutes. At the end of the run, one membrane was removed and stained with ponceau S solution to see if the transfer was successful. After visualization of bands, the stain was removed by washing in Tris-buffered saline-0.1% Tween 20 (TBS-T).

3.5.5 Immunodetection of protein

The nitrocellulose membrane was incubated with 50 mL TBS-T containing 5% (w/v) Bovine serum albumin (BSA), for 1 hour at room temperature to block non-specific binding sites. The membrane was washed 3 times for 15 min with TBST before the primary antibody was added. The nitrocellulose was incubated with gentle shaking at 4°C overnight. The concentration (1:1000) of primary antibody was determined by the manufacturer's (Cell Signalling Technology, USA) data sheet and was diluted to the required concentration in TBST. After the incubation with primary antibody was completed, the nitrocellulose was washed 3 times for 15 min with TBST and the nitrocellulose incubated with secondary antibody for 1 hour. The secondary antibody used was species-specific and HRP-linked raised to the Fc-region of the primary antibody IgG molecule and were used at a 1:5000 dilution in TBST at room temperature with continuous agitation. At the end of incubation with the secondary antibody, the nitrocellulose was again washed 3 times with

TBST. Immunolabelled proteins were visualised using the Bio-Rad Clarity™ Western Enhanced Chemiluminescence (ECL), system. Substrate kit components were mixed in a 1:1 ratio (Prepared 0.1 mL of solution/cm^2 of membrane: For a mini-sized membrane (7x8.5 cm), 7 mL of solution and for a midi-sized membrane (8.5 x 13.5 cm), 12 mL of solution is sufficient). The membrane was placed on a clear surface with protein side up and substrate added to blot for 5 min. Finally, the membrane was viewed using the ChemiDoc™ MP imaging system (Bio-Rad, USA) and the image analysed with Image Lab™ version 6.0.0 build 25, Standard Edition software (Bio-Rad, USA).

3.6 Silencing AMPK expression by small interference RNA (siRNA)

Mechanism of RNA interference: siRNA is a technique that silences gene expression by introducing a double-stranded RNA (dsRNA) homologous to the target mRNA. The dsRNAs in different cell types are cleaved by the host RNase III enzyme (dicer) which in turn form what is called siRNAs. They typically contain 21-23 nucleotides (Hannon, 2002; Sharp, 2001).

Protocol: The AMPK-siRNA(Prkaa2) (Ambion, Life Technologies) which was used in this experiment is a pool of 3 target-specific 21 nucleotides designed to knock down the PRKAA2 gene (Ambion, USA). These siRNAs were then incorporated into an RNA-induced silencing complex (RISC), where the duplex siRNA is unwound, so the antisense strand can guide RISC to the target mRNA having the complementary sequence. The target mRNA was cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA (Takasaki *et al.*, 2004). Lastly, RISC was released to additional mRNA molecules, whereas the cleaved mRNA was degraded by the cellular ribonucleases. An example is given in (Figure 3-4).

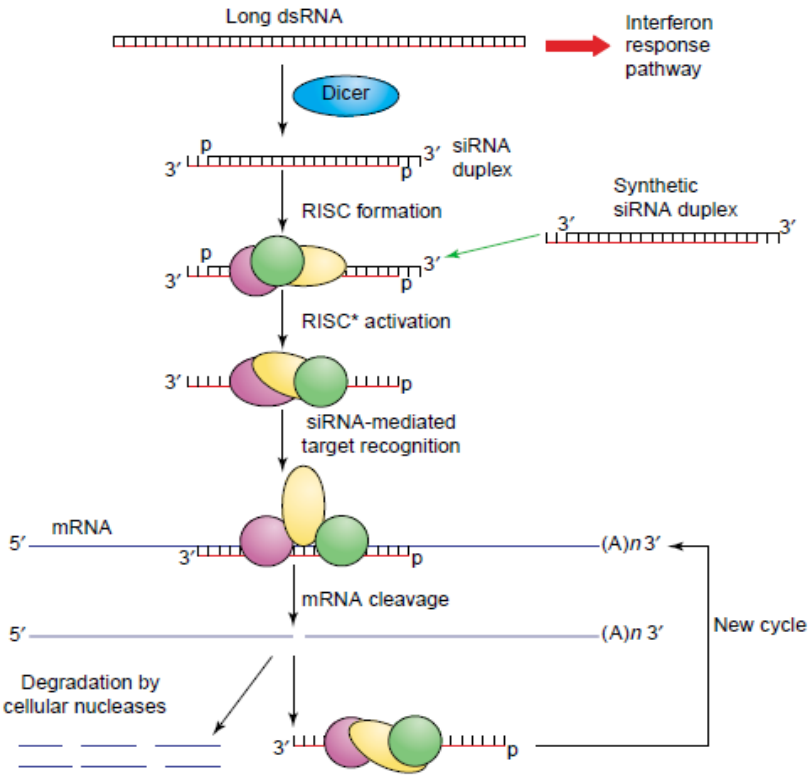


Figure 3-5: Schematic representation of a gene-silencing pathway. In Caenorhabditis elegans and embryonic mammalian cells, for example, the RNase III Dicer degrades long double-stranded RNA (dsRNA) to generate small-interfering RNAs (siRNAs) that trigger the RNA interference (RNAi)-specific pathway adapted from (Sioud, 2004).

3.6.1 Transfection protocol

C2C12 cells were grown in 6-well plates at a seeding density of 0.3 x 10^6 until they were 70-90% confluent. Lipofectamine® 2000 reagent 9 µL (Invitrogen) was diluted in 150 µL Opti-MEM® medium. This was followed by the addition of 10 µM PRKAA2-siRNA diluted in Opti-MEM® medium. The diluted PRKAA2-siRNA 3µL (30 pmoL) was added to the diluted Lipofectamine® 2000 reagent in the 1:1 to form a siRNA-lipid complex and incubated at room temperature for 5 min. Finally, the PRKAA2-siRNA (250 µL) Lipofectamine® complexes formed were introduced into the cells dropwise using a pipette and incubated for 1-3 days at 37ºC. The transfected cells were visualised using the EVOS microscope FL (Life technologies, USA) as illustrated in Figure 4-11 and 4-14 in chapter 4. Cells were further incubated for 6 hours in growth medium without serum followed by total RNA extraction using the Cells-to-CT ™ TaqMan® Kit (Section 3.6.3) followed by qRT-PCR to assess gene expression.

3.6.2 Preparation of transfected cells for RT-PCR using the Cells-to C_T™ kit to assess gene expression.

Preparation of cells for lysis was done according to manufacturer's guidelines. Firstly, chilled 1X PBS was placed on ice, which was sufficient for 50 µL per 10^5cells. Then followed by Preparation of DNase treatment i.e. Lysis solution (at room temperature) 49.5 µL and DNase 0.5 µL mixture. Cells were grown in 6 well plates and detached using 500 uL of 0.05% Trypsin/EDTA (1X) (Life technologies, USA) and trypsin action was halted by adding 3 mL of growth medium. Cells were counted using the TC 20™ Automated cell counter (BIORAD, USA), and gently centrifuged to pellet the cells. Growth medium was discarded, and the pellet resuspended in 50 µL of chilled 1X PBS followed by pelleting the cells. PBS was aspirated without disturbing the pellet. The pellet was resuspended in 5 µL of cold 1X PBS and pipetted up and down thoroughly to resuspend the cells. 5 µL of cells was distributed in a 96-well PCR plate.

3.6.3 Preparing the Cells-to-C_T ™ lysate

To the prepared cells, 50 µL of room temperature lysis solution or DNase/Lysis solution was added and pipetted up and down 5 times to mix well and incubated at room temperature for 5 min. After the incubation, 5 µL of stop solution was added and pipetted up and down again to mix well and followed by 2 min of incubation. The lysates were now placed on ice and proceeded with qRT-PCR. (*Note: this is also a stopping point and lysates can be stored on ice for up to 2 hours or at or below -20ºC for up to 5 months*).

3.6.4 RT-PCR for Cells-to C_T

All reagents, including previously frozen Cells-to-CT™ lysates, were thawed on ice. Adhering to manufactures protocol, TaqMan®1-Step qRT-PCR master mix was prepared whilst on ice, for the number of reactions required plus 10% overage. Table 3-4 illustrates a typical example. On ice, appropriate volume (18-19 µL) of RT-PCR Master mix was added to each sample or control well of an optical reaction plate. This was followed by addition of an appropriate volume (1-2 µL) of lysate or Nuclease-Free Water for the non-template control (NTC) to each well (20 µL total). Finally, Real-time PCR was performed as indicated below:

I. Reverse transcription, 1 cycle at 50ºC for 5 min.

II. RT inactivation/denaturation, 1 cycle at 95ºC for 20 min.

III. Amplification, 40 cycles at 95ºC for 15 min, 60ºC for 1 min.

Table 3-4: Reaction set-up for TaqMan 1-Step qRT-PCR. The [1] component volume can be scaled for RT- reaction volumes between 10 µL and 50 µL.

Component	Volume per 20-µl reaction [1]
TaqMan®1-Step qRT-PCR mix	5 µL
TaqMan®Gene Expression Assay(Prkaa2), 20X	1 µL
Nuclease-Free Water	To 19 µL (for 1 µL of lysate) To 18 µL (for 2 µL of lysate)

3.7 The Ferric Reducing Antioxidant Power (FRAP)

***Principle*:** It is based on the reduction of ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous (Fe^{II}) form at low pH. The reduction is monitored by measuring the change in absorption at 593 nm with the formation of an intense blue colour (Benzie, 1996; Liu *et al.*, 1982).

***Procedure**:* The FRAP reagent was a cocktail (10:1:1, v/v/v/) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). Then, 10 µL of the sample was mixed with 300 µL FRAP reagent in a 96-well clear plate. It was incubated at room temperature for 30 min and the plate was read at 593 nm in a Multiskan Go Spectrum plate reader (ThermoFisher Scientific, USA) in triplicates. The standard used was Ascorbic acid (AA) and the results were expressed in µmol AAE/g sample.

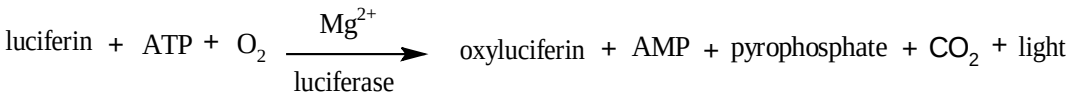
3.8 The Trolox Equivalent Antioxidant capacity (TEAC)

Principle: This assay is based on the scavenging of the 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical converting it into a colorless product. The degree of decolorization induced by a compound is related to that induced by Trolox, giving the TEAC value. The assay is frequently used for structure-activity relationships (SARs).

Procedure: Prior to commencement of the experiment, the blue-green $ABTS^+$ solution was prepared by mixing ABTS salt (8 mM) with potassium persulfate (3 mM) in distilled water. This solution was stored in the dark for 12-24 hours before use. The $ABTS^+$ solution was diluted with distilled water before use to a final absorbance of 0.70 ± 0.02 at 734 nm (van den Berg *et al.*, 1999; Re *et al.*, 1999). Twenty-five microliters (25 µL) of the sample was mixed with 300 µL $ABTS^+$ solution in a 96-well clear microplate. The plate was read after 30 min incubation at room temperature in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) at 734 nm in triplicates. Trolox was used as the standard and result expressed as µmol TE/g sample.

3.9 ATP assay.

Principle: The assay is based on luciferase's requirement for ATP in producing light (emission maximum~560 nm at pH 7.8) from the reaction (Leach, 1981):



Protocol: The assay was performed using the Invitrogen (life technologies, USA) ATP determination kit following manufacturer's recommendations. The experiment was begun by reagent preparation i.e. 1.0 mL of 1X Reaction Buffer by adding 50 µL of 20X Reaction Buffer to 950 µL of deionized water (dH_2O). This volume was sufficient to make 1 mL of 10 mM D-luciferin stock solution. From this, to make 1 mL of a 10 mM D-luciferin stock solution by adding 1 mL of 1X Reaction Buffer was added to one vial of D-luciferin. Then, to prepare a 100 mM DTT stock solution, we added 1.62 mL of distilled water to the bottle containing 25 mg of DTT. We also prepared a low-concentration of ATP standard solutions by diluting the 5 mM ATP solution in dH_2O. Lastly, to make a Standard Reaction solution, we combined the following components of the reaction as follows to make a 10 mL of 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 2.5 µL of firefly luciferase 5 mg/mL stock solution). This was followed by gentle mixing and the reaction was protected from light until use. A standard curve was generated by adding the appropriate volume of the standard reaction solution in the luminometer and measured the background luminescence. The reaction was commenced by adding the desired amount of dilute ATP standard solution followed by reading the luminescence and then subtracted the background luminescence.

Cultured cells were differentiated as described in Section 3.2.6 and treated according to Section 3.3.1. 1X PBS was used to wash the cells and followed by scrapping to collect the cells which are now called (lysates) and transferred to 2 mL Eppendorf tubes (sigma Aldrich, USA) for homogenization. Then 10 µL of the sample and 100 µL reaction mix were placed in 96 well plates. The same was done for the standard i.e. 10 µL Standard and 100 µL of reaction mix. This mixture was kept at room temperature for 2 min before reading the luminescence using the MultiSkan GO plate reader (Thermofisher Scientific, USA).

3.10 Chromatin Immunoprecipitation (ChIP) assay

Principle: Chromatin immunoprecipitation (ChIP) is a technique used to study Protein–DNA interaction and can be applied to the various study of proteins associated with chromatin, such as histone and its isoforms and transcription factors, across a defined DNA domain (Yan *et al.*, 2004).

In this work, cells were treated with formaldehyde to covalently link protein to DNA (X-ChIP), the nucleoprotein complexes were then sheared either mechanically or by enzymatic digestion. The resultant soluble cross-linked DNA–protein complexes were enriched by immunoprecipitation (Dahl and Collas, 2007). The recovered complexes were analyzed by PCR amplification with gene-specific primers shown in Table 3-5, to detect and quantify specific DNA targets.

Protocol: ChIP assay was used to assess the amount of MEF2A that was bound to NRF-1 (MEF2A→NRF-1). Cells were cultured and allowed to differentiate into myotubes and treated with compound b´ as outlined in Section 3.2.6 and 3.3.1 respectively.

After treatments, the cells were washed with 1X PBS and 37% formaldehyde was directly added to the plate in a final concentration of 1% (v/v) to crosslink protein to DNA and protein to protein, at 37ºC for 10 min. Crosslinking was stopped by adding glycine to the medium to a concentration of 0.125M. The cells were washed three times with cold 1X PBS and lysed with lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris pH 8.0, distilled water, 10 mM $Na_4P_2O_7$, 20 mM NaF, 0.15 µM Okadaic acid, Na_3VO_4 and 1X Roche Complete Protease Inhibitors) and kept on ice for 10 minutes. Cells were scraped into an Eppendorf tube and sonicated with 10-15 sec bursts (1 min rest on ice) at 33% maximum on ice. The supernatant was centrifuged at 13000 rpm for 10 min. Thereafter, the supernatant was transferred to a new microtube. Then 100 µL of supernatant was diluted 9-fold with ChIP dilution buffer (0.01 SDS,1.1% Triton X-100,1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl and distilled water) to make 1 mL. The resultant supernatant was now referred to as ‘INPUT’ sample (IN) and 30µL was collected and kept at -80ºC until used for de-crosslinking. The remaining supernatant (970 µL) now called ‘Immunoprecipitation’ (IP) was used immunoprecipitation.

3.10.1Pre-clearing

To clear the sample of any endogenous antibodies, 30 µL of 50 % slurry agarose beads/salmon sperm (SCBT) was added to the IP and incubated the sample at 4ºC on a rotating platform for 1-2 57 hours. The beads were pelleted by centrifugation at 2000 rpm/2 min at 4ºC and the supernatant

was transferred to a new tube. MEF2A antibody (cell signaling, USA) was added and immunoprecipitation allowed for 36-48 hours, then after incubation 40 µL of 50% slurry agarose/salmon sperm beads was added to the sample and rotated gently for 4-6 hours at 4ºC. Once more, the beads were pelleted by centrifugation at 5000 rpm for 1 minute at 4ºC. The pellet was in sequence with the following buffers: Lower salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and distilled water), High salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl and distilled water), Lithium wash buffer (0.25M LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% deoxycholic acid-Na and distilled water) and twice washed with TE buffer (10 mM Tris, pH 8.0, 1mM EDTA and distilled). These washing steps were done by adding 1 mL of each buffer, rotating the tubes for 3-5 min and 2000 rpm at 4ºC except for the last wash with TE buffer which was performed at room temperature.

Table 3-5: List of primers used in ChIP assay.

Designed primer name	Amplicon size	Primer Sequence direction
MEF2A-NRF-1 (+VE)	315bp	**Forward:** CCT TCC TGT GCC GGG TGA TCT **Reverse:** CTA TTT TTA GGA GTC AGG CCC GG
MEF2A-NRF-1 (-VE)	250bp	**Forward**: AGT TGT GCC ACC TGT CCC A **Reverse:** CAA TGT CAG CTC ACA CTCA

3.11 Statistical Analysis

The results in this report were expressed as a mean ± Standard deviation (SD). Statistical analyses of the data were subjected to a one-way ANOVA. Data with a p-value of <0.05 was considered statistically significant in all cases. The Dunnett multiple comparison tests was employed when ANOVA indicated a significance difference. Every other testing and graphing was done using the GraphPad Prism 6 software.

CHAPTER 4

4.0 RESULTS

4.1 Optimization of thiosemicarbazone-triazole hybrid compound b´ concentration for treatment of C2C12 cells using the MTT assay.

C1C12 cells were cultured and allowed to differentiate into myotubes before the hybrid compound b´ was introduced to assess cell viability, cytotoxicity and determine an optimal working concentration. Each sample was mixed, and absorbance read at 540 nm using the Multiskan-Go plate reader. The cells were then treated with four (4) different concentrations of the drug, 0.005 g/L, 0.008 g/L, 0.014 g/L and 0.035 g/L. The concentration of 0.005 g/L showed that the drug worked optimally indicating that it had the least number of dead cells. Therefore, 0.005 g/L was used to treat the cells in all subsequent experiments.

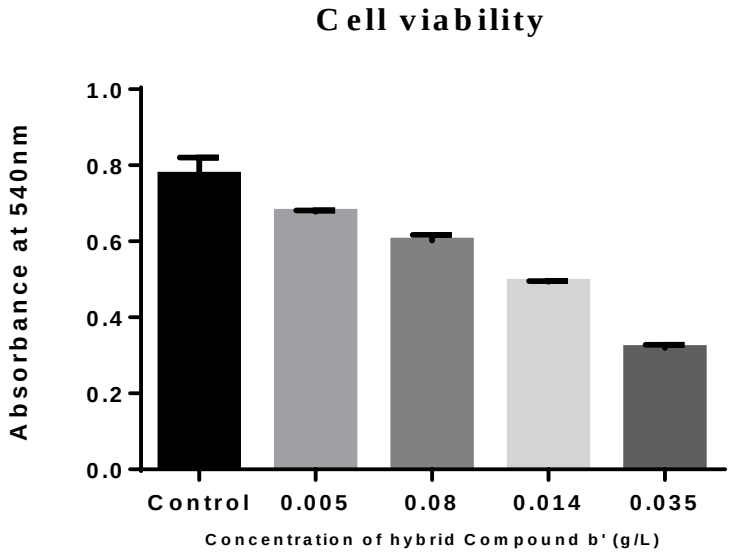


Figure 4-1: Cell viability in response to treatment with hybrid compound b´.

4.2 Glucose consumption test

To determine glucose consumption of palmitate induced insulin-resistance and subsequent treatment with the thiosemicarbazone-triazole hybrid (compound b´), As is shown in Figure 4-7, glucose consumption was markedly increased in the control followed by the TZD group. Surprisingly, compound b´ had no significant effect over the PA+MET and PA+TZD groups, which had a slight increase in glucose consumption over the PA group. The low level of Glucose utilized can be used to confirm that palmitate had induced insulin resistance in C2C12 cells when compared to the normal control cells.

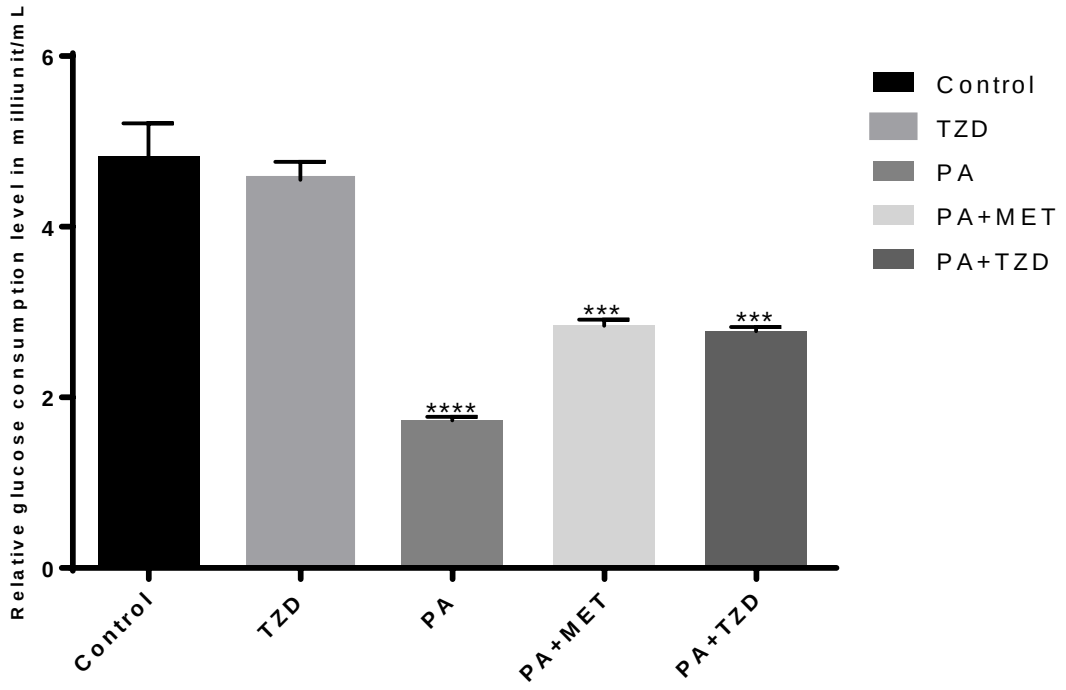


Figure 4-2: Glucose oxidase activity. Differentiated C2C12 cells were treated with without 0.75mM Palmitate for 12-16hours and followed by 5µL of compound b´ hybrid (10mg/mL) for 12-24hours or 100nM metformin for 4hours respectively. Glucose oxidation was measured by the Glucose oxidase buffer GOx. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, Control vs PA+MET $P\leq0.001$(*), and Control vs PA+TZD $P\leq0.001$ (*), control vs PA $P\leq0.05$

4.3 Assessing the effect of Compound b´ on energy production (ATP determination) in palmitate-induced insulin-resistant C2C12 muscle cells.

Following exposure of C2C12 muscle cells to palmitate for 16 hours, it was necessary to determine the energy production using ATP luminescence kit as described in Section 3.9. Results obtained indicate that ATP content was increased in Control vs PA+TZD by almost 3-fold when compared to all other combination of treatment, i.e. Control vs TZD, Control vs PA+MET and Control vs PA, respectively Figure 4-19. The PA group had the lowest content of ATP.

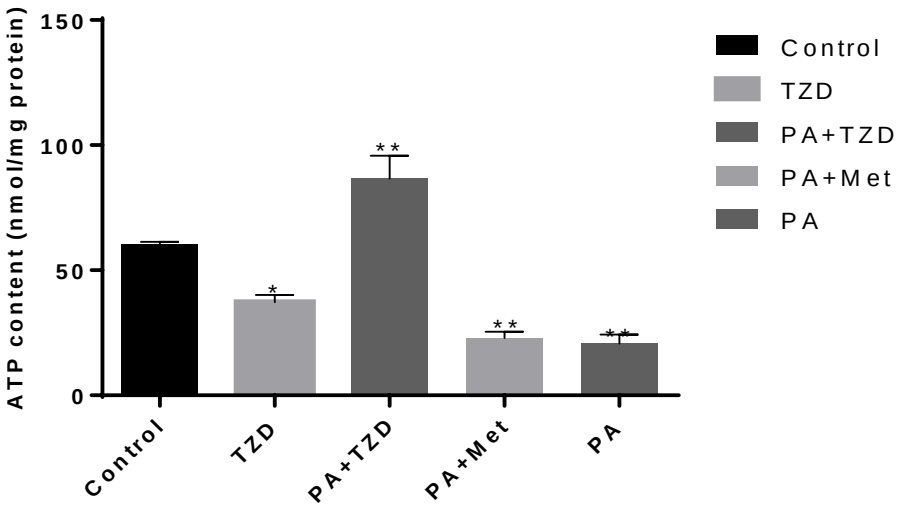


Figure 4-3: ATP assay. Effect of compound b´ on ATP production. C2C12 cells were cultured in 0.75mM palmitate for 16h and then treated with either Compound b´ or metformin as described in section. The percentage ± SD is expressed relative to the control at 100% statistical analysis ANOVA with Dunnet's post hoc test; (*P<0.05 control vs TZD, **P<0.01 control vs PA+TZD, and **P<0.01 control vs PA+MET and **P<0.01 control vs PA respectively).

4.4 qRT-PCR assessment of AMPK gene expression

The experimental treatment groups were assessed for their expression of total AMPK gene, which is a master regulator of cellular energy status. In this study, we show that AMPK gene transcription is high in C2C12 cells which are insulin-resistance induced by palmitate. In Figure 4-4 below, TZD administered to C2C12 cells without palmitate-induced insulin resistance increased AMPK expression by almost 3-fold when compared to Control. A similar result is seen in the combination of Palmitate and metformin (PA+MET), and Palmitate + hybrid compound b´) (PA+TZD) treatment groups, although the major difference is that these groups had cells that were insulin resistant brought about by palmitate. AMPK expression was reduced in the Palmitate (PA) group as expected since the cells were insulin resistant by Palmitate. In the control subject there was no observable change.

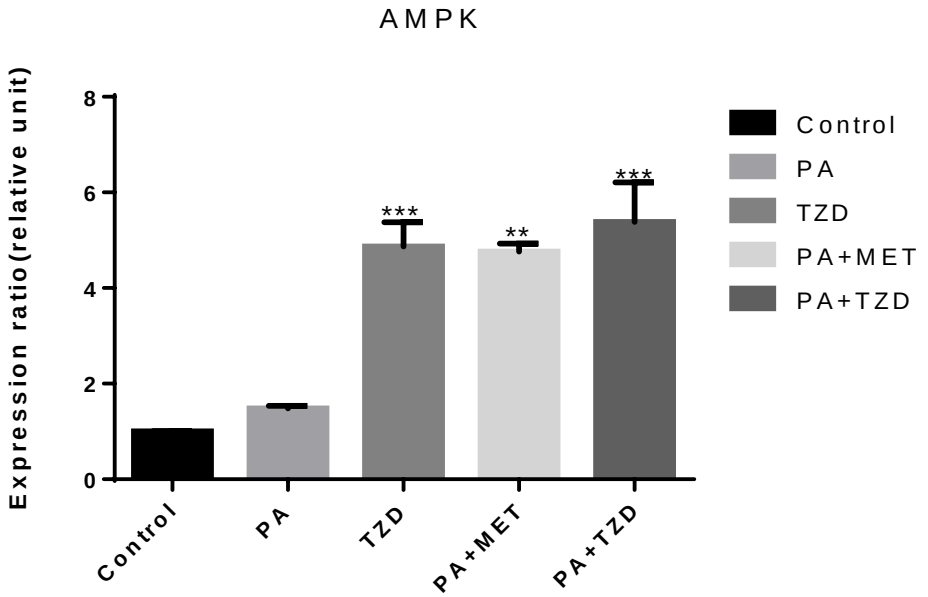


Figure 4-4: Expression of AMPK in response to treatment with Compound b.´ Differentiated C2C12 cells were treated with 0.75mM palmitate for 12-16hours followed by 5µL of compound b´ hybrid (10mg/mL). for 12-24hours or 100nM metformin for 4hours respectively. The expression was analysed by quantitative real-time PCR using actin as a reference gene and calculated according to the relative standard method. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, Control vs TZD $P\leq0.001$(***), Control vs PA+MET $P\leq0.01$(**) and Control vs PA+TZD $P\leq0.001$(***).

4.5 qRT-PCR assessment of LKB1 gene expression

In this experiment we sought to find out if Compound b´ had any effect on the upstream kinase that activates AMPK, the results in Figure 4-3 below, indicate that untreated cells (control) had the highest expression of LKB1 when compared to all other groups. Palmitate (PA) treated group had the lowest expression. LKB1 is one of the major upstream kinases that activate AMPK and phosphorylates AMPK on the activation loop threonine (Thr172) within the catalytic subunit and activates AMPK in vitro (Hong *et al.*, 2003; Woods *et al.*, 2003b).

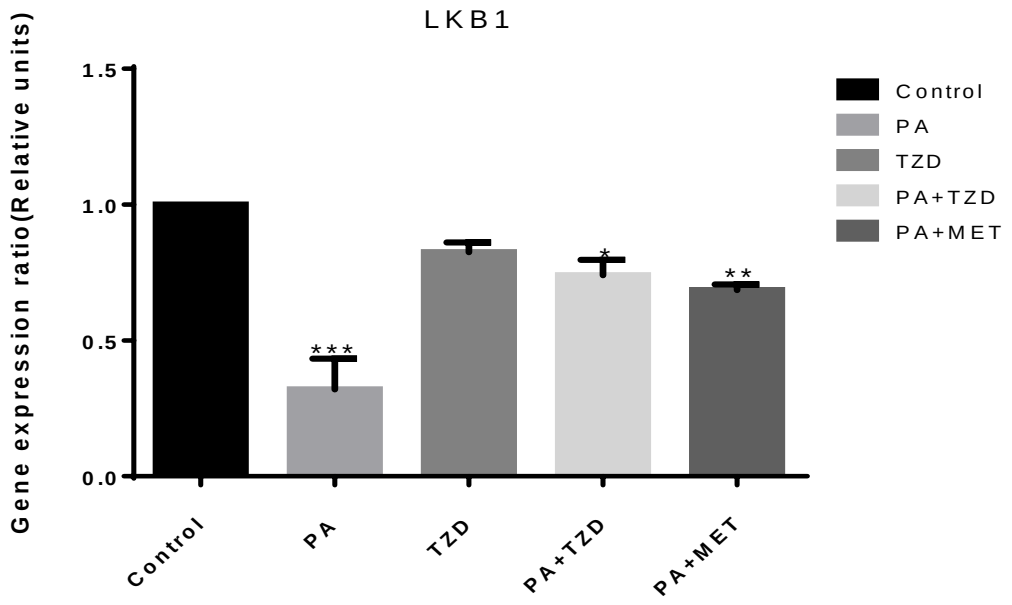


Figure 4-5: Expression of LKB1 gene in response to treatment with compound b´. Differentiated C2C12 cells were treated with 0.75mM palmitate for 12-16hours followed by 5µl of compound b´ hybrid (10mg/mL). for 12-24hours or 100nM metformin for 4hours respectively. The expression was analysed by quantitative real time PCR using actin as a reference gene and calculated according to the relative standard method. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, control vs PA $P\leq0.001$(***), Control vs PA+TZD $P\leq0.05$(*) and Control vs PA+MET $P\leq0.01$(**).

4.6 qRT-PCR assessment of MEF2A gene expression

The result obtained is shown in Figure 4-4, indicates that the hybrid compound had a positive effect on the treatment groups i.e. TZD, and PA+TZD, except for PA group which had low expression level. The PA+MET group was level with the control group, suggesting that there was gene transcription was not up-regulated. There was a slight increase in gene transcription in the PA+TZD group when compared to the positive control of PA+MET. This further reinforces the fact that MEF2A is a much-needed transcriptional gene is glucose transport.

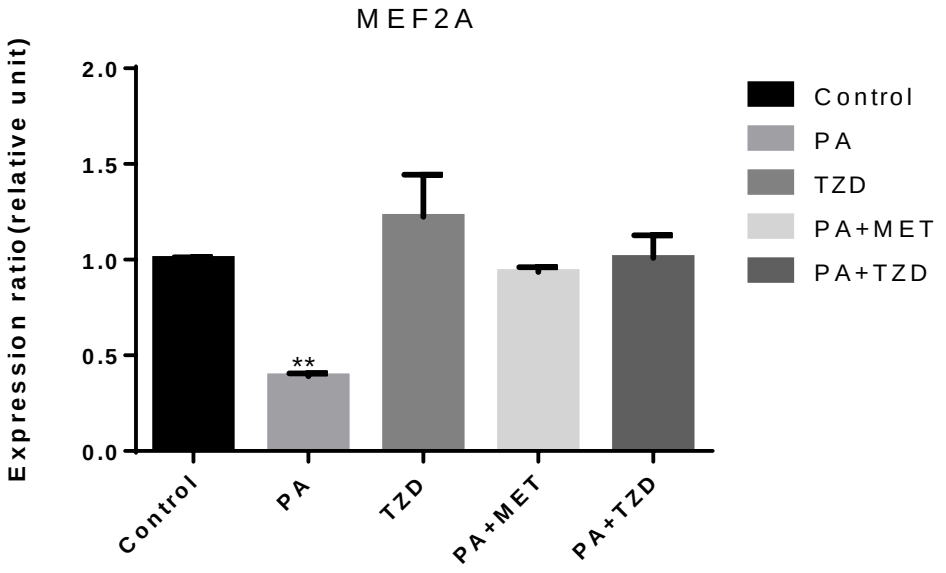


Figure 4-6: Expression of MEF2A gene in response to treatment with Compound b.´Differentiated C2C12 cells were treated with 0.75mM palmitate for 12-16hours followed by 5µL of compound b´ hybrid (10mg/mL). for 12-24hours or 100nM metformin for 4hours respectively. The expression was analysed by quantitative real-time PCR using actin as a reference gene and calculated according to the relative standard method. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, control vs PA $P<0.01$(**).

4.7 qRT-PCR assessment of GLUT-4 gene expression

The result in Figure 4-5 indicates that the hybrid compound b´ increased GLUT-4 expression in relation to that of the standard drug (metformin) used to treat T2D and known to induce GLUT-4 expression. The TZD alone group had the highest expression of GLUT-4, followed by the PA+TZD and PA+MET. As expected, the PA group had the lowest expression of Glut-4. The disease burden of T2D reduces the levels of GLUT-4.

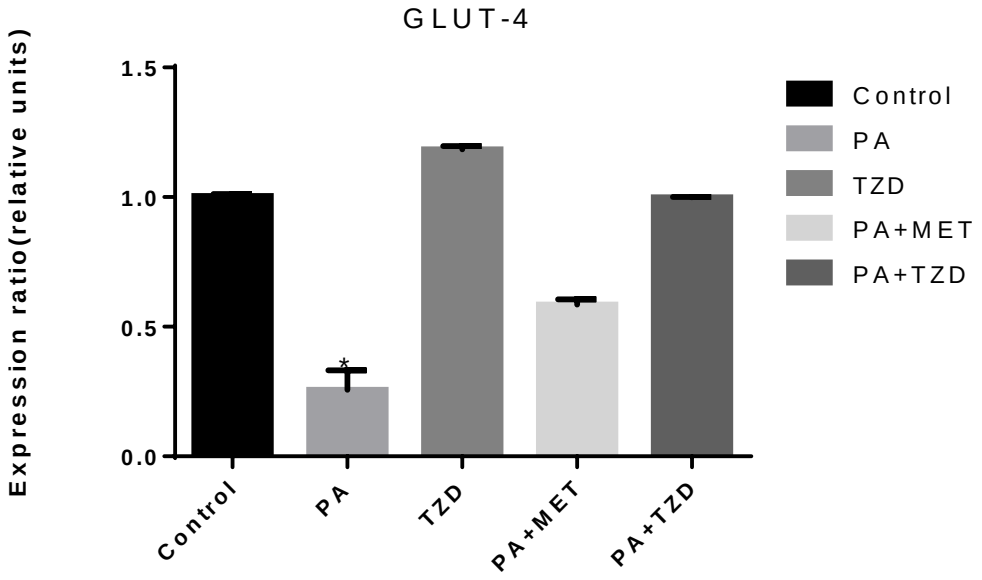


Figure 4-7: ´Expression of GLUT-4 gene in response to treatment with compound b´. Differentiated C2C12 cells were treated with 0.75mM palmitate for 12-16hours followed by 5µL of compound b´ hybrid (10mg/mL). for 12-24hours or 100nM metformin for 4hours respectively. The expression was analysed by quantitative real-time PCR using actin as a reference gene and calculated according to the relative standard method. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, control vs PA $P<0.05$(*).

4.8 qRT-PCR assessment of PPARα gene expression

To investigate the effect towards pro-lipid oxidation, we assessed the peroxisome proliferator-activated receptor α (PPARα), a member of nuclear receptors that play a key role in the regulation of metabolic homeostasis and inflammation (Cariou *et al.*, 2012). The results obtained, as is evident in Figure 4-6, show that the hybrid compound did not have any advantage in up-regulating PPARα. The TZD group had the highest expression of PPARα, cognizance taken that the cells in this group were not insulin-resistant. On the other hand, the PA (Palmitate induced-insulin resistance) group had low expression level of PPARα as the PA+MET group and the PA+TZD group. This result indicates that the drug (Compound b´) had no advantage in rescuing the cells with palmitate induced insulin resistance.

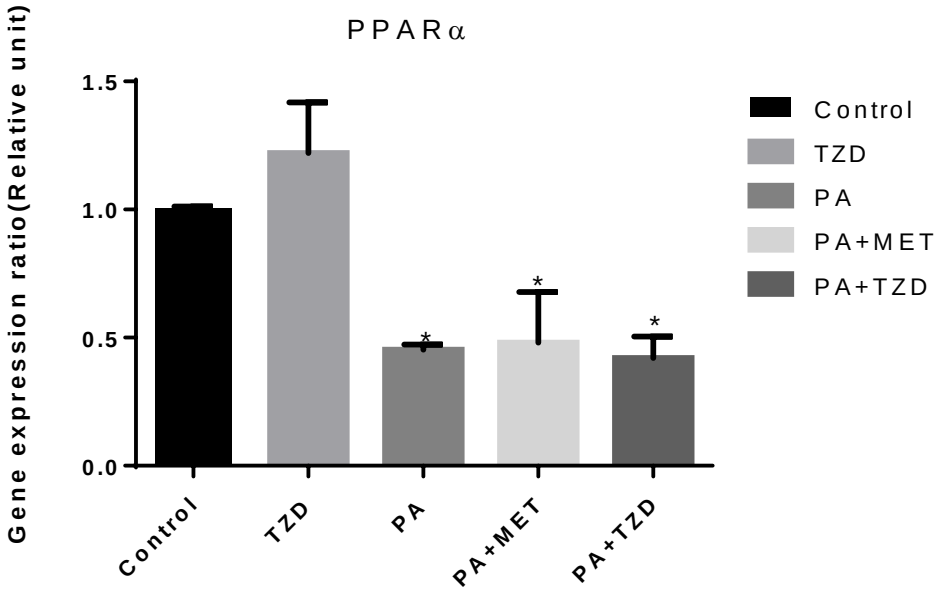


Figure 4-8: Expression of PPARα in response to treatment with Compound b´. Differentiated C2C12 cells were treated with 0.75mM palmitate for 12-16hours followed by 5µL of compound b´ hybrid (10mg/mL) for 12-24hours or 100nM metformin for 4hours respectively. The expression was analysed by quantitative real-time PCR using actin as a reference gene and calculated according to the relative standard method. Results were presented as means ± SD. Level of significance was accepted at $P<0.05$. The P value, Control vs PA+MET $P\leq0.05$(*), Control vs PA+MET $P\leq0.05$(*), and Control vs PA+TZD $P\leq0.05$ (*).

4.9 Assessment of NRF-1 binding MEF2A in palmitate induced insulin resistant C2C12 cells treated with compound b´

ChIP assay was carried out to determine the amount of NRF-1 bound to MEF2A and hyperacetylation of histones in region of NRF-1 binding site on MEF2A gene. The results in Figure 4-9 indicates that there was binding of NRF-1→MEF2A, in the Control, TZD alone group, PA+MET, PA+TZD and PA in both IP and IN categories. The band intensity is more pronounced in the control group as indicated in Figure 4-9 while there is less band intensity in TZD and PA+MET. It would be sufficient to say that there was binding in these two groups as compared to PA+TZD and PA groups.

Figure 4-9: ChIP assay images. A pictomicrograph of agarose gel showing a section of the NRF-1 bound to MEF2A binding site that was amplified by PCR from DNA that was co-immunoprecipitated (IP) with acetylated histone H3 or histone H3 antibody and from corresponding input (IN) samples.

4.10 Protein expression

In this experiment, western blotting was employed to analyse protein expression of total AMPKα following treatment with compound b´. Figure 4-10 shows that the expression of AMPK protein was significantly reduced in Control and TZD. On the other hand, the treatment groups involving TZD+PA, PA, and PA+MET showed a marked expression of AMPK as expected because the cells were under stress after inducing insulin resistance.

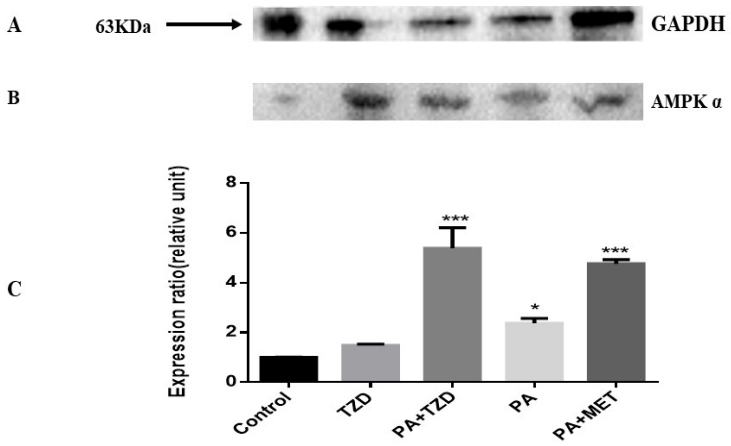


Figure 4-10: Western blot. Cell lysates were analyzed by western blot using AMPK α and anti-mouse IgG, HRP-linked anti-bodies. **A:** GAPDH at 63KDa. **B:** Representative protein expression of C2C12 cells with palmitate induced insulin-resistance in groups PA+TZD, PA, and PA+MET and AMPK protein expression in response to treatment with hybrid. The data were represented as the mean ± SD of experiments. The P values. Control vs PA+TZD P≤0.001 (***), Control vs PA P≤0.05 and Control vs PA+MET P≤0.001(***).

4.11 Assessing the transfection efficiency, the siRNA in C2C12 cells.

To assess the transfection efficiency, we used the Silencer Select (Ambion by Life Technologies, USA) pre-designed siRNA (Prkaa2) and alpha 2 catalytic subunit to knockdown the expression of the Prkaa2α subunit, a Negative Control siRNA (Ambion by Life Technologies, USA) to determine transfection efficiency and to control effects of siRNA delivery and a Block-iT Alexa flour red fluorescent oligo (Invitrogen by Life Technologies, USA) as a positive control siRNA which is an indicator of lipid-mediated transfection efficiency (Figure 4-11). The transfected cells were then subjected to qRT-PCR for further confirmation of transfection efficiency. Figure 4-11 indicates the relative expression of each siRNA assessed.

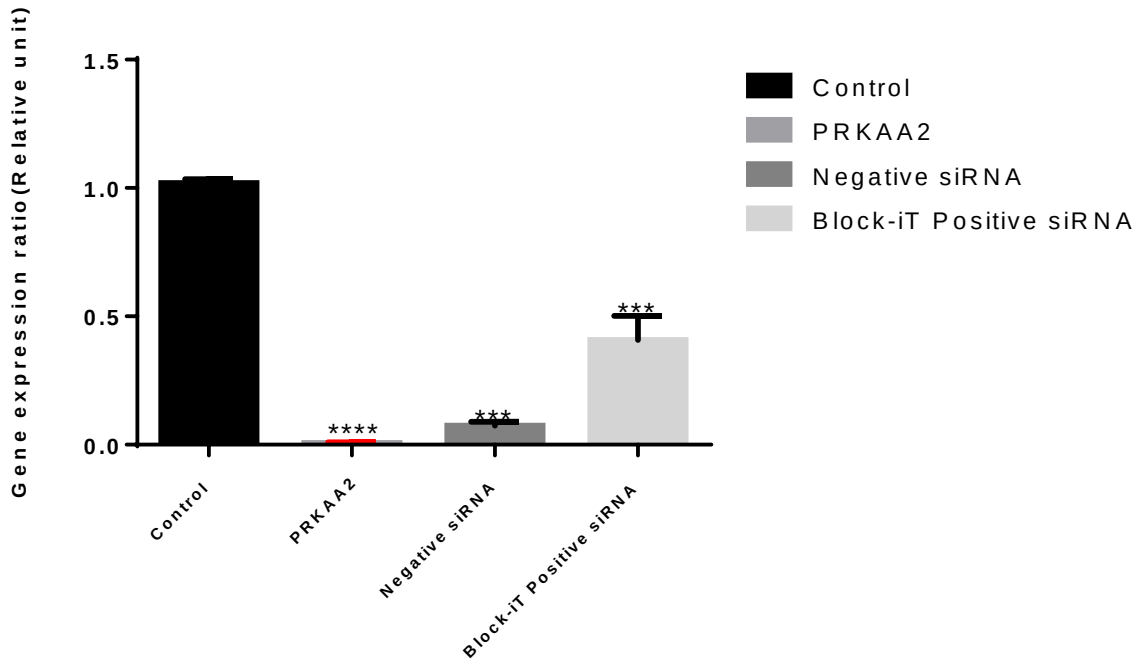


Figure 4-11: siRNA Transfection levels. Shows the level of transfection efficiency of the Prkaa2 siRNA in C2C12 cells. Differentiated C2C12 cells were transfected with AMPK siRNA(Prkaa2) for 24 h or 48h. Quantitative real-time PCR was used to determine the gene expression levels of AMPK by the C2C12 cells that had been treated with AMPK siRNA. Results were presented as means ± SD. Level of significance was accepted at (****P≥0.05 control vs Prkaa2, ***P<0.001 control vs negative SiRNA and ***P<0.001 control vs Block-It positive siRNA respectively).

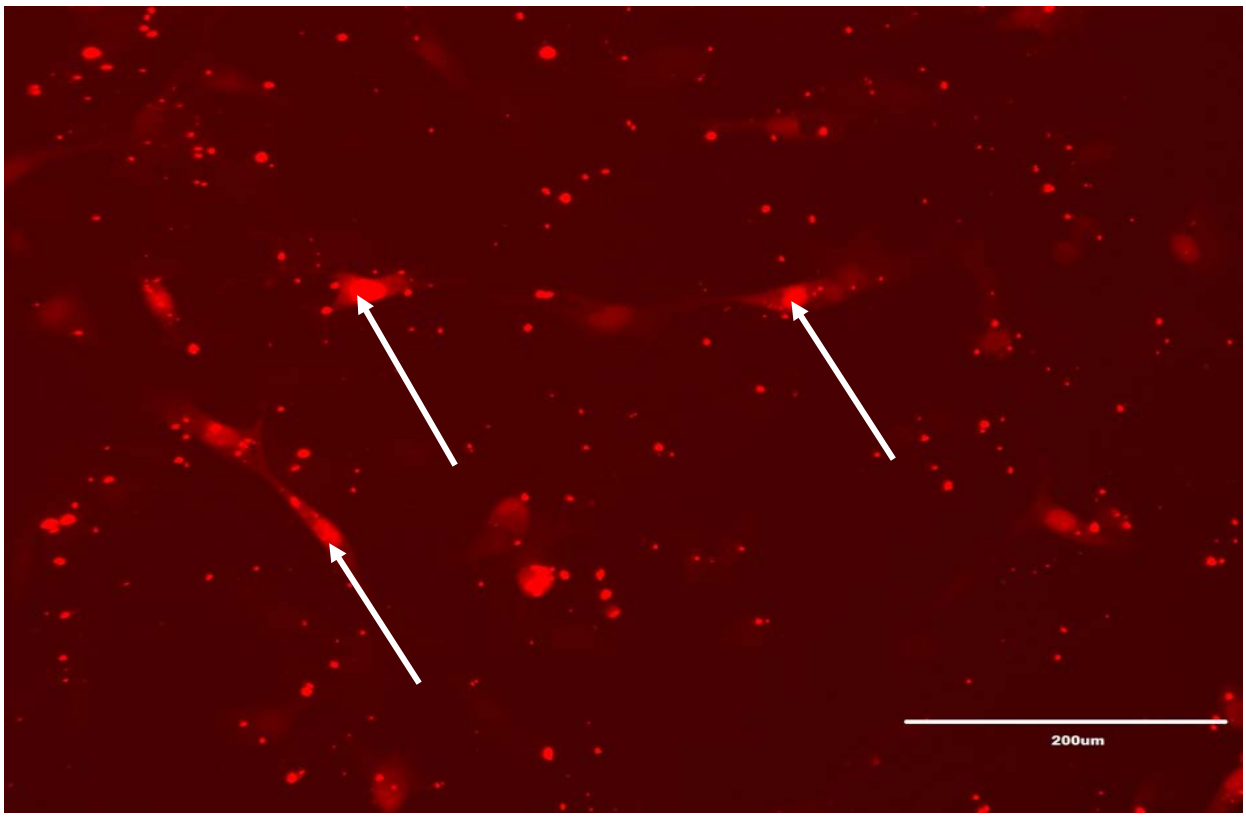


Figure 4-12: Transfection efficiency. C2C12 cells were transfected with lipofectamine 2000 reagent and cells were stained with Block-iT Alexa Flour red and visualised using the Evos fluorescent light microscope (Life Technologies, USA). Micrographs were taken 24hrs after transfection. The white arrow indicates fluorescent cells, suggesting transfection occurred.

4.12 qRT-PCR analysis of siRNA mediated knockdown of Prkaa2 gene

To investigate the effects of compound b´ on C2C12 cells were the main catalytic isoform (α2) of AMPK is knocked out. The cells were treated in the following groups: Control, TZD alone, Metformin alone, TZD+Prkaa2 siRNA, Metformin with siRNA and PRKAA2 siRNA. The results indicate that AMPK (PRKAA2) was silenced in TZD+PRKAA2, MET+PRKAA2 and PRKAA2 alone. But PRKAA2 was not silenced nor was the expression inhibited in the control group, the TZD alone group has almost a two-fold increase and expression of PRKAA2.

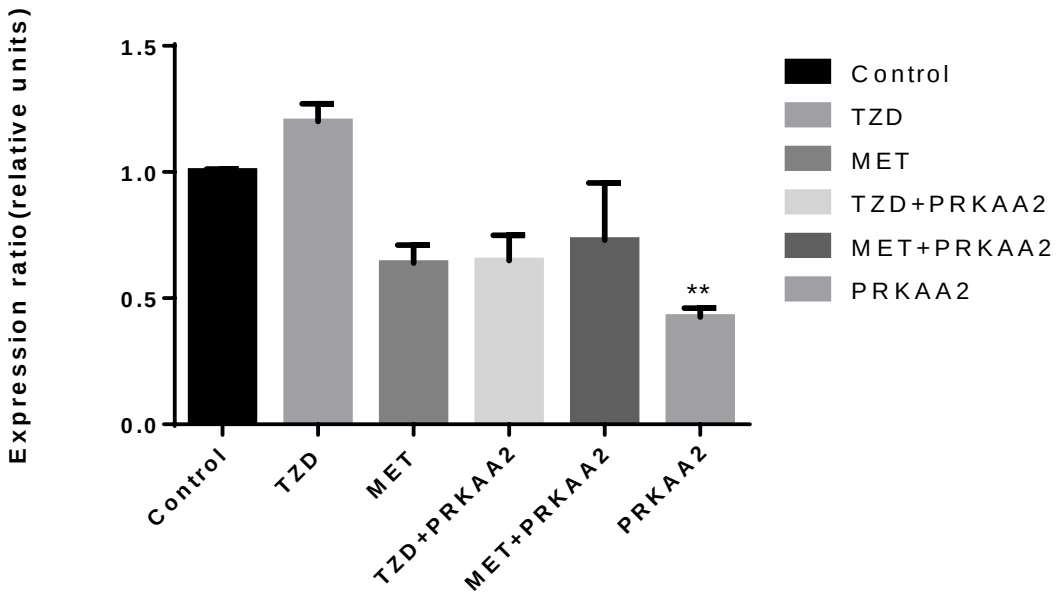


Figure 4-13: Expression of AMPK after silencing AMPKα2 and treatment with Compound b´. Differentiated C2C12 cells were transfected with AMPK siRNA(Prkaa2) for 24 h or 48h. Quantitative real-time PCR was used to measure the gene transcription levels of AMPK by the C2C12 cells that had been treated with AMPK siRNA. Results were presented as means ± SD. Level of significance was accepted at (**P<0.01 control vs TZD, **P<0.01 control vs TZD+Prkaa2, and **P<0.01 control vs metformin+Prkaa2 respectively).

4.13 Glut 4, NRF-1 and MEF2A are downregulated when AMPK is silenced

To corroborate the role of AMPK in glucose transport investigations were made on the effect it has on genes involved in glucose transport i.e. Glut4, NRF-1, and MEF2A. Firstly, differentiated C2C12 myotubes were treated with 200µM of silencer select siRNA and Lipofectamine 2000 was used as a vehicle to silence AMPK expression, followed by 5µL compound b´ (10mg/mL). The expression of AMPK was significantly decreased by culturing the transfected cells at 37ºC for 24-48h. Figures 4-14 to 4-16 illustrate the various levels of expression.

Figure 4-14 indicates that there was significant silencing of GLUT-4 expression after siRNA PRKAA2. The hybrid compound could not up-regulate GLUT-4 expression, i.e.TZD+PRKAA2 had the least expression of GLUT-4 than MET+PRKAA2. In the Groups that were not treated, TZD and MET the expression of GLUT-4 was higher but not more than the control group.

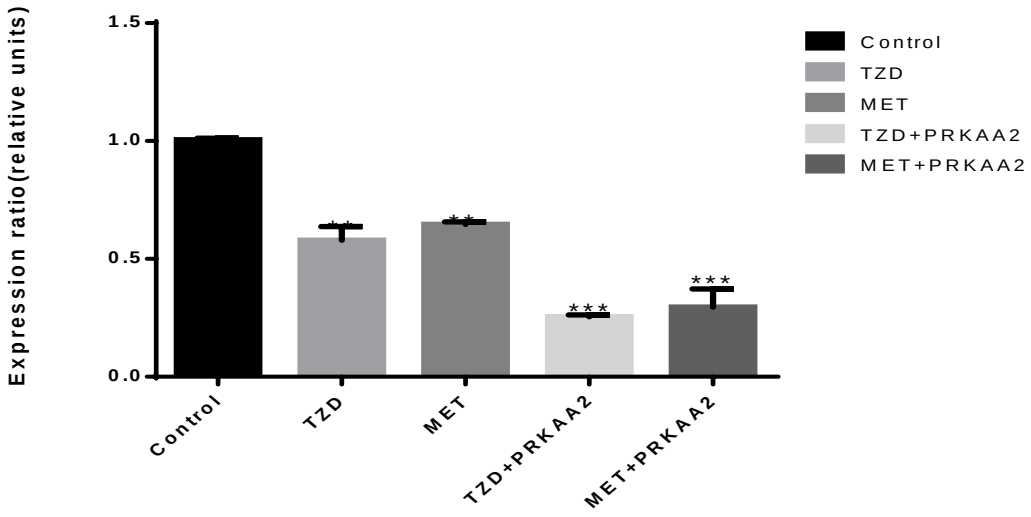


Figure 4-14: Expression of GLUT-4 gene after silencing AMPK and treatment with Compound b´. Differentiated C2C12 cells were transfected with AMPK siRNA(Prkaa2) for 24 h or 48h. Quantitative real-time PCR was used to determine the gene expression levels of AMPK by the C2C12 cells that had been treated with AMPK siRNA. Results were presented as means ± SD. Level of significance was accepted at (**P<0.01 control vs TZD, **P<0.01 control vs metformin, ***P<0.001 control vs TZD+Prkaa2 and ***P<0.001 control vs metformin+Prkaa2 respectively.

To investigate if compound b´ can express MEF2A gene when AMPKα2 has been silenced by siRNA (PRKAA2). Figure 4-15 illustrates that the MEF2A expression was reduced in the TZD+PRKAA2 group and MET+PRKAA2 group but was up-regulated in the TZD alone and MET alone group by ~100-fold. These results further reinforce that MEF2A gene expression was reduced after AMPKα2 was silenced, indicating that MEF2A possibly operates via an AMPK dependant pathway. There was no significant change in the control group since AMPKα2 was not knocked out.

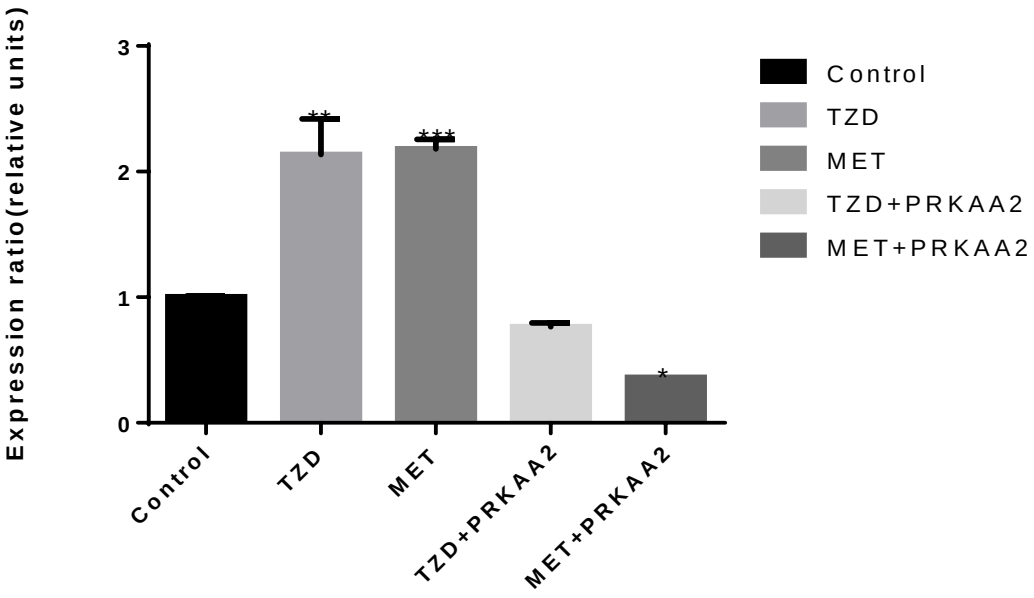


Figure 4-15: Expression of MEF2A after silencing AMPKα2 and treatment with Compound b´. Differentiated C2C12 cells were transfected with AMPK siRNA(Prkaa2) for 24 h or 48h. Quantitative real-time PCR was used to determine the gene expression levels of AMPK by the C2C12 cells that had been treated with AMPK siRNA. Results were presented as means ± SD. Level of significance was accepted at (**P<0.05 control vs TZD, ***P<0.001 control vs metformin and *P<0.05 respectively).

The results in Figure 4-16, illustrate that NRF-1 expression was silenced when AMPK was silenced using siRNA i.e. PRKAA2+MET and PRKAA2. While the TZD had the highest expression of GLUT-4, in agreement of the previous finding in Section 4.7.

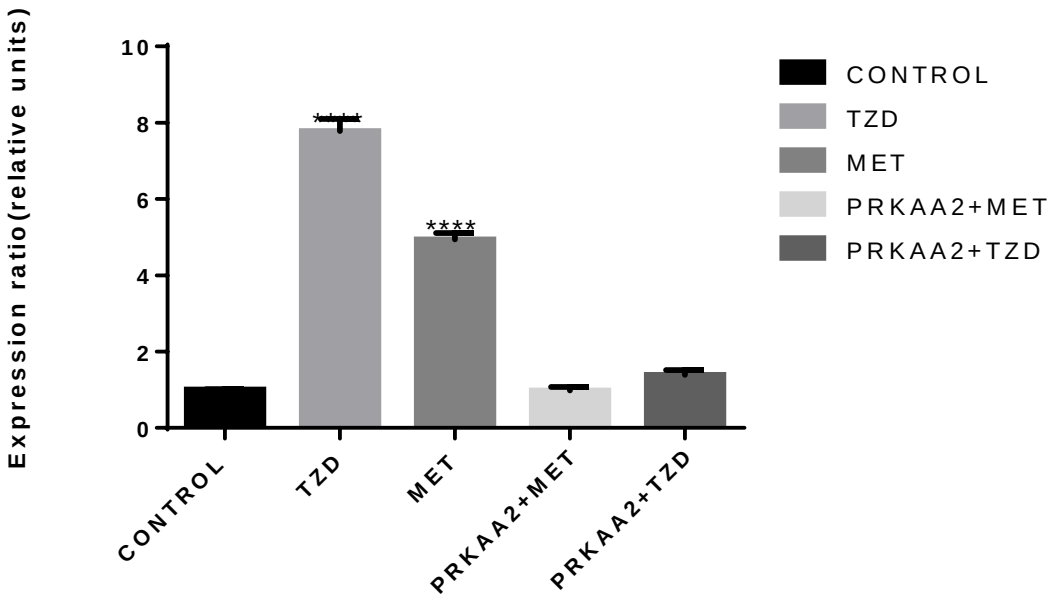


Figure 4-16: Expression of NRF-1 after silencing AMPK*α*2 and treatment with Compound b´. Differentiated C2C12 cells were transfected with AMPK siRNA(PRKAA2) for 24 h or 48h. Quantitative real-time PCR was used to determine the gene expression levels of AMPK by the C2C12 cells that had been treated with AMPK siRNA. Results were presented as means ± SD. Level of significance was accepted at (****P≥0.05, control vs TZD, ****P≥0.05 control vs metformin, respectively).

4.13.1 Assessment of Ferric reducing antioxidant power assay (FRAP) on palmitate-induced insulin resistant C2C12 muscle cells and treated with compound b´.

In this study, the hybrid (compound b´) showed FRAP potential ability through its reducing capacity (Figure 4-17), as was evident in the conversion of Fe^{3+} to Fe^{2+} resulting in the formation of a blue colour. Results in the TZD only group had the highest increase followed by the PA+MET treatment. Finally, the PA+TZD had also an increase without much statistical difference indicating that the drug was potent in rescuing the cells under stress of induced insulin resistance by PA. The PA group had the lowest ratio as there was no drug to cushion the effects of free radicals.

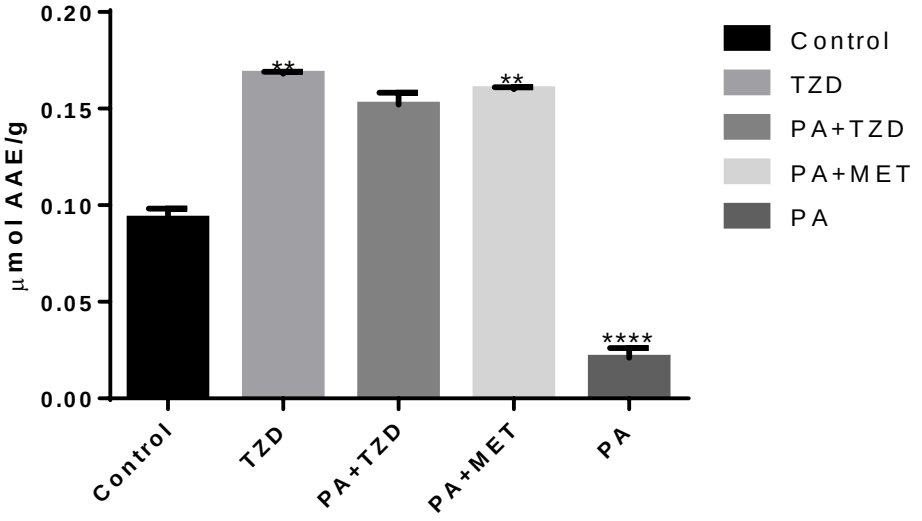


Figure 4-17: Ferric reducing antioxidant power assay (FRAP). The graph shows the response of the treatment groups to the effects. The percentage ± SD is expressed relative to the control at 100% statistical analysis ANOVA with Dunnet's post hoc test; (****P<0.0001 control vs Palmitate, **P<0.01 control vs TZD, and ****P<0.0001 control vs palmitate + metformin respectively).

4.13.2 Assessment of the Trolox equivalent antioxidant capacity (TEAC) on palmitate-induced insulin resistant C2C12 muscle cells and treated with compound b´

This experiment the results show that the hybrid (compound b´) had ABTS radical scavenging properties as is shown in Figure 4-18, i.e. control vs TZD P≤0.01 and Control vs PA+TZD. The highest fold increase was observed in the PA+TZD treatment group. This increase could be attributed to the ability of the hybrid to scavenge free radicals.

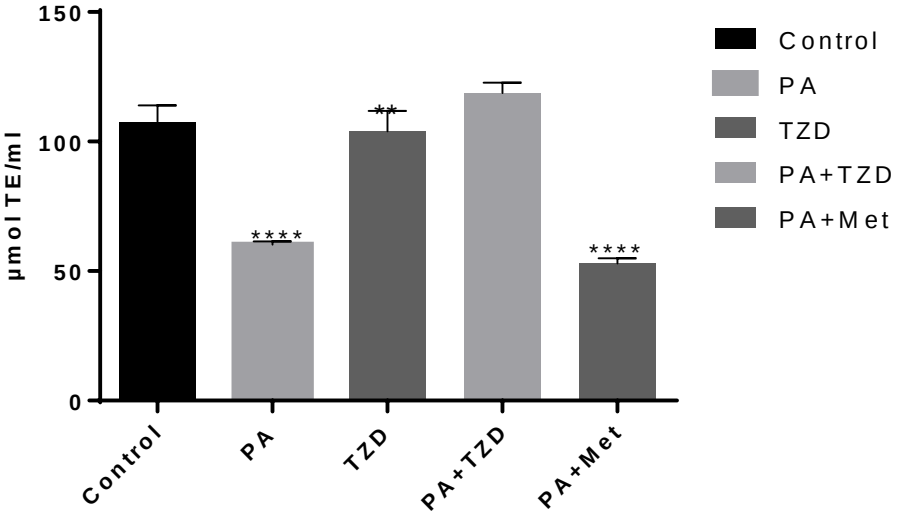


Figure 4-18: TEAC assay. ABTS radical scavenging activity of the treatment groups. The percentage ± SD is expressed relative to the control at 100% statistical analysis ANOVA with Dunnet's post hoc test; (****P<0.0001 control vs Palmitate, **P<0.01 control vs TZD, and ****P<0.0001 control vs palmitate + metformin respectively).

CHAPTER 5

5.0 DISCUSSION

AMP-activated protein kinase (AMPK) is a cellular energy sensor activated by metabolic stresses that either inhibit ATP synthesis or accelerate ATP consumption (Fogarty and Hardie, 2010b). It is for these reasons that we sought a drug with two or more drug pharmacophores in one molecule with the intention to exert multi-drug action (Morphy *et al.*, 2004; Kinfe and Belay, 2013) that a can activate AMPK resulting in a positive effect on T2D.

In this study, a novel hybrid compound derived from thiosemicarbazone joined to a triazole was used, generating a thiosemicarbazone-triazole hybrid with the general structure shown in Section 2.2.7. The C2C12 mouse muscle cells where used because they closely mimic the normal physiological function of normal muscle to investigate the effects of the thiosemicarbazone-triazole hybrid. Skeletal muscle accounts for 70-80% peripheral insulin-stimulated glucose disposal from circulation (Abdul-Ghani and DeFronzo, 2010). Confluent cells were cultured and differentiated into myotubes as previously described in Chapter 2; Section 3.2.3. Myotube formation was necessary since at the molecular level it is associated with upregulation of GLUT-4 mRNA transcription/expression by 4-5-fold and insulin-stimulated glucose disposal (Hommelberg *et al.*, 2010; Richardson and Pessin, 1993).

AMPK, gene expression was investigated under two different conditions. Firstly, analysis of the effect it had on palmitate induced insulin-resistant C2C12 muscle cells and secondly, in C2C12 muscle cells where AMPK was silenced via siRNA (PRKAA2). Both groups were treated with hybrid compound while metformin was used a as a positive control.

The results obtained in Figure 4.4 indicate that AMPK gene was highly expressed in the treatment groups i.e. TZD alone, PA+MET, and PA+TZD where AMPK gene was not silenced in relation to control and metformin. The hybrid compound had a 5-fold turn over than the groups (control and PA), indicating that the methoxy group added stimulated the expression of AMPK. The hybrid compound even had a high expression of AMPK even in cells that had insulin resistance brought about by palmitate (PA+MET and PA+TZD).

In the second scenario (Figure 4-13), where AMPK expression was silenced by predesigned siRNA (PRKAA2), Gene expression of AMPK, was down-regulated in the PRKAA2 alone group when compared to the control. This was expected since AMPK (PRKAA2 gene function was knocked down). The groups with the combination of TZD+PRKAA2 and MET+PRKAA2 had the highest expression of AMPK(PRKAA2) indicating that the hybrid compound b´ might be working

through the AMPK-dependent pathway since it was able to up-regulate the expression of PRKAA2 in C2C12 with knocked out activity of AMPKα2 similar to results observed by Shen and colleagues (Shen *et al.*, 2014).

Similarly, the MEF2A gene, a transcriptional regulator of GLUT-4 in the glucose transport system, was also investigated under two different conditions firstly, analysis of the effect it has on palmitate induced insulin-resistance (Figure 4.6) and secondly, in C2C12 cells where AMPK was silenced via siRNA (PRKAA2) Figure 4-14. The MEF2A gene expression in palmitate-induced insulin-resistance cells showed a slight increase compared to the standard drug metformin + palmitate. There was one and half increase in the TZD alone group when compared to the control group where there was no treatment. The Palmitate alone group expressed low levels of MEF2A consistent with similar results found by Huang and colleagues (Huang *et al.*, 2014). The test indicated that the addition of a methoxy group (electron-donating group attached to the aromatic ring) to the hybrid stimulated high expression of MEF2A relative to metformin (Ayeleso *et al.*, 2017). On the other hand, in the cells where AMPK expression was silenced by siRNA, the expression of MEF2A was downregulated as shown in Figure 4-15, suggesting that MEF2A is vital for the activation of AMPK which is involved in genes that control glucose transport.

Evaluation of the expression of GLUT-4 gene (a key player in glucose transport) (Charron *et al.*, 1999) was conducted since T2D is characterized by decreased glucose transport and metabolism in muscles and adipocytes (Shepherd and Kahn 1999). GLUT-4 gene is regulated by myocyte enhancing (MEFA2) transcription factor which binds to its cis-elements as a hetero-dimer (*MEF2A*/D) resulting in GLUT-4 expression (M Oshel *et al.*, 2000; Ayeleso *et al.*, 2017). Studies have shown that treatment of insulin-resistant rodents with thiazolidinediones restores the expression and translocation of GLUT-4 in adipocytes (Hulin *et al.*, 1996; Shepherd and Kahn 1999). Investigation into the expression of Glut-4 gene after treatment with the hybrid compound b´ and expression of GLUT-4 was also evaluated after silencing AMPK in C2C12 myotubes using qRT-PCR. The result in Figure 4.7 indicates that the hybrid compound b´ increased Glut-4 expression in relation to that of the standard drug (metformin) used to treat T2D and known to induce Glut-4 expression. The TZD alone group had the highest expression of GLUT-4, followed by the PA+TZD and PA+MET. The PA group had the lowest expression of GLUT-4. The experimental group that had AMPK silenced equally showed a marked down-regulation of MEF2A gene (Figure 4.6). Compound b´ had the lowest expression of GLUT-4 when compared to the control (not treated) and when compared to the standard drug metformin indicating that, when AMPK is silenced the expression of GLUT-4 is equally reduced.

NRF-1 a mitochondrial transcription factor, which controls MEF2A gene to regulate GLUT-4 (Ramachandran *et al.*, 2008) was also investigated, where AMPK was silenced via siRNA. The results in Figure 4-16 show that NRF-1 expression was down-regulated after AMPK was silenced, when compared to control. The addition of a methoxyl group to the hybrid with a non-polar short straight alkyl chain of the amine moiety group (Ayeleso *et al.*, 2017; Kinfe and Belay, 2013) did not stimulate the expression of NRF-1 as was observed in experiments conducted by Ayeleso and colleagues (2017). However, the cells where AMPK was not silenced showed a high expression of NRF-1 much higher than the stardard drug of choice metformin in the treatment of T2D.

PPARα is one of the many transcriptional genes that AMPK modulates and is involved in controlling the expression of metabolic and mitochondrial genes (McGee *et al.*, 2008a). PPARα also plays a critical role in lipid homeostasis (oxidation and production) (Sugden *et al.*, 2009). The PPARs synthetic ligands are thiazolidinediones and cause insulin-sensitization (Hevener *et al.*, 2001) through a mechanism that is not clearly understood (Lessard *et al.*, 2004). Analysis of the expression of PPARα after treatment (Figure 4.8) with hybrid compound b´ of whether it is up-regulated or down-regulated reveal that the test drug (hybrid compound b´) i.e. PA+TZD nor the control drug metformin PA+MET did not have any significant increase on the expression of PPARα as compared to control (without treatment). The hybrid compound b´ alone had the highest expression of PPARα. This is because the cells had not been under any other kind of stress. However, this came as a surprise as anticipation was made that the hybrid compound might up-regulate the transcription of PPARα to help alleviate the symptoms of T2D.

The hybrid compound b´ was further investigated if it can induce the expression of the LKB1 gene. LKB1 codes for a kinase that directly phosphorylates and activates AMPK and is located upstream the gene loci of AMPK. LKB1 gene product is part of 12 other related kinase that control vital roles in cell growth, metabolism and polarity (Shackelford and Shaw, 2009). The result in Figure 4.5 indicates that the combination of the hybrid drug with palmitate-induced insulin resistance (PA+TZD) had a slightly elevated level of LKB1 than the metformin + palmitate (PA+MET). The hybrid drug alone, as expected, had a higher stimulation of LKB1 and hence only came in second when compared to control (untreated normal healthy cells). The findings correlate with the study conducted by Kinfe and colleagues (Kinfe *et al.*, 2013), where they demonstrated that thiosemicarbazone-triazole hybrids have an influence on genes (CPT-1, ACC-1, and PGC-1) implicated in obesity and subsequently in type two diabetes.

After demonstrating using mRNA that genes involved in transcription and metabolism are up-regulated by compound b´ in palmitate induced insulin-resistant C2C12 myotubes, demonstration was made using the western blotting technique to assess protein level expression that treatment

with compound b´ increased protein expression of the palmitate-induced insulin-resistance. This was expected as numerous studies have confirmed that Palmitate has no effect on protein expression as was demonstrated by (Feng *et al.*, 2012), where they proved that insulin stimulated glucose uptake and reduced the insulin stimulated phosphorylation of Akt at Thr308 and Ser473, but had no effect on protein expression.

Overexpression of NRF-1 has been shown to increase expression of both MEF2A and GLUT-4 (Baar *et al.*, 2003; Joseph *et al.*, 2017; Mukwevho, 2010). ChIP assay was performed to determine the amount of NRF-1 bound to MEF2A in the region of the NRF-1 binding site on MEF2A gene. Figure 4.8 shows the band intensity of both the IP and IN samples as viewed under the agarose gel. The result could indicate that there was a slight increase in the binding of NRF-1→MEF2A, although the band intensity is more pronounced in the control group as indicated in Figure 4.8. While there is less band intensity in TZD and PA+MET in both the IP and IN, it may be sufficient to say that immunoprecipitated samples (IP) contain specific antibody for MEF2A while for the input samples (IN) there was less binding probably due to no-specific binding. Another reason could be that the cells had died or there was less activation of factors such as CaMKII which are normally activated when the cell is under stress (Joseph *et al.*, 2017).

To confirm the effect on glucose utilization on C2C12 cells with palmitate induced insulin-resistance, analysis was made using the glucose consumption as is shown in Figure 4.2. The low level of Glucose utilized can be used to confirm that palmitate induced insulin resistance in C2C12 cells compared to the normal control cells. This results further reinforces the understanding that insulin resistance inhibits glucose uptake in skeletal muscle (Ding *et al.*, 2016) and that the thiosemicarbazone-triazole hybrid (compound b´) was able to reverse or protect palmitate induced insulin-resistance.

To confirm the antioxidant capabilities of the hybrid compound two established assays were used, viz FRAP and TEAC. FRAP assay is a new method of assessing antioxidant where ferric reducing ability is analysed. The results obtained in our experiment as is shown in Figure 4-17 and Figure 4-18, indicate that the compound had ABTS radical scavenging activities. The hybrid was able to reduce Fe^{3+}-TPTZ complex to produce a blue colour Fe^{2+}-TPTZ. These results can be interpreted that the hybrid has the potency to mitigate against oxidative damage in the body. Furthermore, these results can be compared to findings of Ayeleso and colleagues where they tested all the newly synthesized thiosemicarbazone-triazole hybrid (1a-h) compounds of antioxidants and found that the higher the FRAP value, the greater the antioxidant effects. The change in absorbance is directly related to the total reducing power of the electron-donating antioxidants available in a sample (Ayeleso *et al.*, 2017).

CHAPTER 6

6.1 Conclusion

In conclusion, the newly synthesized thiosemicarbazone-triazole hybrid (hybrid compound b´) was evaluated for its effects on adenosine monophosphate-activated protein kinase (AMPK) and genes (AMPK, MEF2A, GLUT-4, LKB1, PPARα) of interest to type 2 diabetes. The results obtained show an overall increase in transcription of glucose and mitochondrial lipid metabolising genes. The addition of a methoxy group to the hybrid with a non-polar short straight alkyl chain of the amine moiety might be important in the observed up-regulation of NRF-1, GLUT-4 and MEF2A genes. The evaluation of antioxidant properties of the newly synthesized thiosemicarbazone-triazole hybrid on C2C12 muscle cells with palmitate induced insulin resistance and the findings confirmed that it has very potent ability to reduce oxidative stress. This hybrid compound could play a vital role in diseases that have free radical onset in that its antioxidant capacity is able to alleviate oxidative stress in diseases such as diabetes mellitus and cancer.

Demonstration was made that after silencing AMPK, the genes viz GLUT-4, NRF-1, and MEF2A were down-regulated leading to a speculation that the newly synthesized drug would be functioning through the AMPK pathway (AMPK dependent mechanism). But this needs further exhaustive experiments/studies to certainly confirm the speculation.

6.2 Recommended further experiments

It would be interesting to evaluate the treatment groups described in this dissertation for determination of 2-deoxy-[^{3}H]-D-glucose uptake to have a holistic view of glucose transport pathway.

Further studies are needed to evaluate the interactions of the thiosemicarbazone-triazole hybrid (compound b´) with free radicals and the mechanism through which it exerts its antioxidant activity.

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6.3 Appendix 1

Table 6-1. List of reagents used.

PRODUCT AND DESCRIPTION	CATALOGUE NUNBER	SUPPLIER & COUNTRY OF ORIGIN
Agarose 100g	IBENA1701	Sigma-Aldrich, USA
Petri dish TC 60x15mm	PGRE62860	LASEC Laboratories, South Africa.
Coverslips mic no.1 rec 24x60mm	GLAS2C29M2460REC	LASEC Laboratories, South Africa.
Slide mic 1st PLN G/E 1 1.2mm	GLAS4S32M3000P	LASEC Laboratories, South Africa.
DMEM high medium, W/I-glut	D5796-1L	Sigma-Aldrich, USA.
Coomassie (r) brilliant blue G250.	27815-25G-F	Sigma-Aldrich, USA.
Bovine Serum Albumin Fraction V, 50g.	10735078001	Roche diagnostics GmbH, Germany.
Glycine	50046-1kg	Sigma-Aldrich, St louis MO, USA.
Okadaic acid ammonium	08010-50UG	Sigma-Aldrich, St louis MO, USA
Proteinase K	P2308-10mg	Sigma-Aldrich, St louis MO, USA
Glycogen type II from oyster	G7851-25G	Sigma-Aldrich, St louis MO, USA

Tergitol Type NP-40 70%	NP40S-100mL	Sigma-Aldrich, St louis MO, USA
Glucose oxidase activity assay kit	MAK097-1KT	Sigma-Aldrich, USA.
Protein-A-agarose	P2545-1mL	Sigma-Aldrich, USA.
Cell proliferation kit (MTT)	11465007001	Sigma-Aldrich, USA.
Sodium dodecyl sulfate(SDS)	L3771-100g	Sigma-Aldrich, USA.
PureLink RNA mini Kit	12183018A	ThermoFisher Scientific, USA
Gibco Fetal Bovine Serum (FBS) gamma irradiated	10499044	ThermoFisher Scientific, USA.
Lipofectamine 2000 reagent.	11668019	ThermoFisher Scientific, USA.
Opti-MEM reduced serum Medium.	31985047	ThermoFisher Scientific, USA.
Acrylamide electrophoresis	A3553-500G	ThermoFisher Scientific, USA
N, N' Methylenebisacrylamide 99%	146072-500G	ThermoFisher Scientific, USA
Methanol	32213-2.5L	Sigma-Aldrich, Germany.
Chloroform 99%	288306-2L	Sigma-Aldrich, St louis MO, USA.
Glycerol 99%	G6279-500mL	Sigma-Aldrich, Malaysia

Sodium palmitate 98%	P9767-50G	Sigma-Aldrich, St louis MO, USA
PowerUp SYBR Green Master Mix	A25742	Applied Biosystems by Life Technologies, USA.
SuperScript Vilo cDNA Synthesis Kit	11754-050	ThermoFisher Scientific, USA.
ATP determination kit	A22066	Invitrogen by ThermoFisher Scientific
Isopropanol 70% in H_2O	563935-1L	Sigma-Aldrich, USA.
Micro AMP optical 96well plate		Applied Biosystems, USA.
Nuclease free water	E476-100ML	AMRESCO, USA.
Penicillin & Streptomycin 100X	L0022-100	Biowest, France.
30% Acrylamide/Bis solution 29:1.1	1610156	Bio-Rad, USA
4x Laemmli sample buffer 30ml, premixed	1610737	Bio-Rad, USA
AMPK alpha-2 polyclonal antibody	PA519367	ThermoFisherScientifi, USA.
Salmon sperm DNA solution	15632011	Invitrogen by life Technologies, Carlsbad.
Blockit Alexa Flour Red Oligo	14750100	Life Technologies, USA.
Taqman gene expression assay	W01826846	Applied Biosystems, USA.

Cells-to-CT 1-step taqman	A25605	Life technologies, Lithuania
N,N,N',N' tetramethylethylenediamine (TEMED)	T9281-100ML	Sigma-Aldrich, USA.

6.4 Appendix 2

Table 6-2:Equipment used

Name of instrument	**Company/Origin**
StepOnePlus™ Real time PCR System	Applied Biosystems, USA.
T100™ Thermal cycler	Bio-Rad, USA.
Multiskan™GO Microplate Spectrophotometer	ThermoFisher Scientific, USA.
Orbit™ 1000 Digital Shaker from	Labnet International, USA.
Thermo Scientific™ SL 8 and 8R Centrifuge	ThermoFisher Scientific, USA.
ChemiDoc™ MP imaging system	Bio-Rad, USA.
EVOS FL digital Microscope	Life Technologies, USA
Turbo-Blot®Turbo Transfer System	Bio-Rad, USA
TC20™ Automated Cell Counter	Bio-Rad USA
NANODROP LITE Spectrophotometer	Thermo Scientific, USA.

General buffers and Preparation of reagents

Palmitate: To prepare a 75mM stock solution, 0.209g palmitate was dissolved in 10ml absolute ethanol (96%) heated to 90°C. From the stock, a 1:100 dilution was done to yield a 0.75mM working solution i.e. 2% BSA in DMEM with 100µL Palmitate for 10cm culture plate

Potent lysis Buffer / RIPA: 56mM Tris -HCl pH 7.4 at 4°C, 20mM NaF, 1% (v/v) Triton X-100, 10mM Na4P2O7, 4mM Na3VO4, 1mM EDTA, 150mM NaCl, 25x Roche complete inhibitor(RCPI), 0.15µM Okadaic acid, 0.1% Sodium dodecyl sulphate(SDS)

Tris-Buffered Saline: Tween-20 (TBST): 20mM Tris-HCl pH 7.4, 137mM NaCl, 0.1% (v/v) Tween-20

Transfer Buffer: 25mM Tris, 190mM glycine, 20% (v/v) methanol

Electrode buffer: 0.25M Tris base, 1.28M glycine, 1% (w/v) SDS

ChIp dilution buffer: 0.01% SDS,1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, 167mM NaCl

Elution buffer: 1% SDS, 0.1M NaHCO3

Lower salt wash buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl and 500mM NaCl.

LiCl buffer: 0.25%M, 1% NP-40, 1mM EDTA, 10mM Tris-HCl, 1% Deoxycholic acid-Na.

Antibodies

AMPK*α* antibody: catalogue number 2532, is a rabbit polyclonal antibody. It detects endogenous levels of AMPKα. It detects both α1 and α2 isoforms of the catalytic subunit (diluted 1:1000 in 5% w/v BSA, incubated at 4°C overnight). Species reactivity: Human, Mouse, Hamster, and Monkey. Molecular weight is 62kDa.

Anti-mouse IgG, HRP-linked antibody: is an affinity purified horse anti-mouse IgG (heavy and light chain) antibody conjugated to horseradish peroxidase (HRP) for chemiluminescent detection. Diluted in 1:5000 with TBS-T for 1 hour described previously in 3.3.5