Adenosine monophosphate-activated protein kinase as a potential target for synthetic chalco-naringenin analogs and putative therapeutic applications

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AMPK as a potential target for synthetic chalco-naringenin analogs and putative therapeutic applications

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As the candidate's supervisor, I have approved this dissertation for submission
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DECLARATION

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PREFACE

The study represents the original work of the author and has not been submitted to another university in any other form. This study has been submitted in fulfilment of the academic requirements for obtaining a Master's Degree in Pharmacy (Pharmacology).The use of information from other sources in this dissertation has been duly acknowledged in the text and reference section.

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Ntsoaki 'Nyane

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2. Christine Driver, Julia Achieeng Hayangah, Ntsoaki Annah ‘Nyane, Peter Mark Oroma Owira. Metformin with insulin relieves oxidative stress and confers renoprotective in type 1 diabetes in vivo: *Journal of Nephropathology*. (doi: 10.25171/jnp.2017.xx)
3. Christine Driver, Kayode Bamitale, Mehnaaz Olla, Ntsoaki Nyane, Peter Owira. Metformin: The magic bullet in Cardiovascular Disease Complications? Updates on current experimental evidence: *Journal of Clinical and Experimental Pharmacology and Physiology.*CEPP-17-0395.

CONFERENCE ABSTRACTS

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1. UKZN College of Health Sciences research symposium (September 2016), Durban, South Africa, (Awarded first-prize *R*30, 000.00 International Travel Voucher) (AMPK as a potential target for synthetic chalco-naringenin analogs and putative therapeutic applications).
2. International Diabetes Federation's Annual Congress 4th - 8th December 2017 Abu Dhabi, UAE, (AMPK as a potential target for synthetic chalco-naringenin analogs and putative therapeutic applications).

LIST OF ABBREVIATIONS

AACE	American Association of Clinical Endocrinologists
ACAT	Acyl-CoA: cholesterol acyltransferase
ACC	Acetyl-CoA Carboxylase
ACEIs	Angiotensin-Converting Enzyme Inhibitors
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AGEs	Advanced Glycation End-products
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate –activated protein kinase
ANOVA	One-Way Analysis of Variance
ARBs	Angiotensin Receptor Blockers
ATP	Adenosine Triphosphate
CAD	Coronary Artery Disease
CaMKKβ	Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase beta
cAMP	Cyclic Adenosine Monophosphate
CBS	Cystathionine β-Synthase
CCM	Cell Culture Medium
CREB	cAMP Response Element-Binding protein
DAG	Diacyl Glycerol
DCM	Diabetic Cardiomyopathy
DKA	Diabetic Ketoacidosis
DKD	Diabetic Kidney Disease
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DN	Diabetic Nephropathy
DPP-4	Dipeptidyl Peptidase 4
DR	Diabetic Retinopathy
DSP	Distal Symmetric Polyneuropathy
EASD	European Association for the Study of Diabetes
ECACC	European Collection of Cell Cultures
EGCG	Epigallocatechin Gallate
eGFR	estimated Glomerular Filtration Rate

ELISA	Enzyme Linked-Immuno-Sorbent Assay
ER	Endoplasmic Reticulum
ESRD	End-Stage Renal Disease
ET-1	Endothelin-1
F-6-P	Fructose-6-Phosphate
FBG	Fasting Blood Glucose
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFA	Free Fatty Acids
FOXO1	Fork Head Box Protein O1
FSH	Follicle Stimulating Hormone
FT-IR	Fourier Transform Infrared spectroscopy
G-6-P	Glucose-6-Phosphate
G6Pase	Glucose 6-phosphatase
GAD	Glutamic Acid Decarboxylase
GBD	Glycogen Binding Domains
GDM	Gestational Diabetes Mellitus
GFAT	Fructose-6 Phosphate Aminotransferase
GFR	Glomerular Filtration Rate
GLP1	Glucagon-Like Peptide 1
GLUT-2	Glucose Transporter Type 2
GLUT4	Glucose Transporter Type 4
GTIs	Genital Tract Infections
HbA1c	Glycated Hemoglobin
HDL-c	High Density Lipoprotein Cholesterol
HHS	Hyperglycemic Hyperosmolar State
HLA	Human histocompatibility
HMG-CoA reductase	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A reductase
HNF	Hepatocyte Nuclear Transcription
HO-1	Heme Oxygenase-1
ICA	Islet Cell Antibody
ICAM-1	Intercellular Adhesion molecule-1
IDF	International Diabetes Federation
IFN-γ)	Interferon gamma

IGF-1	Insulin Growth Factor-1
IL-2	Interleukin-2
IPF	Insulin Promoter Factor
IR	Insulin Resistance
IRS-1	Insulin Receptor Substrate-1
IRS-2	Insulin Receptor Substrate-2
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LKB1	Liver Kinase B1
LVH	Left Ventricular Hypertrophy
MALA	Metformin-Associated Lactic Acidosis
MI	Myocardial Infarction
MMV	Molegro Molecular Viewer
MODY	Maturity Onset Diabetes of the Young
MPTP	Mitochondrial Permeability Transition Pore
mTOR	Mammalian Target of Rapamycin
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltretrazolium bromide
MTTP	Microsomal Triglyceride Transfer Protein
NAD+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NPH	Neutral Protamine Hagedorn
Nrf2	Nuclear Factor erythroid 2-Related factor 2
OCT1	Organic Cation Transporter 1
OGTT	Oral Glucose Tolerance Test
PAI-1	Plasminogen Activator Inhibitor-1
PCOS	Polycystic Ovarian Syndrome
PDGF	Platelet-Derived Growth Factor
PEPCK	Phosphoenol pyruvate carboxykinase
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1-alpha
PI3-k	Phosphoinositide -3- kinase

PI3K/PKB/Akt	Phosphatidylinositol- 3-kinase/protein kinase B/akt
PKA	Protein Kinase A
PKC	Protein Kinase C
PP2C	Protein Phosphatase 2C
PPAR δ	Peroxisome Proliferator-activated Receptor δ
RAAS	Renin-Angiotensin-Aldosterone System
RAGE	Receptor for Advanced Glycation End products
RCC1	Respiratory Chain Complex 1
RISK	Reperfusion Injury Salvage Kinase
RLU	Relative Light Units
RNA	Ribonucleic Nucleic Acids
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
SGLT2	Sodium Glucose co-Transporter 2
SH2	Src Homology 2
SREBP1	Sterol Regulatory Element-Binding Protein-1
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TG	Triglycerides
TGF-1	Transforming Growth factor-1
TGF-β	Transforming Growth Factor-β
Thr	Threonine
TLC	Thin Layer Chromatography
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
TNF-β	Tumor Necrosis Factor beta
TORC2	Transducer of Regulated CREB activity 2
UDP	Uridine Diphosphate
UKPDS	United Kingdom Prospective Diabetes Study
UTIs	Urinary Tract Infections
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoproteins
VSMC	Vascular Smooth Muscle Cells

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ABSTRACT

Introduction: Diabetes mellitus is a multifactorial chronic metabolic disorder that is characterized by defects in endogenous insulin secretion or action, or both, resulting in chronic hyperglycemia, a clinical hallmark of diabetes. Metformin is currently the first-line drug of choice for the treatment of type 2 diabetes (T2D), being prescribed to at least 120 million people worldwide. It exerts its antidiabetic effects by reducing hepatic glucose production and increased peripheral glucose utilization through activation of AMP-activated protein kinase (AMPK). However, despite significant gains with metformin as a monotherapy in T2D, some patients experience gastrointestinal disturbances and lactic acidosis although the latter is very rare. Moreover, some patients still fail to achieve optimum glycemic control when treated on metformin only. Naringenin, a flavonoid exerts its antidiabetic effects by inhibition of gluconeogenesis through upregulation of AMPK, hence metformin-like effects. Because of these similar pharmacological effects between naringenin and metformin, our laboratory synthesized analogs of chalco-naringenin compounds which could be more permeable to the plasma membrane and hence putatively increased pharmacological effects.

Aims: To identify AMPK as a potential target of synthetic chalco-naringenin analogs and putative therapeutic applications.

Methods: A novel series of 4-[(cyclopropylcarbonyl)amino] chalco-naringenin analogs, compound 5a to 5k, were synthesized and characterized by IR, ^{1}H-NMR and ^{13}C-NMR. In silico screening of the compounds was conducted to evaluate potential antidiabetic activity of the novel chalco-naringenin series. Compounds 2-chlorophenyl (5b) and 5k (2,3-dimethoxyphenyl) had highest binding affinity to AMPK hence were chosen for a study. C2C12 and Chang cells were cultured in dulbecco's modified eagle medium (DMEM) and eagle's minimum essential medium (EMEM) media, respectively, allowed to grow to 80% confluence, and then exposed to different concentrations.

MTT assay was used to determine cell viability and chalcones were subjected for 12, 24 and 48 hours at concentrations (10-750 µM). Cells were exposed to metformin (2-10 mM), naringenin (50-500 µM) and chalcones (10-500 µM) for 48 hours and further subjected to phospho-AMPKα (Thr172) sandwich ELISA Kit to determine phosphorylation of AMPK. To measure the amount of glycogen in cells after exposure to metformin, naringenin and chalcones for 48 hours, cells were harvested ($1x10^{6}$ per mL) and the glycogen assay performed according to Seifter *et al*. (1950).

Results: A chalcone series of eleven compounds were successfully synthesized using the Claisen-Schmidt reaction. The absorbance values and peaks observed on the IR spectra

confirmed the different functional groups observed on the compounds. The synthesized compounds were also characterized through ^{13}C-NMR and ^{1}H-NMR spectroscopy.

The ^{13}C-NMR spectras indicated the presence of the CH_2 group of the cyclopropylcarbonyl amide and there were certain distinct peaks on the spectras that identify carbon atoms found on the compounds. In ^{1}H-NMR, the chemical shift for all the CH_2 groups on the cycloalkane resonated around δ 1,16 – 0,80 ppm as multiplets, while the other CH multiplet resonated around δ 1,62 – 1,58 ppm. Docking scores of the chalco-naringenin series suggested a good binding affinity of these compounds to AMPK, with compound 5b showing the highest binding affinity to AMPK. Cell viability as determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays were found to be dose-dependent for all compounds and compound 5k exerted reduced cell viability as compared to 5b. Furthermore, compound 5b presented with higher IC_{50} values compared to 5k. The effects of chalcones on AMPK phosphorylation were potentiated by co-treatment with metformin or naringenin. Metformin, naringenin and chalcone 5b significantly reduced synthesis of glycogen as compared to control ($p < 0.05$).

Conclusions: Chalco-naringenin analogs showed potential in expression of AMPK through computational chemistry, however in the *in vitro* model the effects of chalcones on AMPK were potentiated by metformin and naringenin. The chalcones could further be explored for their potential on AMPK activity on primary hepatocytes and/ or *in vivo* studies.

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Chapter One

1.0 Introduction

1.1 Epidemiology of Diabetes Mellitus

Globally, 642 million people are estimated to have diabetes by the year 2040 compared to 415 million people currently living with diabetes according to International Diabetes Federation (IDF) (Fig. 1) [1]. In sub-Saharan Africa, 14.2 million people aged 20-79 had diabetes in 2015 representing a regional prevalence of 2.1-6.7 % and this figure is expected to increase to 34.2 million by 2040 [1]. The African region has the highest proportion of undiagnosed diabetes, with over 66.7% of people with diabetes being unaware that they have the disease. Recent data from the IDF estimate that 7 % of 3.8 million South Africans aged 21-79 years have diabetes [2, 3].

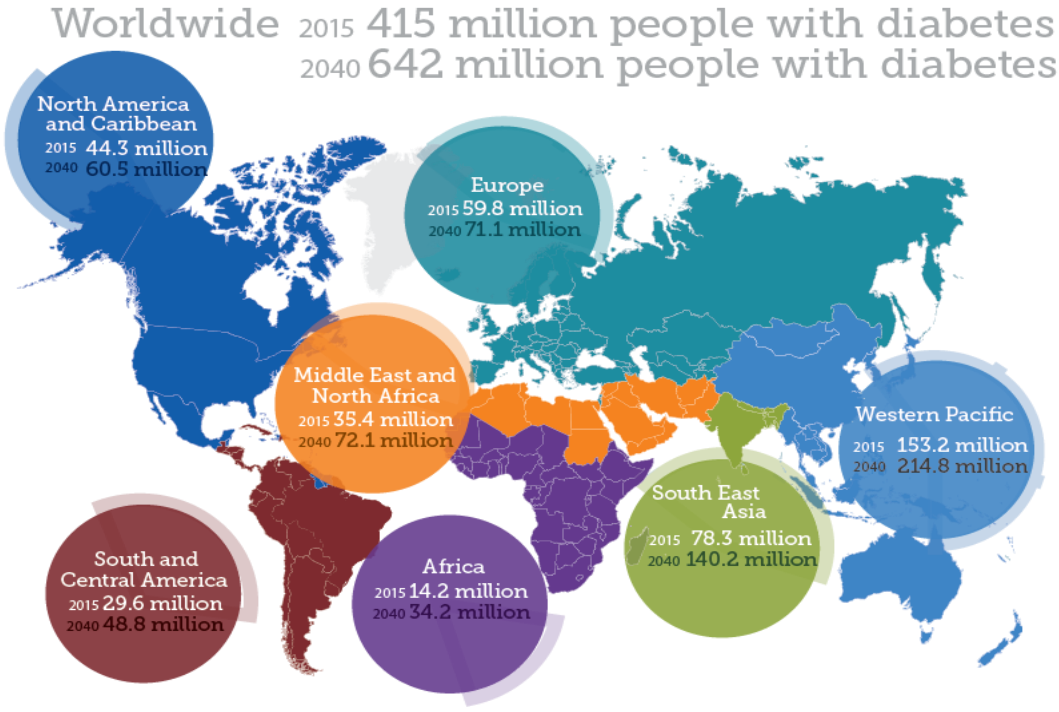


Figure 1: Global burden of diabetes by IDF regions (image adapted from the International Diabetes Federation, Diabetic Atlas 2015) [1].

1.2 **Pathophysiology of Diabetes**

Diabetes mellitus is a heterogeneous group of disorders characterized by persistent hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [4]. The two most common forms of diabetes are Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D) with the latter accounting for 90-95% of all diabetes cases affecting the general population while the former accounts for 5-10% and generally affects children [5, 6]. However, other rare forms of diabetes are Maturity Onset Diabetes of the Young (MODY), Gestational Diabetes Mellitus (GDM) and Idiopathic diabetes [4]. GDM develops during pregnancy causing dysregulation of carbohydrate and lipid metabolism due to acquired insulin resistance resulting in maternal hyperglycemia [7]. MODY is a monogenic form of diabetes that is usually first diagnosed in adolescence or early adulthood characterized by gene mutations that limit the ability of the pancreas to produce insulin [4].

Clinical symptoms associated with diabetes are due to chronic hyperglycemia and corresponding endocrine maladaptations. Glycosuria accompanied by polyuria is common in diabetic patients leading to dehydration that triggers compensatory polydipsia [8, 9]. Diabetes may be diagnosed by glycated hemoglobin (HbA1c) or blood glucose concentrations where the latter is either the Fasting Blood Glucose (FBG), Random Blood Glucose (RBG) or Impaired Glucose Tolerance (IGT) with a 75 g glucose challenge [10].

According to American Diabetes Association (ADA) HbA1c is a marker of chronic glycemia reflecting average blood glucose levels over a 2 to 3-months with the cut-off point of $\geq 6.5\%$ for confirming diabetes as an alternative to FPG ≥ 6.0 mmol/l and RBG of >11.0 mmol/l. Glucose intolerance is defined by failure of blood glucose levels to return to baseline in 2-hours post 75g oral glucose challenge [11, 12]. Patients must have fasted for more than 8 hours before interpretation of OGTT.

The normal blood glucose concentrations range from 3.1 mmol/l – 7.8 mmol/l depending on the fed or fasted state of the person [10, 13].

A minimum concentration of 2.2-3.3 mmol/l is required to provide adequate fuel for the central nervous system. Glucose is its primary energy source and its uptake by the brain is independent of insulin [14]. The glomerulus filters large volumes of urine from the blood stream and glucose is usually reabsorbed in circulation in a multiple step process via the Sodium Glucose co-Transporter 2 (SGLT2) in the proximal convoluted tubule and Glucose Transporter 2 (GLUT-2) transporters at the basolateral membrane back into the blood [15, 16]. When blood glucose

concentrations exceed reabsorptive capacity of the kidney (10 mmol/l), glucose appears in urine resulting in loss of calories and water [17, 18].

Diabetes end-points such as ketoacidosis, neuropathy, retinopathy, nephropathy and cardiovascular diseases contribute to increased morbidity and mortality not only in the developed but also more devastatingly so in developing countries [19]. These end-points are universal to all types of diabetes including T2D, which is the most common in adults but is now increasingly being encountered in adolescents [19].

1.3 **Type 1 diabetes**

Type 1 diabetes (juvenile or insulin-dependent diabetes) is caused by autoimmune destruction of the insulin-producing pancreatic β-cells, following insult by environmental factors, such as viruses or toxins and chemicals [20]. Streptozotocin (STZ) is a glucosamine nitrosourea compound derived from *Streptomyces achromogenes* that was used clinically as a chemotherapeutic agent in the treatment of pancreatic β-cells carcinoma but now used to induce experimental diabetes [21]. It causes permanent diabetes in animal models by damaging pancreatic β-cells through generation of superoxide anions that act on the mitochondria causing an increase in the activity of the enzyme xanthine oxidase causing total or partial destruction of the pancreatic β-cells [22, 23].

The administration of STZ at 60 mg/kg or 45 mg/kg doses has been shown to cause total or partial pancreatic β-cells destruction, respectively [24]. T1D is also closely associated with histocompatibility antigens (Human Leukocyte Antigen (HLA)-DR3 or HLA-DR4) and the presence of circulating insulin antibodies, including Glutamic Acid Decarboxylase (GAD), Islet Cell Antibody (ICA), and islet cell antibody 512 or IA2 (a tyrosine phosphatase antibody) [25, 26]. It was recently documented that antibodies to GAD are sensitive markers for diabetes development but may also be present in genetically susceptible individuals who are unlikely to develop the disease [27].

It has also been shown that T-cells, macrophages and cytokines are implicated in the pathological development of T1D with CD4+ T cells sufficient to induce insulitis while CD8+ T cells contribute to the severity of the damaged β-cells [28, 29]. Failure of the immunoregulatory system causes auto-reactive T-cells to activate Type 1 T helper (Th1 cells) that promote inflammatory immune responses and suppresses Type 2 T helper (Th2), which is a subset of the CD4+ T cells responsible for humoral immunity [30].

Th1 causes the secretion of pro-inflammatory mediators interleukin 2 (IL-2), interferon gamma (IFN-γ), tumor necrosis factor beta (TNF-β) and tumor necrosis factor alpha (TNF-α) [29, 31] (Fig. 2). Clinical onset of T1D is preceded by an extensive asymptomatic period during which β-cells are destroyed because the capacity of normal pancreatic β-cells to secrete insulin far exceeds the normal amounts needed to control carbohydrate, fat and protein metabolism [20]. Patients with insulin deficiency are unable to utilize glucose in peripheral tissues such as skeletal muscles and the adipocytes, therefore resulting in increased secretion of counter-regulatory hormones such as glucagon, adrenaline (epinephrine), cortisol, and growth hormone [32]. The destruction of β-cells may occur rapidly, but is more likely to take place over a period of weeks, months, or even years [33]. Deficiency of insulin leads to uncontrolled lipolysis and elevated levels of free fatty acids (FFA) in the plasma, which suppresses glucose metabolism in peripheral tissues such as skeletal muscles [29].

Impairment of glucose utilization and insulin deficiency also decreases the transcription of a number of genes necessary for target tissues to respond normally to insulin e.g. glucokinase (liver) and glucose transporter 4 (GLUT4) (adipose tissue) [29, 34]. Some patients, particularly children and adolescents have a tendency to develop ketoacidosis as the first manifestation of the disease [4]. Initially only postprandial hyperglycemia is apparent but as insulin secretion gradually diminishes, progressive fasting hyperglycemia is encountered and eventually all T1D patients will require insulin therapy to maintain normoglycemia [4].

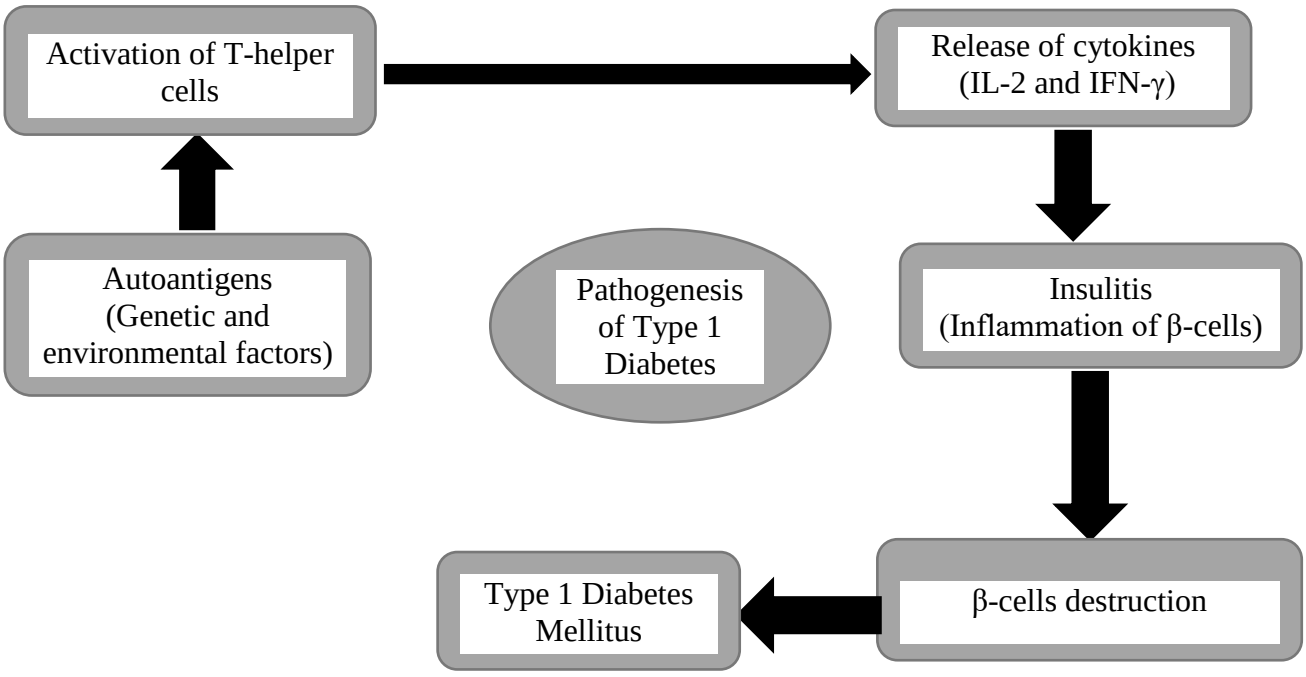


Figure 2. Etiology of Type 1 Diabetes [35].

1.4 **Type 2 diabetes**

T2D is one of the most common public health problems worldwide that is largely influenced by genetic predisposition and environmental factors (Fig. 3) [36]. It is a heterogeneous disorder characterized by impaired glucose tolerance, obesity, dyslipidemia, insulin resistance (IR), increased hepatic glucose production, pancreatic β-cell dysfunction and hyperglycemia [37]. Obesity is generally considered to be a strong risk factor for the development of T2D, and is defined by body mass index (BMI) of greater than or equal to 30 kg/m^2 [38]. Visceral adiposity escalates the development of IR and T2D through excess adipose tissue that contributes to increased circulating FFA and reduced peripheral glucose utilization [39].

Excess fatty acids also result in increased deposition of fat in the skeletal muscles and the liver, increased metabolites such as diacylglycerol that activate isoforms of protein kinase C (PKC) that impede cellular insulin signaling [40]. The detrimental effects of several adipokines such as TNFα, and IL-6 which are produced in excess by an increased adipose mass, and reduced production of adiponectin are further mechanisms through which obesity potentiates the development of T2D [41]. Although there is a strong genetic link in T2D onset there is no association with HLA types and circulating ICAs are absent [42].

The pathophysiology of T2D is poorly understood, however in the early stages of the disorder there is no glucose intolerance, despite insulin resistance because the pancreatic β-cells compensate by increasing insulin output [43]. Insulin resistance and compensatory hyperinsulinemia progress and may lead to downregulation of insulin receptors on the target organs especially the skeletal muscles. Pancreatic β-cells lose their ability to secrete insulin over time, leading to increased loss of glucose control and further hyperglycemia [4, 44, 45].

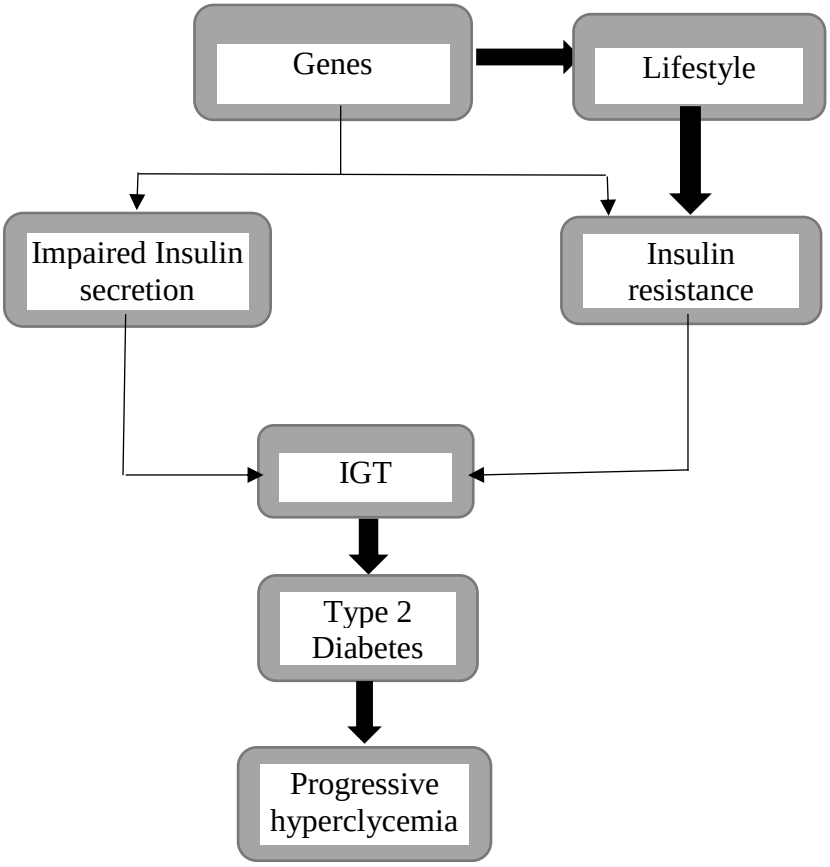


Figure 3. Pathogenesis of Type 2 Diabetes characterized by impaired secretion and insulin resistance [29]; IGT (impaired glucose tolerance).

1.4.2 **Insulin Resistance (IR)**

IR is defined as diminished ability of insulin to exert its usual biological effects on target tissues especially skeletal muscles and adipose tissue [46]. It is a prominent feature of T2D and results from a composite of abnormalities including obesity, glucose intolerance, dyslipidemia and hypertension [47]. Although the precise molecular mechanism leading to IR in T2D have not been elucidated, it is believed to be primarily caused by metabolic abnormalities resulting from defects in glucose uptake due to defective regulation of GLUT-4 protein [48]. The defects in translocation of GLUT-4 protein is caused by reduced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) [49, 50]. Phosphorylated IRS proteins serves as multisite docking proteins for various effector molecules possessing src homology 2 (SH2) domains, including phosphatidylinositol-3-kinase (PI3K) regulatory subunits, the tyrosine kinases as well as several smaller adapter molecules such as the growth factor receptor binding proteins (Fig. 4)

[48, 51, 52]. IRS proteins function as essential signaling intermediates downstream of activated cell surface insulin receptors and play a central role in maintaining basic insulin-mediated cellular functions including glycogen synthesis, protein synthesis, cell survival through glucose uptake, fatty acid synthesis and inhibit gluconeogenesis [53].

IR occurs at multiple levels in cells, from the cell surface to the nucleus including insulin receptor desensitization. Inhibition of IRS-1 and insulin receptor substrate-2 (IRS-2) result in suppression of IRS protein and functionality, inhibition of PI3K cascades, and failure to restrain fork head box protein O1 (FOXO1) activated gene transcriptional profiling. [54, 55]. Insulin stimulates amino acid uptake into cells, inhibits protein degradation and promotes protein synthesis [56]. Its shortage in the diabetic state causes a profound increase in protein catabolism, rather than a decline in protein synthesis [57].

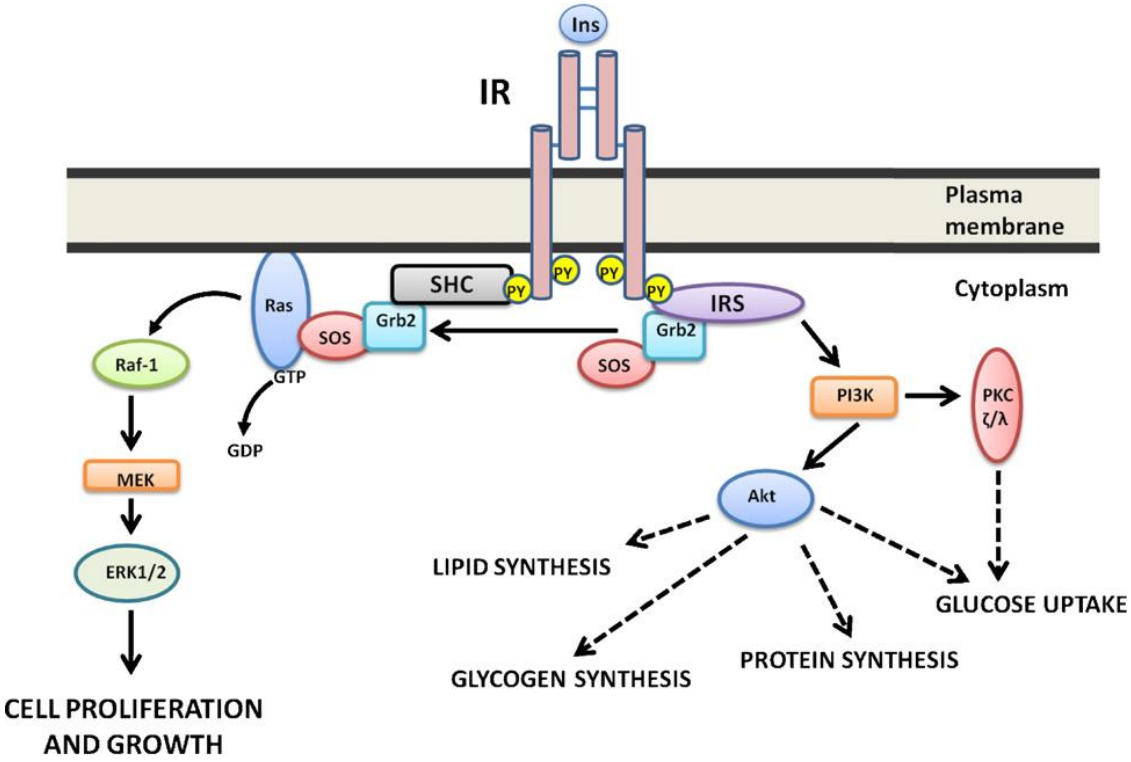


Figure 4. Insulin signaling pathways. The binding of insulin to its receptor leads to autophosphorylation on the insulin receptor (IR) subunit and the Tyr phosphorylation of IRS proteins and other downstream signaling molecules such as Shc. Activation of theses downstream effectors appears to be important for glucose transport, protein, glycogen, and lipid synthesis, whereas activation of Growth factor receptor-bound protein 2 (Grb2) dependent or independent of IRS-1 (but dependent of Shc) leads to activation of MAPK signaling pathways that control cell proliferation and growth [52].

1.5 **Gestational diabetes**

Gestational diabetes is defined as glucose intolerance with onset or first recognition during pregnancy [58]. The onset of diabetes during pregnancy and its duration affect the prognosis for a good obstetric and perinatal outcome [59, 60]. Although early diagnosis and management of GDM are still controversial, they result in beneficial effects on maternal and neonatal outcomes, and if not managed correctly may lead to diabetes postpartum [61]. GDM is diagnosed by OGTT between 24 and 28 weeks of pregnancy [62, 63]. Hyperglycemia develops during pregnancy because of secretion of placental hormones, which cause insulin resistance by preserving pregnancy and blocking actions of insulin (counter-insulin) impairing the mother's insulin secretion capacity [64]. It occurs in up to 7% of pregnant mothers and increases risk of preeclampsia [65]. Initial management includes dietary modification, exercise and blood glucose monitoring [66].

The main goal of treatment is good glycemic control during pregnancy to avoid macrosomia with its attendant risks and if hyperglycemia persists, insulin therapy is the mainstay of treatment according to standard treatment guidelines [67]. However, insulin has several drawbacks including multiple daily injections, the risk of hypoglycemia and maternal weight gain. Although insulin remains first-line treatment for GDM, safe and effective oral therapy would be more acceptable even highly desired for women with GDM. Langer *et al.* [68] and Rowan *et al.* [69] have shown that some oral hypoglycemic agents for treating GDM have gained popularity and can be good alternatives to insulin. Glyburide and metformin could be used as oral treatments for GDM regarding glucose control and treatment failure. Their incorporation in the management of GDM could allow higher efficacy rate with a significantly reduced need for insulin that should be reserved for patients who fail to respond to both oral treatments or who experienced adverse effects because of both [70].

Despite the significant gains shown with glyburide efficacy in GDM management, it was regarded inferior to insulin because of an elevated risk for neonatal intensive care unit admission, respiratory distress, neonatal hypoglycemia, birth injury, and macrosomia [71-74]. However, metformin inhibits hepatic gluconeogenesis and glucose absorption and stimulates glucose uptake in peripheral tissues, with the effect of reducing maternal weight gain, and neonatal hypoglycemia [75]. Obesity is always associated in high risk for metabolic diseases, so less weight gain could lead to reduced incidence of other complications. More observational

studies are needed to confirm that metformin and glyburide can be used as add-on therapy in GDM and standard treatment guidelines could be revised to incorporate both drugs.

According to ADA, targets of blood glucose levels during pregnancy are ≤ 5.8 mmol/l for fasting plasma glucose and ≤ 6.7 mmol/l or less 2 hours after meals [76]. After giving birth, blood glucose levels in the women with GDM are supposed to return to normal. However, many women who have had GDM may develop T2D later in life. Therefore, all women who had GDM should be recommended to maintain their ideal body weight and to exercise regularly to reduce their risk for T2D [77]. Metformin could be used in women with GDM with observations of comparative glycemic control and neonatal outcomes, especially for patients with mild GDM. However, the risk of preterm birth cannot be ignored. Clinicians should weigh the benefits versus risks of metformin in management of GDM according to the specific condition of patients. Further studies with larger sample sizes shall be designed to assess maternal and neonatal complications and to evaluate long-term follow-up of children for the safety of metformin as a universal treatment for GDM patients.

1.6 **Maturity onset diabetes of the young**

MODY is a rare form of diabetes that is associated with a strong family history that suggests an autosomal dominant transmission [78]. This is characterized by a slow onset of symptoms, the absence of obesity, lack of ketosis, and no evidence of β-cells autoimmunity. There are six different variants of MODY, caused by mutations in genes encoding islet enriched transcription factors or glucokinase [79]. MODY 1, MODY 3 and MODY 5 are caused by mutations in the hepatocyte nuclear transcription (HNF) 4α, HNF-1α and HNF-1β factors respectively [80]. These transcription factors are expressed in the liver but also in other tissues including the pancreatic islets and the kidney.

They affect islet development or the expression of genes important in glucose-stimulated insulin secretion and the maintenance of β-cells mass [80]. Individuals with MODY 2 have mutations in the glucokinase gene that plays a key role in glucose metabolism and insulin secretion [81]. MODY 2 patients have mild to moderate, stable hyperglycemia that is present from birth and generally stable throughout life and does not respond to oral hypoglycemic agents [82].

MODY 4 is a rare variant form caused by mutations in insulin promoter factor (IPF) 1, which is a transcription factor that regulates pancreatic development and insulin gene transcription together with other genes involved in glucose metabolism [83]. Patients with MODY may present with moderate to severe symptoms with or without ketosis. Unlike T1D, the disease is

generally mild and controlled easily with diet, hypoglycemic oral agents, or low doses of insulin (<40 units) [83].

1.7 **Idiopathic diabetes**

Some forms of TID have no known etiologies, however, patients may have permanent insulinopenia and are prone to ketoacidosis, although no evidence of autoimmunity [12]. Even though only a minority of patients with T1D fall into this category, of those who do, most are of African or Asian ancestry [12]. Individuals with this form of diabetes experience episodes of ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited, lacks immunological evidence for β-cells autoimmunity, and is not HLA associated [84].

1.8 **Diabetic complications**

Diabetes is associated with acute metabolic and long-term vascular complications [85]. If hyperglycemia is not controlled, it leads to serious metabolic complications [86]. With such metabolic complications there is a reduction in the action of circulating insulin together with an elevation of counter-regulatory hormones such as glucagon, catecholamines, cortisol and growth hormone [9].

The end-result of these acute metabolic complications is ketonemia, metabolic acidosis, glycosuria, osmotic diuresis with loss of water, sodium, potassium and other electrolytes [87]. The long-term vascular complications of diabetes that occur as a result of hyperglycemia are grouped into macrovascular such as coronary artery disease, peripheral arterial disease and stroke, and microvascular complications such as diabetic nephropathy, neuropathy, retinopathy and cardiomyopathy [88].

1.8.1 **Acute Metabolic Complications in Diabetes**

The two most common life-threatening complications of uncontrolled diabetes mellitus include diabetic ketoacidosis (DKA) and hyperglycemic hyperosmolar state (HHS). DKA usually occurs in patients with T1D but HHS is mostly diagnosed in patients with T2D and they both require prompt recognition and aggressive therapy to optimize clinical outcomes [89].

Although there are important differences in their pathogenesis, the basic underlying mechanism for both disorders is a reduction in the effective concentrations of circulating insulin leading to hyperglycemia and severe dehydration [90].

These hyperglycemic emergencies continue to be important causes of morbidity and mortality in patients with diabetes [86].

1.8.1.1 **Diabetic Ketoacidosis**

DKA is an acute but major life-threatening complication of diabetes that mainly occurs in patients with TID even though uniquely frequent among some patients with T2D [91]. It is defined by blood glucose concentrations >11.0 mM, venous blood pH<7.3, bicarbonate<15 mM, glycosuria, ketonemia and ketonuria [92]. DKA is a consequence of absolute or relative lack of insulin with a concomitant rise in plasma levels of insulin counter-regulatory hormones such as catecholamines, glucagon, cortisol and growth hormone leading to hyperglycemia and ketosis (Fig. 5) [91]. The combination of insulin deficiency and hyperglycemia reduces the hepatic level of fructose-2, 6-phosphate, which alters the activity of phosphofructokinase and fructose-1, 6-bisphosphatase and ultimately increase glucose synthesis with a subsequent decrease in glycolysis [93].

Insulin deficiency also reduces levels of the GLUT4, which impairs glucose uptake into skeletal muscles and adipose tissues thus reducing intracellular glucose metabolism [94]. Ketosis results from a marked increase in FFA released from adipocytes, with a resulting shift towards ketone body synthesis rather than utilization in the liver, which leads to accumulation of acetoacetate and β-hydroxybutyrate and eventually induction of anion gap metabolic acidosis. However, hyperglycemia develops as a result of increased gluconeogenesis, accelerated glycogenolysis and impaired glucose utilization by peripheral tissues [93, 95, 96].

The clinical presentation of DKA usually develops rapidly, i.e. over a period of < 24 hours presenting with polyuria, polydipsia and weight loss [91, 97]. With appropriate therapy the mortality in DKA is low (<5%) and is related more to the underlying or precipitating events, such as infections or myocardial infarction [98]. The major non-metabolic complication of DKA therapy is cerebral edema, which most often develops in children [99].

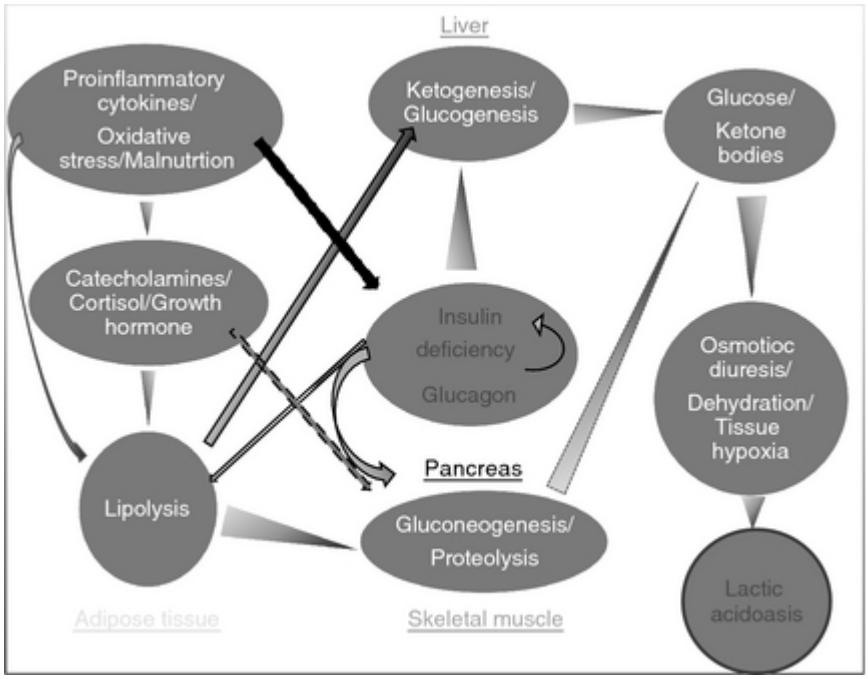


Figure 5. Pathophysiology of diabetic ketoacidosis (DKA). Insulin insufficiency promotes gluconeogenesis in the liver and ketogenesis in the skeletal muscles. Increased production of insulin counter-regulatory hormones promotes lipolysis and proteolysis in the adipose tissue and the skeletal muscles courtesy. Malnutrition, oxidative stress as well as pro-inflammatory cytokines further exacerbate β-cells destruction, lipolysis and proteolysis. Hyperglycemia and increased production of ketone bodies lead to tissue hypoxia, osmotic diuresis, dehydration and hyperlactacidemia [98].

1.8.1.2 **Hyperglycemic hyperosmolar state**

HHS is characterized by a relative deficiency of insulin concentrations to maintain normoglycemia but adequate levels to prevent lipolysis and ketogenesis as determined by residual C-peptide [96, 100]. Insulin deficiency leads to hepatic glucose production (through glycogenolysis and gluconeogenesis) and impairs glucose utilization in the skeletal muscles. Hyperglycemia induces osmotic diuresis that leads to intravascular volume depletion, which is exacerbated by inadequate fluid replacement.

Although the fundamental difference between HHS and DKA is small, HHS is characterized by a greater severity of plasma glucose elevation (>33.3 mmol/L), marked increase of plasma osmolality (>320 mOsm/kg), absence of or mild ketosis, and altered mental status [91, 97]. It is associated with glycosuria, leading to osmotic diuresis, loss of water, sodium, potassium,

and other electrolytes. General treatment of DKA and HHS requires frequent monitoring of patients, correction of hypovolemia and hyperglycemia, replacement of electrolyte losses, and attention to precipitating factors [90].

Treatment of DKA and HHS is initiated with intravenous (IV) fluids, normal saline (0.9% NaCl) at the rate of 15-20 mL/kg body weight during first hour unless the patient has cardiac dysfunction and then followed by 0.45% normal saline at a rate of 4-14 [101]. Once serum glucose reaches 11.1 mmol/l, IV fluids should be changed to 5% dextrose with 0.45% NaCl at 150-250 mL/hr.

Then continuous insulin infusion should be administered at bolus dose of 0.1 unit/kg IV and maintenance dose of 0.1 unit/kg/hr IV. Adequate renal function should be established (urine output ~50 mL/hr). If K is <3.3 mEq/L, insulin can be stopped and 20-40 mEq/hr of K should be given until K >3.3 mEq/L. If K is >5.5mEq/L, it should not be given but, if K is between >3.3 and <5.3 mEq/L, K at 20-30 mEq should be given in each liter IV fluid to maintain K between 4-5 mEq/L [101].

1.8.2. **Metabolic causes of complications of diabetes**

Although chronic hyperglycemia is an important etiologic factor leading to complications of diabetes, the mechanisms by which it leads to such diverse cellular and organ dysfunctions are unknown [93]. Increased glucose levels lead to accumulation of glycolytic metabolites and glucose modified proteins that are otherwise referred to as glucotoxins [102]. In general, the adverse-effects of hyperglycemia can cause vascular dysfunctions either by generating toxic and reactive metabolites or by altering intracellular signaling pathways [102]. Several molecular pathways have been proposed in an effort to explain the pathological developments of these complications and include the hexosamine pathway, polyol pathway, PKC activation, enhanced oxidative pathways, and formation of Advanced glycated end-products (AGEs) (Fig. 6) [103].

1.8.2.1 **Advanced Glycation End-products**

AGEs are formed by increased intracellular glucose via the non-enzymatic glycosylation of intra and extracellular proteins, which may change protein conformation and permanently impair their functions [104]. Intracellular production of AGE precursors appear to damage cells by mechanisms such as modification of intracellular proteins including those involved in the regulation of gene transcription [105, 106]. AGE precursors diffuse out of the cell, modify circulating proteins in the blood such as albumin and as a result circulating proteins

bind to AGE receptors and activate them. This causes the production of inflammatory cytokines and growth factors such as TNF-α and IL-1, which in turn induce a degenerative and proliferative cascade in mesenchymal and endothelial cells (Fig. 6) [107, 108]. AGEs can also alter cellular functions by binding to their receptors, such as the receptor for advanced glycation end-products (RAGE) or other receptors, including the macrophage scavenger receptor, p60, p90 and galectin-3 [109, 110].

RAGE is a transmembrane protein that belongs to the immunoglobulin family expressed in several cells and tissues, including endothelial cells and upon binding to AGEs [111] it initiates multiple cascades of cellular signaling pathways, including p44/42 MAPK and PKCs, and further disrupts cellular homeostasis [112, 113]. AGEs can be detected in the serum and glomerular tissues, therefore increased levels in these locations are strong indicators of tissue damage. The serum level of AGEs correlates with the level of glycemia and these products accumulate as glomerular filtration rate declines [85, 114]. AGEs block nitric oxide activity in the endothelium and cause the production of ROS, and early glycation and oxidation processes result in the formation of Schiff bases and Amadori products [115].

1.8.2.2 **Oxidative stress**

Hyperglycemia increases oxidative stress through several pathways. A major mechanism appears to be the over production of the superoxide anion $O_2^{\bullet -}$ by the mitochondrial electron transport chain [116]. Physiological generation of species $O_2^{\bullet -}$ (particularly the superoxide radical) occurs during normal electron shuttling by cytochromes within the electron transport chain [116]. Hyperglycemia leads to an increased production of electron donors (NADH and $FADH_2$) by the tricarboxylic cycle that as a result generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane [117]. Therefore, the voltage gradient across the mitochondrial membrane will increase until a critical threshold is reached, and electron transport inside complex III is blocked [118]. This increases the half-life of free radical intermediates of coenzyme Q (ubiquinone) which reduces O_2 to superoxide, and markedly increases the production of superoxide [119, 120]. ROS generation from various organs/tissues has been associated with decline in the pancreatic β-cells function as observed in T2D (Fig. 6) [121].

1.8.2.3 **The Polyol Pathway**

Intracellular glucose is predominantly metabolized by phosphorylation and subsequent glycolysis. However, when blood glucose concentrations increase it is converted to sorbitol by aldose reductase [122], which reduces toxic aldehydes in the cell to inactive alcohols, but when the glucose concentrations in the cells increases, it also reduces that glucose to sorbitol reductase through the cofactor Nicotinamide Adenine Dinucleotide Phosphate (NADPH) [122]. NADPH is an essential cofactor for regenerating critical intracellular antioxidants, and eventually reduced to glutathione [123]. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase, which uses Nicotinamide Adenine Dinucleotide (NAD+) as a cofactor. As glutathione levels decrease, cellular antioxidant capacity becomes compromised, resulting in elevated levels of ROS that can attack macromolecules and induce oxidative damage [123, 124]. Sorbitol is not easily metabolized and therefore will accumulate intracellularly. Hence increased sorbitol concentrations alters redox potential, increases cellular osmolality, generates ROS and eventually leads to other types of cellular dysfunction [122, 125, 126].

1.8.2.4 **Protein Kinase C Pathway**

Hyperglycemia increases the synthesis of DAG mainly from phosphoinositol diphosphate (PIP_2), which is a critical activating cofactor for the classic isoforms of protein kinase β 1/2, γ, and α [127]. DAG levels are elevated chronically due to an increase in the glycolytic intermediate dihydroxyacetone phosphate that is reduced to glycerol-3-phosphate and subsequently increases the synthesis of DAG [128, 129]. When PKC is activated by increased intracellular glucose, it has a variety of effects on gene transcription for fibronectin, type IV collagen, contractile proteins, and extracellular matrix proteins in endothelial cells and neurons [129]. Endothelial dysfunction is accompanied by a decreased production of prostacyclin, Nitric Oxide (NO) that is responsible for maintaining blood vessel integrity and increasing endothelin-1, vascular endothelial growth factor and cyclooxygenase dependent vasoconstrictors [130]. Some studies showed that inhibition of PKC prevented early changes in the diabetic retina and kidney thus ameliorating the detrimental effects mediated by the activation and action of PKC [131, 132] (Fig. 6).

1.8.2.5 **Hexosamine pathway**

Increased intracellular glucose is usually metabolized in glycolysis through glucose-6-phosphate (G-6-P) and fructose-6-phosphate (G-6-P). However, F-6-P can also be diverted into a signaling pathway where glutamine: fructose-6 phosphate aminotransferase (GFAT) converts the F-6-P to glucosamine-6-phosphate (G-6-P) and finally to uridine diphosphate (UDP) N-acetyl glucosamine [103, 133]. Inhibition of GFAT, the rate-limiting enzyme in the conversion of glucose to glucosamine blocks the increase of transforming growth factor-1 (TGF-1) and plasminogen activator inhibitor-1 (PAI-1) transcription [134, 135]. N-acetyl glucosamine results in pathologic changes which increases modification of the transcription factor Sp1 leading to an increase in the expression of TGF-1 and PAI-1 both of which are harmful for blood vessels in diabetic individuals [136, 137] (Fig. 6).

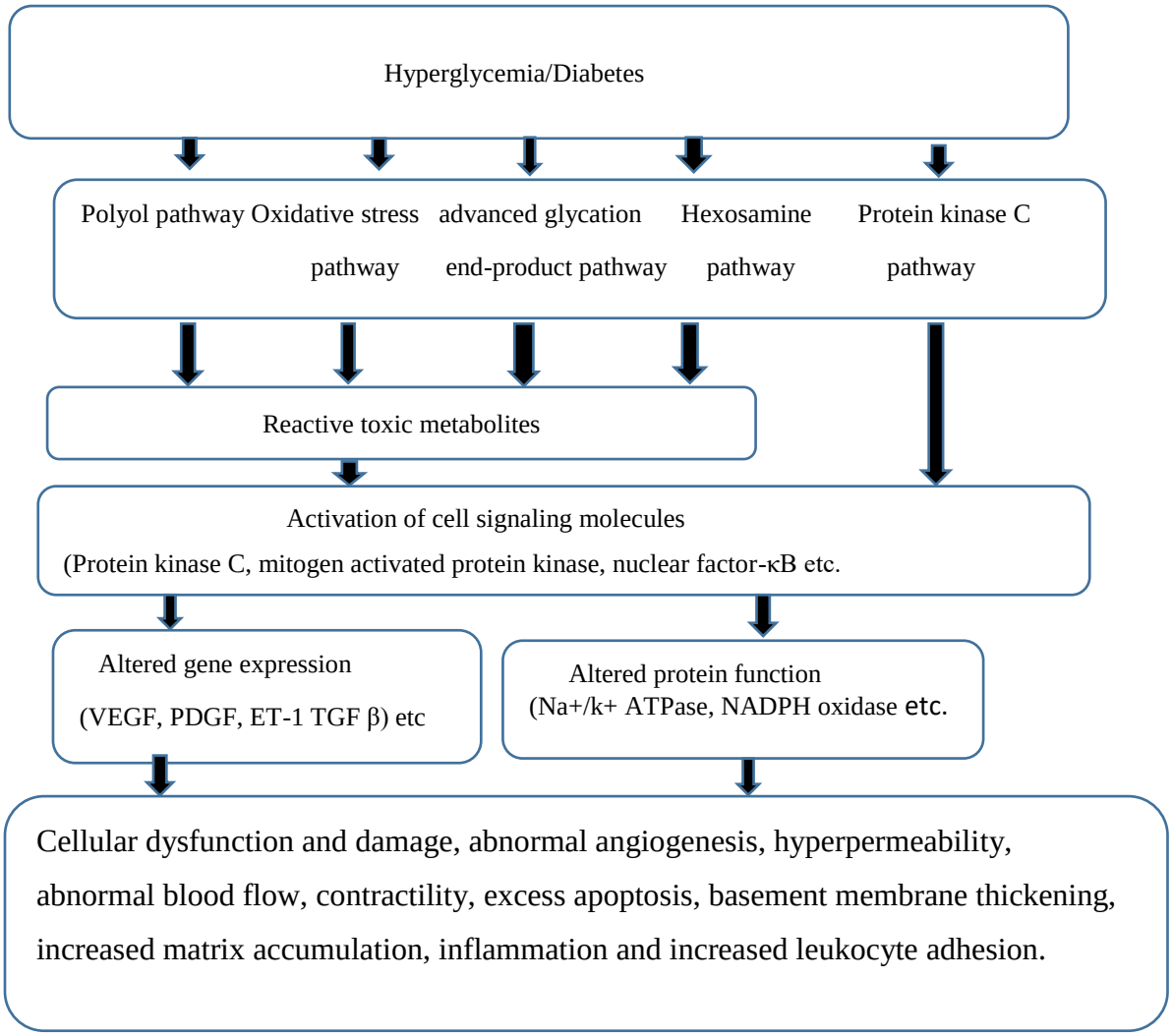


Figure 6. Mechanisms by which hyperglycemia induces diabetic vascular complications. ET-1 (Endothelin-1); NADPH (Nicotinamide Adenine Dinucleotide Phosphate); PDGF (Platelet-Derived Growth Factor);TGF-β (Transforming Growth Factor-β); VEGF Vascular Endothelial Growth Factor [136].

1.8.3 **Long-term Complications of Diabetes**

Diabetic patients develop end-point complications such as cardiovascular diseases, stroke, atherosclerosis, nephropathy, neuropathy, retinopathy and cardiomyopathy [85]. These complications can be categorized into two major groups namely microvascular and macrovascular complications.

1.8.3.1 **Microvascular Complications of Diabetes**

1.8.3.1.1 **Diabetic Retinopathy**

Diabetic Retinopathy (DR) is the most common microvascular complication of diabetes. Globally, diabetic retinopathy affects 2 million people and the prevalence is approximately 35% of all cause cases of blindness [138]. In industrialized countries, DR is the leading cause of blindness in people aged 15 - 64 years [139]. It is defined as a microvascular complication that affects the peripheral retina, macula, or both leading to visual impairment and blindness in people with diabetes [85]. Biochemical alterations such as oxidative stress, activation of PKC and formation of AGEs have been shown as responses of the retina to hyperglycemia [104, 140]. DR is generally classified as either background (nonproliferative retinopathy) or proliferative whereby background retinopathy develops first and causes increased capillary permeability, microaneurysms, hemorrhages, exudates, macular ischemia, and macular edema [141]. Proliferative retinopathy is characterized by the formation of new blood vessels on the surface of the retina and can lead to vitreous hemorrhage [142]. If proliferation continues, blindness can occur from vitreous hemorrhage and traction retinal detachment intervention is made [139]. These changes cause glaucoma and cataracts and if not treated could lead to total blindness.

1.8.3.1.2 **Diabetic nephropathy**

Diabetic nephropathy (DN) is a major cause of end-stage renal disease (ESRD) which is characterized by glomerular hemodynamic abnormalities that result in glomerular hyperfiltration, leading to glomerular damage as evidenced by microalbuminuria (albumin excretion of 30–299 mg of albumin/g of creatinine) [143]. As glomerular function continues to decline, overt proteinuria (presence of proteins of greater than 0.5g/24 h) increases, glomerular filtration rate (GFR) declines and ESRD escalates [144]. DN is also characterized by the elevation of blood pressure and progressive renal insufficiency due to glomerular lesions and loss of GFR in the absence of albuminuria [143]. Dyslipidemia with increased low-density lipoprotein (LDL) cholesterol and triglycerides are independently associated with diabetic kidney disease [145, 146]. The pathophysiological mechanisms underlying DN involve generation of ROS, AGEs, and activation of intracellular signaling moieties such PKC [147, 148]. Glycemic control and renin-angiotensin-aldosterone system (RAAS) inhibition have long been mainstays of therapy in patients with Diabetic kidney disease (DKD) [149]. Clinically, Angiotensin-converting enzyme inhibitors (ACEIs) and Angiotensin Receptor Blockers (ARBs) have been a cornerstone for management of DKD because of their effectiveness in delaying the progression of diabetic nephropathy [150, 151]. Conversely, poor blood pressure control can accelerate the progression of diabetic nephropathy [152].

Multiple large-scale clinical trials have demonstrated that improved glycemic control in patients with T1D and T2D have reduced microalbuminuria, macroalbuminuria and progression to DKD and ESRD [153-155]. ACEIs and ARBs have been extensively studied, and are considered superior to other antihypertensive drug categories in the treatment of DN because of their capacity to reduce both intraglomerular pressure and proteinuria by preferentially dilating the efferent arteriole [152]. Furthermore, it has been reported that renoprotective effects of treatment with ACE inhibitors and ARBs, which appear to be present regardless of their blood pressure-lowering effects, possibly because of decreasing intraglomerular pressure. Which further decreases the risk of progression of kidney disease independent of the blood pressure-lowering effects [152, 156].

1.8.3.1.3 **Diabetic neuropathy**

Diabetes causes both acute and chronic neuropathies characterized by signs and symptoms of peripheral neural impairment in patients with diabetes distinct from other causes of neural damage [157]. Diabetic neuropathy is thought to be caused by both direct hyperglycemia-

induced damage to the nerve parenchyma and neuronal ischemia resulting from hyperglycemia-induced decreases in neurovascular flow [158]. The risk of developing diabetic neuropathy is directly proportional to both the duration and magnitude of hyperglycemia [85]. The precise nature of injury to the peripheral nerves from hyperglycemia is not known but is likely related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress [125, 159]. The most common chronic neuropathic complication of diabetes is distal symmetric polyneuropathy (DSP) [160]. It frequently presents with loss of peripheral sensation which, when coupled with impaired microvascular and macrovascular function in the periphery, can contribute to non-healing ulcers the leading cause of nontraumatic amputation [161]. Although up to 50% of patients do not have symptoms of neuropathy, DSP is manifested clinically by paresthesia, dysesthesia, pain, impaired reflexes, and/or decreased vibratory sensation [160, 161].

DSP can either be typical or atypical, whereby typical DSP is chronic, distal, symmetric, sensory dominant and often painful. Any variations that may be proximal or asymmetric suggest an atypical neuropathy [162]. An estimated 20% of patients with DSP experience severe pain and it is the leading cause of foot ulceration and limb amputations [163, 164].

Diabetic patients also tend to develop autonomic neuropathy, including cardiovascular autonomic dysfunction, which is characterized by abnormal heart rate and vascular control [165]. Diabetic cardiomyopathy has been associated with cardiac autonomic dysfunction and patients with diabetic cardiomyopathy have diastolic filling and relaxation abnormalities, which are then commonly followed by systolic dysfunction and heart failure [166].

1.8.3.2 **Macrovascular complications of diabetes**

In macrovascular complications, the injurious effects of hyperglycemia lead to chronic inflammation and injury to the arterial walls in the peripheral or coronary vascular system therefore resulting in atherosclerosis, which in turn narrows the arterial walls [145]. Oxidized lipids from LDL particles then respond to endothelial injury and inflammation by accumulating in the endothelial wall of the arteries [167, 168]. Foam cells eventually form as a result of immune response activation whereby the stimulation of macrophage proliferation and T-lymphocyte attraction occurs and thereby inducing collagen accumulation in the arterial walls [169]. The result of this pathogenesis is the formation of lipid-rich atherosclerotic lesions with fibrous caps. This explains why diabetic patients are at increased risk of developing

cardiovascular diseases such as myocardial infarction (MI), making diabetes an independent risk factor for ischemic diseases, coronary artery disease, stroke and death [170].

1.8.3.2.1 **Diabetic cardiomyopathy**

Diabetic cardiomyopathy (DCM) refers to diabetes-associated structural and functional myocardial dysfunction not related to other confounding traditional factors such as coronary artery disease (CAD), hypertension, congenital heart diseases or valvular heart diseases [171, 172]. It affects approximately 12% of diabetic patients globally [173], leading to overt heart failure and death. DCM is characterized by myocardium structural abnormalities leading to left ventricular hypertrophy (LVH), diastolic and systolic dysfunction or a combination of both [174]. These events follow metabolic alterations induced by hyperglycemia, insulin resistance and dyslipidemia, which alter signal transduction pathways that regulate cardiac function [175]. One of the principle abnormalities is the excess generation of AGEs that deactivate NO and impair coronary artery vasodilation [176].

Sustained hyperglycemia causes excess formation of mitochondrial ROS that results in cardiac glucotoxicity, mitochondrial failure and apoptosis, fibrosis and eventually contractile dysfunction [177]. Although the increase in HF and cardiovascular mortality are mainly due to accelerated atherosclerosis, compelling epidemiological and clinical data indicate that diabetes mellitus increases the risk for cardiac dysfunction and heart failure independently of other risk factors such as CAD and hypertension [178]. In this regard, an early and specific detection of DCM could be worthwhile for appropriate therapeutic interventions.

1.9 **Treatment of Type 2 Diabetes: The current Antidiabetic Agents**

Currently available therapeutic agents for diabetes include insulin and various oral anti-diabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, and glinides used as monotherapy or in combination to achieve better glycemic control (Table 1) [179].

Table 1: Drugs clinically used for treatment of T2D [180].

Class	**Example**	**Mechanism of action**	**Side effects**

Biquanides	Metformin	Lowers blood glucose concentrations by decreasing hepatic gluconeogenesis and increasing glucose uptake by skeletal muscles through activation of AMPK.	Gastrointestinal intolerance (diarrhea and other GI disturbances such as nausea, abdominal discomfort, metallic taste, and anorexia.
Nonsulfonylureas secretagogues (Meglitinides)	Repaglinide, Nateglinide.	Blocks ATP-dependent potassium channels, stimulates insulin release from pancreatic β-cells.	Hypoglycemia, associated with rare hepatotoxicity and weight gain.
Sulfonylureas	Glimepiride, Glipizide Chlorpropamide, Glyburide, Tolazamide, Tolbutamide.	Stimulate the release of insulin from β-cells through interaction with ATP sensitive K channel and enhance β-cells sensitivity to glucose.	Weight gain, hypoglycemia.
α-Glucosidase Inhibitors	Acarbose, miglitol.	Inhibits enzymes that break down carbohydrates into glucose and subsequent delay in glucose absorption.	Flatulence, diarrhea, and abdominal pain.

Thiazolidine diones	Pioglitazone, Rosiglitazone.	Bind to and activate a nuclear receptor Peroxisome proliferator-activated receptor gamma (PPAR-γ), increase glucose transport into adipose, skeletal muscles and hepatocytes. Stimulation activates GLUT4 gene transcription.	Hepatotoxicity, risk of myocardial infarction and weight gain.
Amylin-Receptor Agonists	Pramlintide, Acetate.	Suppresses glucagon release, delays gastric emptying.	Hypoglycemia and gastrointestinal symptoms including nausea, vomiting, and anorexia.
SGLT2 inhibitors	Dapagliflozin, canagliflozin, empagliflozin.	Decrease renal glucose reabsorption and thereby enhancing urinary glucose excretion and subsequent. reductions in plasma glucose and glycosylated hemoglobin concentration.	Urinary and genital tract infections more common with SGLT2 inhibitors, hypotension.
Dopamine D2-R antagonist	Bromocriptine	Mechanism of action unknown.	Hypotension, Hypoglycemia.
Insulin	Rapid-acting (aspart, glulisine, lispro) Long-acting	Binds to insulin receptor, stimulate glucose transport across cell membrane by ATP dependent translocation of GLUT4 to the plasma	Hypoglycemia, weight gain and hypersensitivity reactions.

	(glargine,detemir) Intermediate-Acting Insulin (Neutral Protamine Hagedorn (NPH).	membrane. PIP3 and tyrosine phosphorylated guanine nucleotide exchange proteins facilitates GLUT4 translocation from cytosol to plasma membranes.	

1.10 **Pharmacotherapy with metformin**

Biguanides have mostly been prescribed to patients whose hyperglycemia is due to ineffective insulin action (insulin resistance) [181]. Newly diagnosed T2D patients are currently treated with metformin as a first-line drug that has been shown to be associated with clinical benefits such as reduced insulin resistance, decreased hepatic glucose output, improved glycemic control and dyslipidemia. In addition, there is also reduction in weight gain and reduced risk of cardiovascular morbidity and mortality [182].

Metformin is an insulin-sparing agent and does not increase weight or provoke hypoglycemia, it offers obvious advantages over insulin or sulfonylureas in treating hyperglycemia in T2D [183]. In the past 3 decades, metformin has been recommended as the first-line therapy for T2D by the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD) and the American Association of Clinical Endocrinologists (AACE) treatment guidelines, respectively [184, 185].

The United Kingdom Prospective Diabetes Study (UKPDS) reported that metformin therapy decreases the risk of macrovascular and microvascular complications of diabetes, which is in contrast to the other therapies that only modify microvascular morbidity [186]. However, despite significant gains with metformin as monotherapy in T2D, a considerable number of patients fail to achieve optimum glycemic control and are either maintained on metformin with insulin or insulin secretagogues, or switched to insulin monotherapy.

Biguanides are also indicated for use in combination with insulin secretagogs or thiazolidinediones for T2D where oral monotherapy is inadequate. Repeated insulin injections

make some patients less compliant and some of the patients become intolerant to metformin because of associated side-effects such as GI disturbances, vitamin B_{12} malabsorption and rare lactic acidosis [187]. Metformin is controversially contraindicated in patients who have developed end-point diabetes complications such as heart or renal failure due to perceived risk of lactic acidosis [188, 189]. Phenformin is a biguanide with similar actions to metformin but was withdrawn from the markets because of increased incidence of lactic acidosis [190]. Phenformin has powerful inhibitory effects on functioning of the mitochondrial respiratory chain and inhibits lactate oxidation thus increasing plasma lactate concentrations leading to lactic acidosis [189].

New agents such as carbohydrate absorption inhibitors, glucagon-like peptide 1 (GLP1) agonists (analogs) or SGLT-1 and SGLT-2 antagonists despite their significant effects in controlling glycemia are not devoid of unwanted side-effects [191]. Recent reports indicated that the use of SGLT-2 inhibitors is associated with risk of DKA by increasing FFA and possibly reducing ketones clearance [192]. Other side-effects include an increase in incidence of genital tract infections (GTIs) and in some studies a numerical excess of urinary tract infections (UTIs) and bone fractures has been reported [193, 194].

1.10.1 **Metformin-associated lactic acidosis**

Although metformin is a drug of choice for the treatment of T2D, it is contraindicated in some patients due to the risk of lactic acidosis [195]. Metformin-associated lactic acidosis (MALA) is an extremely rare condition although cases continue to be reported and are associated with mortality rates of 30 to 50% [196]. Lactic acidosis is a life-threatening condition characterized by low blood pH (< 7.35) and elevated arterial lactate (> 5.0 mmol/L) levels [197]. It can occur in patients with predisposing factors such as renal insufficiency, hepatic disease, congestive heart failure and sepsis [198]. Lactic acidosis occurs during conditions of excessive lactate production and/or impaired hepatic lactate clearance. It is divided into two categories namely type A lactic acidosis which results from the accumulation of lactate through glycolysis in the absence of oxygen and type B lactic acidosis which is known to be associated with metformin [195].

Metformin has been shown in *in-vitro* studies to reduce oxygen consumption and glucose production in isolated rat hepatocytes in a dose-depend manner, by inhibiting Respiratory Chain Complex 1 (RCC1) and mitochondrial glycerophosphate dehydrogenase that impairs conversion of cytosolic lactate to pyruvate leading to increased plasma lactate levels and

reduction in ATP production [197]. Metformin stimulates conversion of glucose to lactate in the intestinal mucosa, blocks mitochondrial oxidative metabolism, decreases hepatic gluconeogenesis from lactate, pyruvate and alanine resulting in anaerobic glycolysis and lactate accumulation [199].

It would be expected that this inhibition causes oxidative stress, which may lead to conversion of pyruvate to lactate in the cytosol and hence increased lactate levels [198, 200]. Although metformin is controversially contraindicated in patients who have developed end-point diabetes complications such as heart failure and renal failure due to perceived risk of lactic acidosis, the FDA stipulated that metformin may be initiated in patients with an eGFR >45 mL/min/1.73 m^2, but renal function should be assessed at regular intervals (3–6 months) depending on risk of developing renal impairment [201].

eGFR not serum creatinine should be used to estimate kidney function in patients with renal impairment because serum creatinine is dependent on several factors. Patients already initiated on metformin treatment can continue cautiously on such treatment but depending on benefits and risks if eGFR >30-45 mL/min/1.73 m^2 and appropriate monitoring in patients is required [202, 203]. Therefore, it should be envisioned that in CKD stage 3 or 4 there is a further renal functional decline and consequently metformin may accumulate leading to metabolic/lactic acidosis [204].

The FDA recommended that metformin could be associated with reduced readmissions and mortality in patients with congestive heart failure (CHF) [205]. Based on findings from observational evidence, metformin was found to be associated with a lower risk of all-cause mortality when compared to non-metformin treatment in patients with treated T2D and CKD or CHF.

Furthermore, there were no new cases of MALA for patients with CHF and there was no data to suggest higher incidences of lactic acidosis with metformin use in older adults. Based on limited available evidence, the comparative risk of MALA in patients with CKD and CHF does not appear to be higher than the risks with the use of other hypoglycemic medications, hence the benefits of metformin use in CKD and CHF outweigh the risks [205].

1.10.2 **Metformin and vitamin B_{12}**

Long-term use of metformin is associated with vitamin B_{12} malabsorption that may be due to its effects on calcium-dependent membrane action and the cobalamine-IF uptake by ileal cell receptors leading to reduced vitamin B_{12} absorption [206]. Metformin interferes with vitamin

B_{12} intrinsic factor complex hence vitamin B_{12} absorption. However, there is evidence that dietary calcium supplementation reverses metformin-induced vitamin B_{12} malabsorption [207]. Although clinical significance of this is not yet clear, it is believed that bacterial overgrowth could also cause reduction of vitamin B_{12} absorption through increased binding of bacterial cells to vitamin B_{12}-IF complexes [208] .

T2D is a progressive and complex disorder that is difficult to treat effectively in the long term. The majority of patients are overweight or obese at diagnosis and will be unable to achieve or sustain normal blood glucose without oral antidiabetic agents [209]. Recently new drugs have markedly expanded and improved therapeutic options, however, blood glucose control is not optimized despite the presence of this new medication. Adverse-events especially hypoglycemia and weight gain, are still frequent and decrease treatment adherence.

1.10.3 **Antihyperglycemic effects of metformin**

Metformin exerts its antihyperglycemic effects primarily by inhibiting hepatic gluconeogenesis and by enhancing effects of insulin in certain target organs like muscles and adipose tissues [210, 211]. It has been shown to reduce glycosylated hemoglobin by 1.1–3.0% in T2D patients [212]. Metformin acts in the liver to suppress gluconeogenesis mainly by potentiating the effects of insulin, reducing hepatic metabolism of lactate and opposing the effects of glucagon [213].

Additionally, metformin can reduce the overall rate of glycogenolysis through AMPK activation, which leads to suppression of gluconeogenic enzymes. Metformin improves insulin sensitivity by increasing insulin-mediated insulin receptor tyrosine kinase activity which activates post-receptor insulin signaling pathways. This has been attributed in part to increased translocation of insulin-sensitive glucose transporters to the plasma membrane [214, 215].

However, loss of glycemic control with traditional combinations with insulin, sulfonylureas or thiazolidinediones has recently led to combination of metformin with newer agents such as SGLT-2 antagonists (e.g. gliflozin, dapagliflozin, empagliflozin, ertugliflozin, ipragliflozin, luseogliflozin, tofogliflozin), GLP-1 analogs or Dipeptidyl peptidase 4 (DPP-4) inhibitors (such as exenatide, liraglutide, linagliptin, saxagliptin, sitagliptin, teneligliptin) with concomitant amplification of adverse effects not commonly experienced with metformin monotherapy [216, 217].

1.11 **Non-glycemic effects of metformin**

1.11.1 **Inflammation and hematological effects of metformin**

Metformin has recently been shown to prevent atherosclerosis by improving endothelial-dependent vasodilation, inhibiting vascular inflammation by AMPK activation in diabetic rats [218]. AMPK has recently been shown to ameliorate anemia when activated by metformin [219, 220]. Activation of AMPK leads to phosphorylation of eNOS, thus stimulating the release of NO that is important for vascular function [221]. In agreement with this, metformin improved endothelial function *in vivo*, by reducing endoplasmic reticulum stress, superoxide production and increasing NO bioavailability in mice on a high-fat diet [222], an effect that involved AMPK or Peroxisome Proliferator-activated Receptor δ (PPAR δ) pathways [223]. Furthermore, metformin has been shown to suppress inflammatory response in an otitis media model by relieving oxidative stress prevent thrombosis and inhibiting platelet activation by reducing mitochondrial free radical overload [224, 225].

1.11.2 **Antidyslipidemic effects**

Dyslipidemia is major contributor to increased cardiovascular risk associated with T2D and it encompasses abnormalities in all lipoproteins [226]. Patients usually present with elevated total cholesterol, low Density lipoprotein cholesterol (LDL-c), triglycerides (TG) and reduced high density lipoprotein cholesterol (HDL-c) which significantly increase the risk of cardiovascular disease in these patients [227, 228]. Metformin has pleiotropic effects and can be useful in diabetic dyslipidemia, diabetic cardiomyopathy and vascular dysfunctions [229].

It has been shown to decrease total cholesterol, LDL-c, plasma free fatty acids and TG and may even increase HDL-c [230, 231]. Favorable effects on HDL-c and LDL-c in people who do not have diabetes have also been reported with metformin therapy. In a large retrospective study analysis, metformin has been shown to lower TG and increase HDL-c in diabetic patients [232] and has been reported to enhance therapeutic outcomes on atherogenesis with statins in patients with T2D [232]. These effects of metformin appear to be exerted by activation of AMPK which switches energy balance from anabolic to catabolic state leading to inhibition of glucose, protein and lipid synthesis but increased fatty acid oxidation and glucose uptake [233].

1.11.3 **Metformin effects on hypertension and cardiovascular morbidity**

Cardiovascular morbidity and hypertension are common end-points of diabetes complications [234]. Diabetes is associated with increased progression of asymptomatic left ventricular dysfunction and symptomatic HF, increased hospitalizations for HF, and an overall increased mortality risk in patients with chronic HF [235, 236]. Metformin has been shown by the UKPDS study to reduce cardiovascular disease risks in T2D patients [186, 236]. A prospective observational study as well as a retrospective cohort study have provided evidence that metformin use was associated with decreased incidence of atherosclerosis and myocardial infarction or stroke [237, 238].

Clinical and experimental evidence so far presented suggest that non-glycemic cardioprotective effects of metformin are mediated by activation of AMPK and eNOS and several kinases of the reperfusion injury salvage kinase (RISK) signaling pathway that prevent mitochondrial permeability transition pore (MPTP) opening and reperfusion [239, 240]. Despite initially conflicting reports on the effects of metformin on blood pressure, it has now become apparent that metformin actually normalizes blood pressure in hypertensive individuals but does not affect blood pressure in normotensive individuals [188].

These effects of metformin on blood pressure are thought to be related to the lowering of plasma norepinephrine levels leading to a decrease in cardiac sympathetic tone and reduced workload and myocardial fat oxidation [241, 242]. These effects are consistent with decreased myocardial oxygen consumption ($mVO_{2)}$ and decreased myocardial sympathetic activity [188].

1.11.4 **Polycystic ovarian syndrome (PCOS)**

Polycystic ovarian syndrome (PCOS) described, as a syndrome of ovarian dysfunction is the most common endocrine disorder affecting 4‑12% of women of reproductive age [243, 244]. PCOS is characterized by chronic oligo- or an-ovulation presenting with menstrual irregularities, hirsutism, infertility and elevated serum testosterone and androstenedione [245, 246]. Obesity, insulin resistance and hyperinsulinemia are the hallmarks of PCOS [247]. Insulin resistance appears to be the link between PCOS and T2D, with other recognized cardiovascular risk factors such as dyslipidemia and hypertension, as well as with anatomical and functional cardiovascular derangement [244]. Metformin has been shown to reduce systolic blood pressure, hyperinsulinemia and follicle stimulating hormone (FSH) in women with PCOS [248]. Furthermore, metformin may increase ovulation, improve the menstrual cycle, reduce serum androgen levels and may improve hirsutism [244].

1.11.5 **Antineoplastic effects of metformin**

Patients with T2D have an increased risk of malignancy compared to non-diabetic patients particularly liver, pancreas, endometrium, colon, rectum, breast, and bladder cancer [249, 250]. Persistent insulin resistance and hyperinsulinemia in T2D activate Insulin Growth Factor-1 (IGF-1) which is a potent mitogen [251, 252]. Most cancer cells can also overproduce insulin receptors most commonly as the α-isoforms, which can stimulate insulin-mediated mitogenesis even when IGF-1 receptors are deficient.

Activation of the insulin receptor is essential for promoting cell survival and division in cancer cells and these growth-promoting effects of the IGF insulin axis may extend to normal smooth muscle cells, thus establishing a common pathway for accelerated atherosclerosis and accelerated cancer development in diabetes [253, 254]. Metformin usage has been demonstrated to be inversely related to the incidences of many types of cancers such as breast, ovarian, endometrial and colorectal cancers in T2D patients and clinical trials with metformin have now been extended to non-diabetic populations [255, 256].

Antineoplastic effects of metformin have been shown to be mediated by the activation of the LBK1-AMPK signaling pathway which inhibits Mammalian Target of Rapamycin (mTOR),a critical mediator of Phosphatidylinositol- 3-kinase/protein kinase B/akt (PI3K/PKB/Akt) which is the most frequently dysregulated signaling pathway in oncogenesis [257, 258].

Other mechanisms of metformin anti-cancer effects include its ability to reduce circulating plasma insulin and IGF-1 concentrations hence limiting the risk of hepatocellular carcinoma [259] and stimulating tumour specific CD8+ cells which initiate expansion and differentiation of effector cytotoxic T lymphocytes leading to apoptosis [260, 261]. Some *in vitro* and *in vivo* studies strongly suggest that metformin may be a valuable adjuvant in cancer treatment because of lower risk of cancer in patients who use the drug [262].

1.12 **Mechanism of action of metformin**

Metformin uptake into hepatocytes is facilitated by the organic cation transporter protein (OCT1), where it accumulates in the liver. It directly inhibits RCC1 leading to a decrease of both cytosolic and mitochondrial adenosine triphosphate /adenosine diphosphate (ATP/ADP) ratio, serving as an allosteric activator of 5'-AMP-Activated Protein Kinase (AMPK) (Fig. 2) [263]. Decreased electron transport chain activity suppresses tricarboxylic acid cycle flux and decreases mitochondrial ATP synthesis causing cellular energy stress and elevation of the AMP: ATP ratio.

AMPK is a primary metabolic sensor that is phosphorylated by the upstream kinases LKB1 and Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase beta (CaMKKβ) [264]. LKB1 is an important upstream kinase of AMPK cascade in mammalian cells that phosphorylates Threonine (Thr) 172 on the activation loop of AMPK catalytic subunit and subsequently activates AMPK *in-vitro* [265]. In addition, inhibition of LKB1 activity in cells simultaneously abolishes the activation of AMPK by different stimuli.

Metformin most likely does not directly activate either LKB1 or AMPK, as it does not influence the phosphorylation of AMPK by LKB1 in a cell-free assay [266]. Rather, there is evidence that AMPK activation by metformin is secondary to its effect on mitochondria, the primary target of the drug [267]. At cellular level, metformin activates AMPK by phosphorylation, which suppresses glucagon-stimulated glucose production, and causes increased glucose uptake by hepatocytes and skeletal muscles [268].

AMPK activation in the skeletal muscles also increases glucose consumption and is another potential site of metformin action. Both of these consequences of metformin lower blood glucose and contribute to therapeutic benefits in T2D [265, 269].

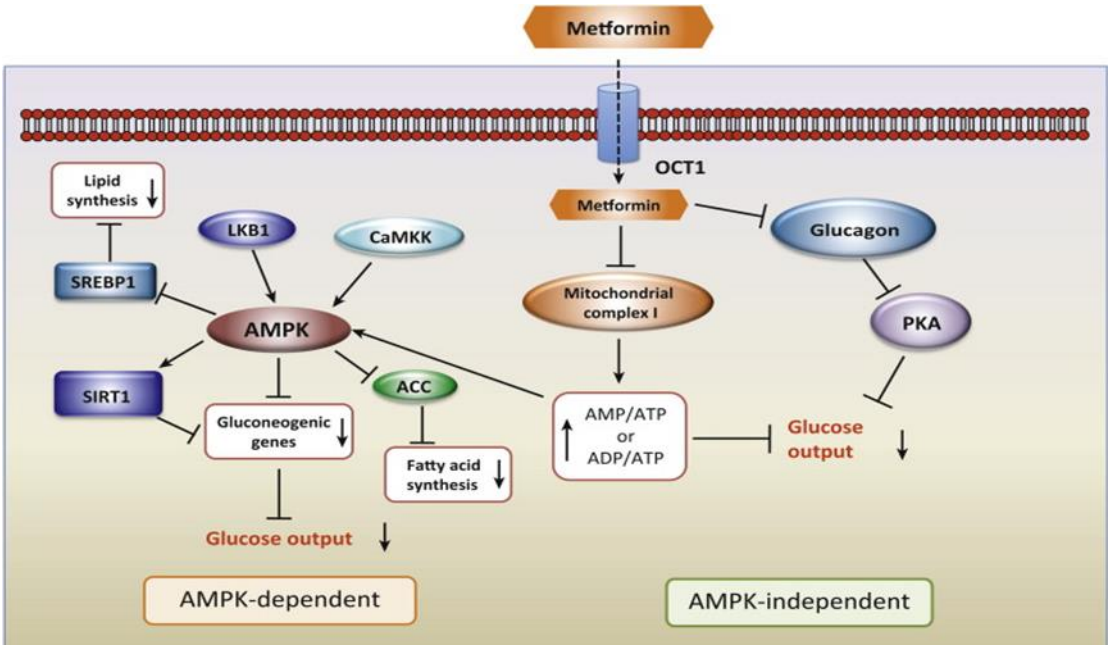


Figure 7. Known and proposed mechanisms of action of metformin in hepatocytes. AMP (Adenosine Monophosphate Protein); AMPK (Adenosine Monophosphate-Activated Protein Kinase); ATP (Adenosine Triphosphate); OCT1 (Organic Cation Transporter 1); PKA (Protein Kinase A); ACC; (Acetyl-CoA Carboxylase); SIRT1; SREBP1, (Sterol Regulatory Element-Binding Protein-1); LKB1 (Liver Kinase B1) [268].

Metformin's primary benefit in T2D has been in its ability to slow down the accelerated basal rates of hepatic gluconeogenesis without an apparent effect on lactate turnover for gluconeogenesis or increases in insulin secretion. Metformin suppresses hepatic glucose output through the down-regulation of the transcription of the rate-limiting gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [270, 271].

Metformin activates LKB1 thereby resulting in the phosphorylation of AMPK and cytosolic isolation of the cAMP Response Element Binding protein (CREB) transcription factor transducer of regulated CREB coactivator 2 (TORC2) [272]. It is then trapped in the cytosol of the hepatocyte CREB within the nucleus that is efficient at transcribing a transcriptional cofactor named PGC1α [273]. Resultant low amounts of PGC1 α causes less transcriptional activation of G6Pase and PEPCK thereby leading to a slowing down of the accelerated basal rates of hepatic gluconeogenesis (Fig. 7) [267].

Therefore, the success of metformin in T2D and cardiovascular diseases is dependent on AMPK activation despite its limited drawbacks. However, some patients still fail to obtain optimum glycemic control with metformin. The most common side-effects of metformin are GI disturbances, metformin associated lactic acidosis and vitamin B_{12} malabsorptions. Would phytochemicals with similar pharmacological effects but devoid of side-effects therefore be more beneficial? What are the potential implications of concurrent consumption of naringenin with metformin?

1.13 **Plant-derived alternatives**

Flavonoids constitute the most abundant bioactive constituents of the grapefruit and four types of flavonoids (flavanones, flavones, flavonols and anthocyanins) have been identified in the citrus fruits [274, 275]. The chemical skeleton structure of flavonoids is based on a 15-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C) (Fig.8) [276]. Chalconoids commonly known as chalcones are a group of compounds that are classed as minor flavonoids as they also contain the C6-C3-C6 backbone [277]. The flavonoids are well-known by many biological activities which includes protective effects such as anti-oxidant, vasorelaxant and anti-thrombotic properties [278]. It has been suggested that flavonoids decrease the risk of coronary heart disease by three major actions which are improving coronary vasodilatation, decreasing the ability of platelets in the blood to clot, and

preventing LDLs from oxidizing [275, 279]. Naringin is the major flavanone found in grapefruit which possesses the distinct bitter taste with other flavanone hesperidin [280].

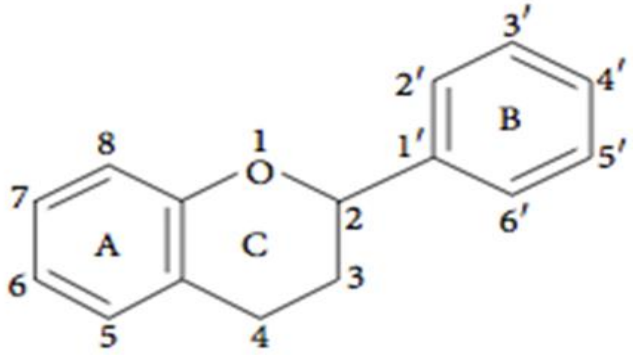


Figure 8. Skeleton structure of flavonoids.

Naringin has two rhamnose units attached to its aglycone portion. On the other hand, naringenin (4′, 5, 7-trihydroxyflavanone 7-rhamnoglucoside) like all flavonoids has a chemical structure based on a 15-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C) (Fig. 8) [276]. Upon ingestion, naringin is hydrolyzed by intestinal bacterial naringinase complex to its aglycone naringenin, which appears to be biologically more potent [280, 281]. Naringin is rapidly converted to naringenin whether administered orally or IV by either intestinal bacterial or hepatic metabolism, respectively [281]. We have recently reviewed the pharmacological evidence that show similarities between metformin and naringenin and proposed that naringenin can be used as adjunct or therapeutic substitute to metformin given their similarities in pharmacological actions (Fig. 9) [282].

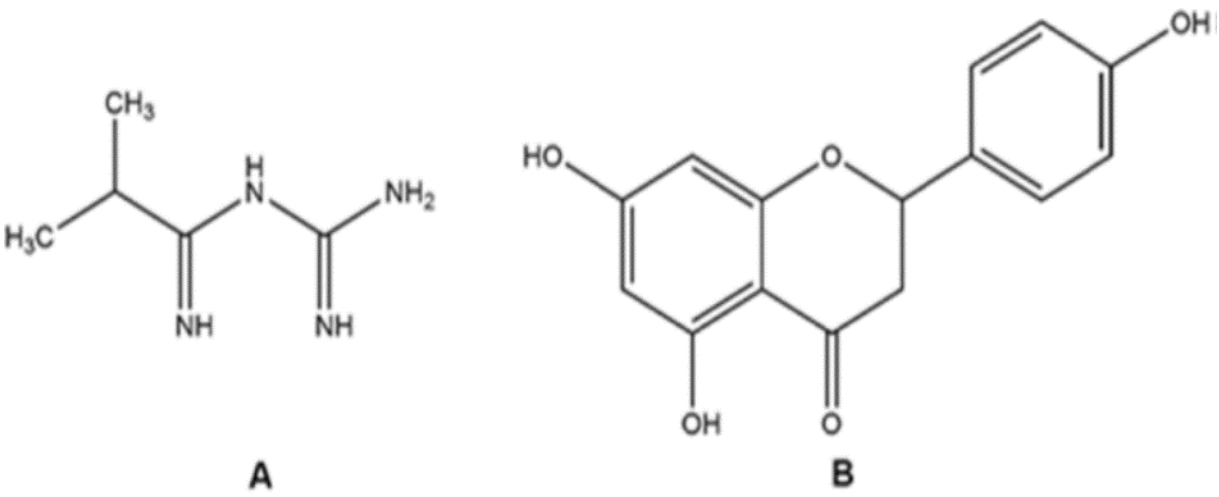


Figure 10. Chemical structures of A: metformin and B: naringenin. ChemDraw, PerkinElmer®

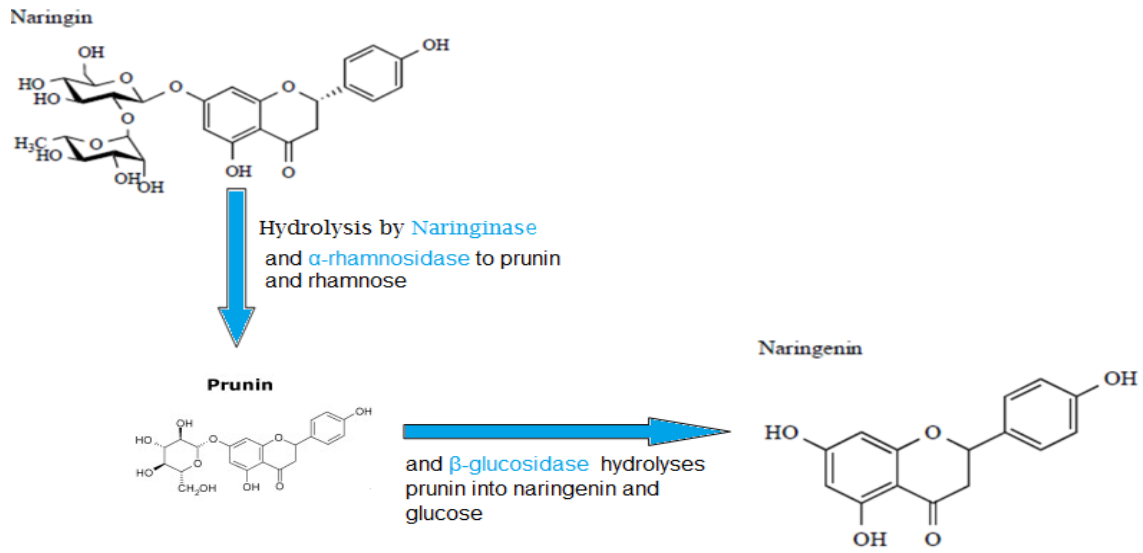


Figure 9. Enzymatic hydrolysis of naringin. Naringinase is an enzymatic complex with α-rhamnosidase activity which hydrolyses naringin to prunin and rhamnose, and β glucosidase activity which in turn hydrolyses prunin to naringenin and glucose, respectively [278, 283].

1.13.1 **Antihyperglycemic effects of flavonoids**

Citrus derived flavonoid, naringin or its aglycone naringenin have been shown to have antidiabetic, antidyslipidemic and cardioprotective effects and may reduce the risk of age-related chronic diseases [278, 280, 284, 285]. The antidiabetic properties of flavonoids are mainly through their effects on a number of molecular targets and regulation of several pathways such as reducing apoptosis, improving proliferation of pancreatic β-cells and

promoting insulin secretion [284]. Moreover, flavonoids regulate glucose metabolism in hepatocytes with subsequent improvement of hyperglycemia and decrease in insulin resistance [286]. Uptake of glucose by the cells is an important phenomenon in normalizing blood glucose levels and there are pharmacological evidences regarding beneficial effects of flavonoids on peripheral glucose uptake in both insulin sensitive and non-insulin sensitive tissues [49].

Antihyperglycemic effects of naringenin have been described in experimental animal models [287, 288]. This is in tandem with observations that grapefruit or its main flavonoid naringin, a naringenin glycone, has antihyperglycemic effects mediated by downregulation of hepatic PEPCK and G6Pase, respectively [289].

Furthermore, naringin has been reported to increase insulin sensitivity by enhancing tyrosine phosphorylation [290] suggesting its ability to relieve insulin resistance and also enhance glucose uptake in skeletal muscles perhaps by antagonising glucagon signaling pathways similarly to metformin, amongst others [278, 291, 292].

These observations, therefore, strongly suggest that naringenin alone or synegistically with other flavanoids have potential antidiabetic activity which could be mediated by AMPK, which is known to increase glucose uptake, fatty acid uptake and utilisation, and glycolysis in the heart and other peripheral tissues. Furthermore, six homoisoflavonoids and one dihydrochalcone have been reported to have antidiabetic effects by activating AMPK [293].

1.13.2 **Antidyslipidemic effects of naringenin**

It was previously reported that naringin, improved diabetic dyslipidemia in experimental animal models by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) and Acyl-CoA: cholesterol acyltransferase (ACAT) which catalyze rate-limiting steps in cholesterol biosynthesis [294], similarly to previously published studies [295, 296]. Naringenin has been shown to prevent atherosclerosis in *Ldlr*$^{-/-}$ mice by preventing production and increasing oxidation of FFA, preventing hepatic steatosis, and overproduction of very low density lipoproteins (VLDL) [297].

Furthermore, naringenin ingestion was associated with lipid-lowering effects, reduced plasma markers of endothelial dysfunction and improved insulin sensitivity [298, 299]. Naringenin has further been shown to prevent dyslipidemia by preventing apoprotein B overproduction through activation of PPARα gene transcription and upregulation of its fatty acid oxidation downstream effectors [300, 301].

It has also been demonstrated that naringenin inhibits secretion of apoB-containing lipoproteins primarily by limiting the accumulation of TG in the endoplasmic reticulum (ER) lumen [295],

secondary to microsomal triglyceride transfer protein (MTTP) inhibition and activity leading to increase in the expression and activity of the LDLR [301, 302]. Naringenin protects against intercellular adhesion molecule-1(ICAM-1), suppress macrophage inflammation, inhibit leukotriene B4, monocyte adhesion and foam cell formation, induce of Heme Oxygenase1 (HO-1) and G0/G1 cell cycle arrest in vascular smooth muscle cells (VSMC) [303]. These events eventually downregulate atherosclerosis-related genes which are believed to play crucial roles in atherosclerosis [303]. Like metformin, antidyslipidemic and antiatherogenic effects of naringenin therefore appear to be effected by AMPK-mediated pathways [301].

1.13.3 **Antioxidant effects of naringenin**

Antioxidant activities of naringenin and other flavanones have intensively been investigated. Naringenin has previously been shown to boost antioxidant capacity *in vivo* by increasing hepatic concentrations of catalase, superoxide dismutase and glutathione reductase [304] to confer renoprotective effects by relieving oxidative stress in streptozotocin-induced diabetes [305] and to ameliorate daunorubicin-induced nephrotoxicity by mitigating AT1R, ERK1/2-NFκB p65 mediated inflammation [306]. Antioxidant effects of naringenin appear to be largely mediated by free radical scavenging effects due to the phenolic hydroxyl groups attached to the flavonoid structure [306, 307]. It appears from experimental evidence adduced so far that antioxidant effects of metformin and naringenin could be the convergence point of their overlapping pharmacological effects.

1.13.4 **Naringenin effects on hypertension and cardiovascular morbidity**

It has been have reported that naringin, a naringenin glycone, relieves hyperglycemia-induced cardiac fibrosis and cardiac hypertrophy in diabetic rats [308] and has general cardioprotective effects under experimental conditions [309, 310]. Recently, protective effects of naringenin on cardiorenal syndrome have been reported where it mitigated cardiac remodeling, cardiac dysfunction and left ventricular diastolic pressure in experimental animals [311]. Naringenin supplementation was found to improve hypertension in high-carbohydrate fed rats and stroke-prone hypertensive rats. Calcium-dependent K channels are important regulators of vascular relaxation and naringenin has been shown to activate large conductance of Ca^{2+}activated K^{+} currents in a concentration-dependent manner in rat tail artery myocytes [280].

These actions of naringenin are suggested to be consequence of its antioxidant effects that activate AMPK, PPARα–, MAP kinase-mediated signaling pathways [308, 312].

1.13.5 **Antineoplastic effects of flavonoids**

On the other hand, antineoplastic potential of naringenin is currently gaining recognition. Naringenin has recently been shown to have pro-apoptotic and chemo-sensitizing effects that halt the cell cycle, upregulate pro-apoptotic genes and inhibit procell survival signaling pathways *in vitro* [313]. It has been shown that naringenin inhibits proliferation of colon cancer and melanoma cells and reduces migration of neoplastic hepatoma and pancreatic cancer cells [314] amongst others.

Other studies have suggested that naringenin may have tamoxifen-like effects on breast cancer cell lines and induces apoptosis by activating STAT3 and NF-κB [315]. However, detailed molecular mechanisms of its anti-proliferative effects and apoptosis induction are yet to be determined although experimental evidence currently suggest that anti-neoplastic effects of naringenin similarly to metformin are mediated by PI3K/AKT and MAP kinase signaling pathways [316]. Even though experimental evidence is largely based in *in vitro*, naringenin appears to have the potential to be a useful adjunct to chemotherapeutic agents in the treatment of human cancers [313].

1.13.6 **Inflammation and hematological effects of naringenin**

Naringenin has been shown to inhibit leukocyte recruitment, reduce oxidative stress and boost antioxidant capacity by activating macrophage Nuclear Factor erythroid 2-Related factor 2 (Nrf2) which induces HO-1 expression and also suppress activation of Nuclear Factor-κB (NF-κB) in macrophages which causes production of pro-hyperalgesic cytokines such as Interleukin- 33 (IL-33), TNF-α, Interleukin-1 β (IL-1β) and Interleukin-6 (IL-6) [317, 318]. Naringenin has further been reported to prevent cholesterol-induced systemic inflammation and atherosclerosis, suppress production of pro-inflammatory cytokines in murine endotoxaemia model by inhibiting the NF-κB and MAP kinase signaling pathways and also reducing platelet activation [239, 319, 320]. Both metformin and naringenin therefore appear to exert anti-inflammatory effects through similar molecular signaling pathways by relieving oxidative stress in both *in vivo* and *in vitro* studies [320].

1.14 **AMPK activation, an attractive target in T2D**

AMPK is a major cellular regulator of lipid and glucose metabolism. Recent evidence shows that pharmacological activation of AMPK improves blood glucose homeostasis, lipid profiles,

blood pressure and insulin-resistance making it a novel therapeutic target in the treatment of T2D [321]. It is worth mentioning that the gold standard first-line treatment of T2D, metformin acts by activating AMPK through inhibition of RCC 1 leading to a decrease in both cytosolic and mitochondrial ATP/ADP ratio [266, 322]. Animal model studies with metabolic syndrome phenotypes have exhibited decreased AMPK activity in the muscles and evidence exists that AMPK activity is diminished in the skeletal muscle or adipose of humans with T2D or obesity [321, 323, 324]. Therefore, AMPK seems to be an attractive and promising target for the pharmacological treatment of T2D.

AMPK is a serine/threonine enzyme that mediates cellular homeostasis and controls key players of metabolic pathways [272]. AMPK exists as a complex of three subunits, the catalytic α1&2 subunit with an N-terminal kinase domain (KD) and the regulatory subunits β and γ; the α catalytic subunit has a Ser/Thr domain that has to be phosphorylated to ensure enzyme activity (an α1 catalytic subunit that is active in the absence of γ & β subunits has been constructed; identifying an auto-inhibitory sequence between amino acid 313-392) [325].

The β subunit has glycogen-binding domains (GBD) which are suggested to facilitate the binding of AMPK to its substrate glycogen synthase and also serve as glycogen concentration sensors in the cell to mediate the synthesis of glycogen in response to glycogen and energy levels of the cell. The γ subunit regulates the activity of the enzyme by sensing cellular concentrations of AMP, ADP and ATP in the cell [272]. The γ subunit of AMPK also contains four copies of the Cystathionine β-synthase (CBS) sequence. Alexander Bateman first recognized CBS sequence by looking at internal sequence duplications within proteins of *methanococcusganaschii* genome [326].

AMPK activation is due to phosphorylation of Thr172 in the activation loop of α subunit and it is achieved by upstream kinases that include LKB1 and CaMKKβ [327]. Catalysis of AMPK is attributable to LKB1:STRAD:MO25 complex in response to increase in the AMP/ATP ratio which comes as a result of elevated Ca^{2+} levels by CaMKKβ (Fig. 11). Binding of AMP and ADP at the γ subunit results in activation of AMPK thereby promoting phosphorylation of Thr172 by upstream kinases and inhibiting dephosphorylation by protein phosphatases thus allowing AMP and ADP as allosteric activators of AMPK [272].

Thr172 is dephosphorylated by Protein Phosphatase 2C (PP2C) switching active AMPK to the inactive form [328].

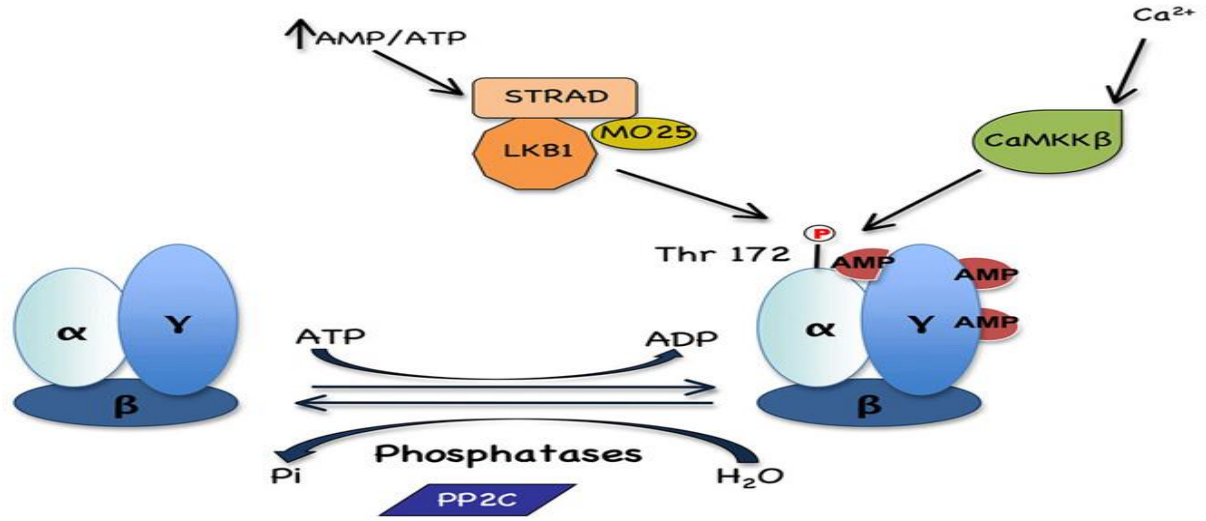


Figure 11. Regulation of AMPK activation [328].

Role of AMPK in regulation of lipid and glucose metabolism in the skeletal muscles. AMPK activity may be increased by an altered AMP or by hormonal action resulting in increased glucose transport as well as increased fatty acid oxidation as shown (Fig. 12) [328].

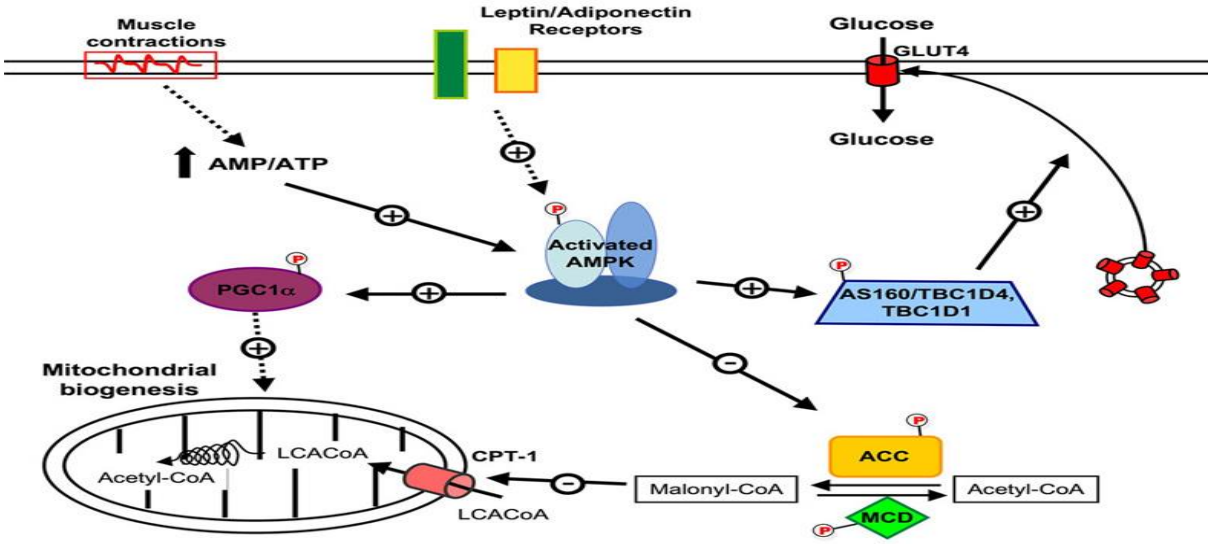


Figure 12. AMPK regulation of metabolism in the skeletal muscle; ACC, acetyl-CoA carboxylase; AMPK; (AMP-activated protein kinase); AS160, (Akt substrate of 160 kDa); CPT1-α, (Carnitine Palmitoyl Transferase-1); Glut4, (Glucose transporter 4); MCD, Malonyl-CoA Decarboxylase); PGC1α, PPARγ co-activator 1α; LCACoA, (Long Chain Acyl CoAs) [328]. In T2D, insulin fails to suppress gluconeogenesis and hepatic glucose production. AMPK suppresses gluconeogenesis by inhibiting the transcription of key regulatory

gluconeogenic enzymes PEPCK, G6Pase, and further inhibiting the synthesis of glycogen by deactivating glycogen synthase in hepatocytes. It will then downregulates the expression of gluconeogenic enzymes by inhibiting the transcription factors ChREBP and HNF-4 α [272].

1.15 **Rationale and motivation**

Naringenin has been shown to cause AMPK activation in L6 myotubes resulting in the enhancement of insulin sensitivity and a decrease in blood glucose levels in diabetic animal models [329]. The pharmacological and molecular similarities between metformin and naringenin have shown that naringenin can be used as an adjunct or substitute to metformin given the similarities, however the efficacy of naringenin could be limited due to presence of 3-OH groups attached to benzene rings [330].

Recent evidence shows that pharmacological activation of AMPK improves blood glucose homeostasis, lipid profiles, blood pressure and insulin-resistance making it a novel therapeutic targets in the treatment of T2D [322]. It has been shown that naringenin exerts its antidiabetic effects by inhibition of gluconeogenesis through upregulation of AMPK hence metformin-like effects. Naringin has further been shown to have non-glycemic affects like metformin that mitigate inflammation and cell proliferation.

Because of these similar pharmacological effects between naringenin and metformin, our laboratory has synthesized analogs of chalco-naringenin compounds which could be more permeable to the plasma membrane and hence putatively increase pharmacological effects. These compounds have nitro-groups attached to the benzene ring that are highly electron withdrawing and hence have shown good binding affinity to their target enzymes *in silico*. The structures of compounds were characterized, based on their spectroscopic data obtained from proton and carbon NMR and FT-IR. We propose that the lipophilic nature of these compounds could enhance permeation of cell membrane and therefore improve the binding affinity to AMPK.

If the compounds bind to AMPK, we hypothezise that they can activate it hence metformin-like effects [282].

The evidence of potential AMPK activation by these compounds was obtained through computational chemistry techniques by docking to enzyme. The synthesized compounds were shown to fit very well in the hydrophobic binding pockets of AMPK and presented the good binding affinity to their enzymes through computer simulation. Therefore, we hypothesize

those synthetic analogs of chalco-naringenin would possesses antidiabetic activity, and could be potential agents for the treatment for T2D.

1.16 **Aim**:

To investigate the effects of synthetic chalco-naringenin analogs on the expression of AMPK *in-vitro*.

1.17 **Objectives:**

i. To determine effects of chalco- naringenin analogs on cell viability and apoptosis.
ii. To determine the effects of chalco-naringenin analogs on quantitative expression of AMPK protein *in-vitro*.
iii. To determine the effects of chalco-naringenin analogs on glycogen synthesis in C2C12 and Chang cell lines.

Chapter Two

Experimental

2.1 **Materials and Chemicals**

The skeletal muscle cell lines (C2C12) and Chang were purchased from European Collection of Cell Cultures (ECACC). Unless otherwise stated, all chemicals and reagents used were purchased from Sigma-Aldrich™ (SA). The chalco-naringenin analogs were synthesized in Department of Pharmacology and Pharmaceutical chemistry UKZN. Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit was purchased from Biocom Diagnostics Africa.

2.2. **Synthesis of the minor flavonoids (chalcones)**

Chalcones/chalconoids are primarily synthesized using Claisen-Schmidt reaction; in this reaction, acetophenone is reacted with benzaldehyde in the presence of a strong base such as sodium hydroxide (NaOH), potassium hydroxide (KOH) or sodium hydride (NaH) as a catalyst in a polar solvent (scheme 1) [331].

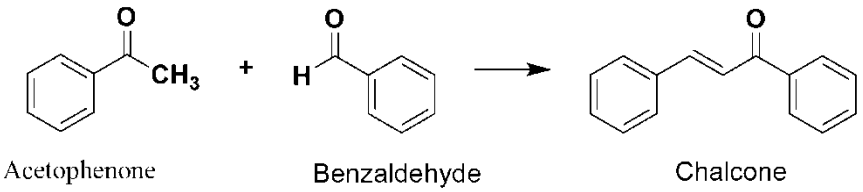


Figure 13: Claisen-Schmidt reaction, chalcone synthesis.

A series of 11 compounds of 4-[(cyclopropylcarbonyl) amino] chalco-naringenin analogues were synthesized using Claisen-Schmidt and characterized by ^{1}H-NMR and ^{13} C-NMR using a BrukerAvance III 400MHz spectrometer, the solvent that was used to dissolve compounds for NMR submission was deuterated chloroform ($CDCl_3$) and Infrared spectroscopy using a Perkin Elmer spectrum 100 FT-IR spectrophotometer . Characterization of compounds was also done using melting point determination by electrothermal 9300 digital melting point apparatus. The progress of reactions and their completion was monitored using analytical Thin Layer Chromatography (TLC) performed on plates pre-coated with silica gel (Merck $60F_{254}$, 0.25mm). Computer docking simulation studies against AMPK were done.

2.3 **In silico evaluation: Docking**

The 3-D complex structures of AMPK were obtained from the protein data bank using PDB code **4CFH**. The enzyme was then isolated from the ligand using Molegro Molecular Viewer

(MMV) software suite (www.nlcbio.com).The free enzymes were then used in all subsequent computational calculations. The 2D structures of compound **5a-5k** were drawn using the chemical structure drawing package - Chemdraw Ultra. Docking was done on Auto-dock Vina version 4, ligand interaction was done on Maestro software and visualization of poses and conformations was done on chimera software.

To this end, all these ligands were docked into the active site of AMPK. The docked compounds were then ranked according to the binding affinities against the target protein.

To validate the docking approach used in this study, the same docking procedure was applied to the experimentally determined compounds with known inhibitory properties of the respective enzymes. They were removed and re-docked into their respective enzymes and their binding affinities have been reported.

2.4 **Cell culture**

The C2C12 and Chang cells were cultured within 40 passages in 75 cm^3 flasks in DMEM with 4500 mg/L glucose and EMEM media containing supplemented with 10% fetal bovine serum (FBS), 10% penicillin-streptomycin, 10.0 mM HEPES and 1.0 mM sodium pyruvate. The cultures were maintained at 37°C in humidified air with 5% CO_2.

2.5 **Trypsinization and cell counting**

Trypsinization was done to detach cells from the culturing vessel once they reached 80% confluency, and to sub-culture and seed the cells for the various biochemical assays. This process involved rinsing the cells three times with warm 0.1M PBS (3 ml; 37°C) and incubating the cells with trypsin-EDTA (1 ml) for 2 min. The cells were then observed for roundness using an inverted light microscope (Olympus IXSI; 20 x). Once they were found to be round, trypsin was discarded and Cell Culture Medium (CCM) (5 ml) added to the flasks which were agitated to remove cells from the culturing vessel and the cell suspension was counted using hemocytometer. Trypan blue (0.4%) was applied in a dye exclusion procedure for cell counting. The principle of dye exclusion is based on damaged/dead cell membranes that allow entry of the dye into the cells and the cells stain blue whereas sustainable cells remain unstained.

2.6 **Chalcones and positive controls preparation**

Metformin and naringenin were dissolved in 100 % Dimethyl sulfoxide (DMSO) suitable for cell culture to a stock concentration of 4.0 mg/ 2 mls (15 mM) and 4.1 mg/ml (15 mM), respectively. Chalco-naringenin analogs (2-chlorophenyl and 2,3-dimethoxy phenyl) were dissolved in DMSO to a final concentration of 10 mg/ 2 mls and 10.8 mg/ 2 mls to 15 mM, respectively.

2.7 **Cell Exposure**

Cells were exposed to metformin (2, 5, 10, 12.5 mM), naringenin (50, 100, 200, 300, 500 μM), 2-chlophenyl (5b) (10, 20, 50, 100, 200 and 500 μM) and 2,3-dimethoxyphenyl (5k) (10, 20, 50, 100, 200 and 500 μM for 12 hours, 24 hours and 48 hours for viability and IC_{50} determination using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretrazolium bromide (MTT) assay. The doses of compounds used were similar to naringenin doses used in the previous studies. We decided to use similar doses to naringenin because these newly synrhisezed compounds are derived from naringenin although some chemical properties may differ somehow. To determine the effects of chalcones on AMPK, the cells were plated in 6-wells plates (5.0×10^5 cells/well) in the presence of 11 mM glucose only and allowed to grow to 80% confluence, followed by treatment with metformin (2, 10, mM), naringenin (50, 200, 300,500 μM), 2-chlophenyl (5b) (10, 20, 50, 100, 200 and 500 μM) and 2,3-dimethoxyphenyl (5K) (10, 20, 50, 100, 200 and 500 μM) for 24 hours. Co-treatment of cells with positive controls (naringenin and metformin) plus test compounds was also done. Cells were harvested and stored at -80 °C for glycogen determination and a glucose uptake assay. Vehicle controls constituted Cell Culture Medium (CCM) with or without chalcones were applicable. After the treatments, cells were harvested, protein determination was done and further biochemical analyses (Fig. 4).

2.8 **3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay**

Cell viability was assessed by MTT assay that is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. Cells (1.5×10^4) were aliquoted into a 96-well plates and incubated in wells containing 100 μl cell culture medium with 11.0 mM glucose, 20 μl of MTT (stock solution 8.0 mg/1.6 mls MTT in PBS) at 37°C for 4 hours. The cells were previously treated with metformin (2-12.5 mM), naringenin (50-500 μM) and chalcones (10-500 μM) at three different time intervals (12, 24, and 48) hours. MTT salt solutions were discarded and

100 µl of DMSO added to each well then incubated for 1.0 hour at 37° C to solubilize formazan crystals. All experiments were performed independently in triplicates. The absorbance was read at 570 nm with EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK). The percentage of cell viability was obtained using the optical density readings of chalcones treated cells compared to those of untreated cells (control), where percentage viability = $[A]_{test}$ / $[A]_{control}$ *100, where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of control sample.

2.9 **Flow diagram of experimental protocol**

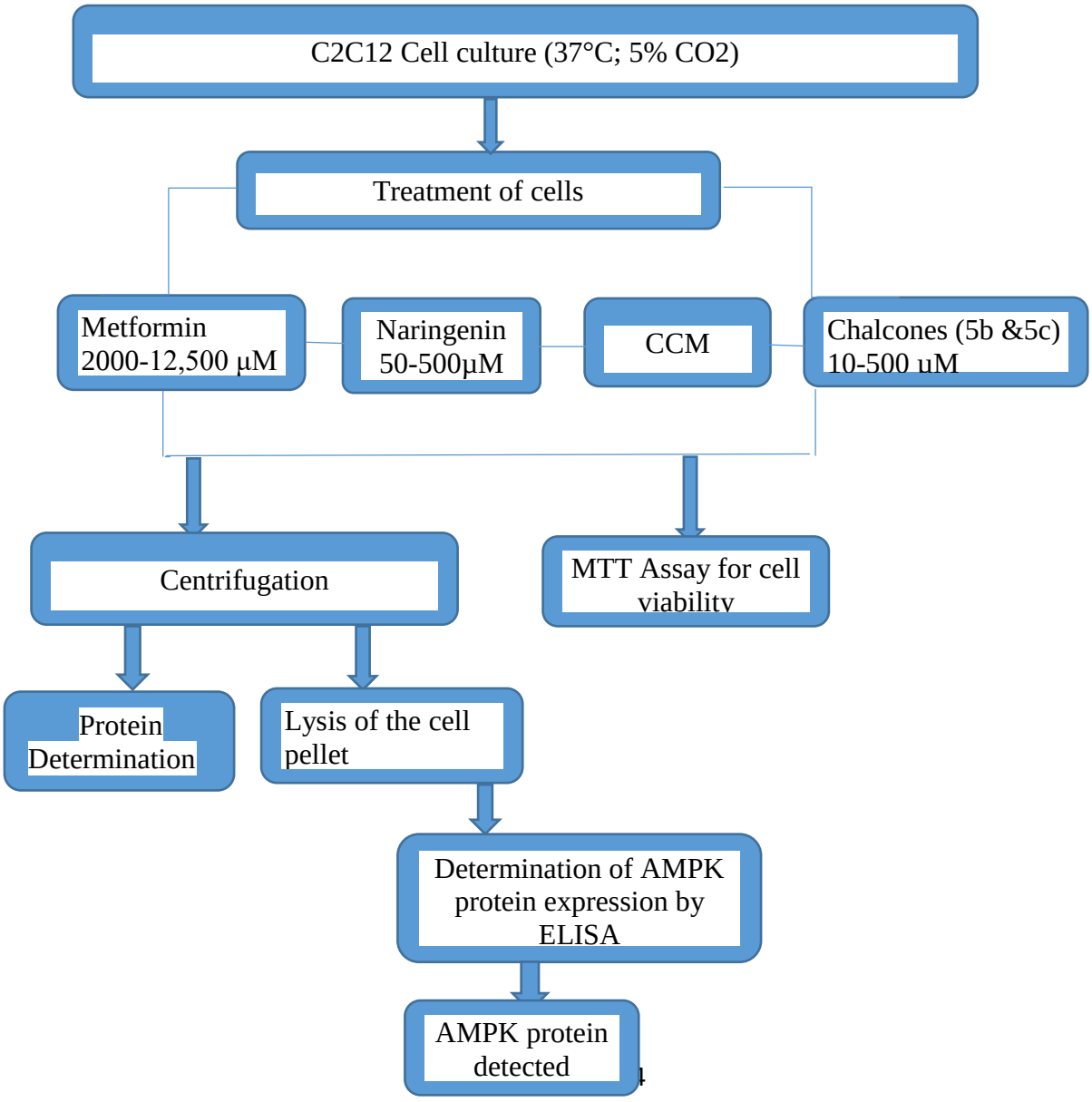


Figure 14. Flow diagram of experimental protocol.

2.10 **Glycogen Determination**

Glycogen measurement was performed in the liver (Chang) and muscle (C212) cell lines harvested after 24 hours incubation. According to Seifter *et al*. (1950), harvested cells ($1x10^6$ per mL) were put into a test tube and heated with 2 ml of 30% KOH at 100°C for 30 minutes [332]. The reaction was stopped by adding Na_2SO_4 (10%, 0.194 mL) into the test tube and then allowed to cool. The cooled mixture (200 µl) was aspirated and mixed with (95%, 200 µl) ethanol for glycogen precipitation. The precipitated glycogen pellet was washed and allowed to solubilize in distilled water (1.0 mL). Thereafter, anthrone (0.5g dissolved in 250 mL of sulphuric acid, 4 mL) was added and boiled for 10 minutes and allowed to cool down. After cooling, the absorbance was read using the spectrostar Nano spectrophotometer at 620 nm. Glycogen concentrations were calculated from the glycogen standard curve (Glycogen concentration (µg/ml) = CSF - (y-intercept)/ Slope * Sample dilution
CSF= sample fluorescence (CSF) for each sample

2.11 **Determination of AMPK by Phospho-AMPK α (Thr172) Sandwich ELISA kit**

Treated cells were collected by adding fresh media with low speed centrifugation (1200 rpm). Cells were washed three times with 5-10 ml ice-cold 1X PBS. Cells were then harvested from 5 mls of growth media and then lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM Phenylmethylsulfonyl Fluoride (PMSF). Lysates were sonicated on ice for 2 minutes. Cell lysates were micro centrifuged for 10 min at 14,000 rpm at 4°C and supernatant was transferred to a new tube. Cell lysates were stored at −80°C in single-use aliquots. Cell lysates were then diluted with sample diluent. An aliquot of 100 µl of each diluted cell lysate was aspirated in the appropriate well in the 96-well plate. The plate was sealed with tape and firmly pressed on top of micro wells. The plate was incubated for 2 hours at 37°C. The tape was then gently removed and each well was washed 4 times with 1X Wash Buffer, 200 µl each time. Reconstituted detection antibody (100 µl) was added to each well and plate sealed with tape and incubated at 37°C for 1 hour. The wells were washed and 100 µl of reconstituted Horseradish Peroxidase (HRP) linked secondary antibody was added to each well. The plate was sealed with tape and incubated for 30 min at 37°C. 3,3′,5,5′-Tetramethylbenzidine (TMB)

Substrate (100 µl) was added to each well, sealed with tape and incubated for 10 min at 37°C or 30 min at 25°C. Lastly, 100 µl of stop solution was added to each well. Absorbance was read at 450 nm within 30 min after adding stop solution using spectrophotometer.

3.0 **Statistical analysis**

Experiments were done in triplicates and all data presented as mean ± SEM. Statistical analysis was done using nonparametric test, Mann-Whitney U test or One way Analysis of Variance (ANOVA), to determine differences between groups and Dunnett's test for post-hoc analysis, at 95% confidence interval, where p-value of $p<0.05$ was considered statistically significant, using Graph Pad Prism® Software version 7.0 San Diego, California.

Chapter Three

RESULTS

3.1 **Chemistry**

A chalcone series of eleven compounds was successfully synthesized using the Claisen-Schmidt reaction. An intermediate compound, *N*-(3-acetylphenyl) cyclopropane carboxamide, was first synthesized by reacting 3-amino acetophenone with cyclopropane carbonyl chloride. The intermediate was reacted with differently substituted aldehydes to yield compounds **5a – 5k**.

The synthesized compounds fit very well in the hydrophobic binding pockets of AMPK (Fig. 15).

Table 2: Binding free energies of ligands docked onto AMPK (PDB code: 4CFH).

Compound	ΔG binding (kcal/mol)
5b	-9.2
5k	-8.7
Naringenin	-8.7
AMP	-11.8

The synthesized compounds showed characteristic peaks on IR, ^{1}H-NMR and ^{13} C-NMR. They also presented good binding affinity to the enzyme as shown by binding free energies suggesting potential metformin-like antidiabetic effects (Table 2).

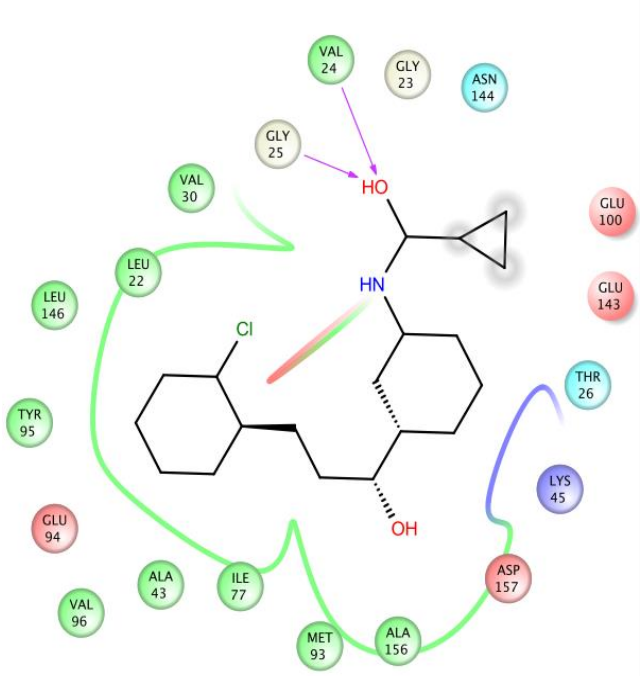
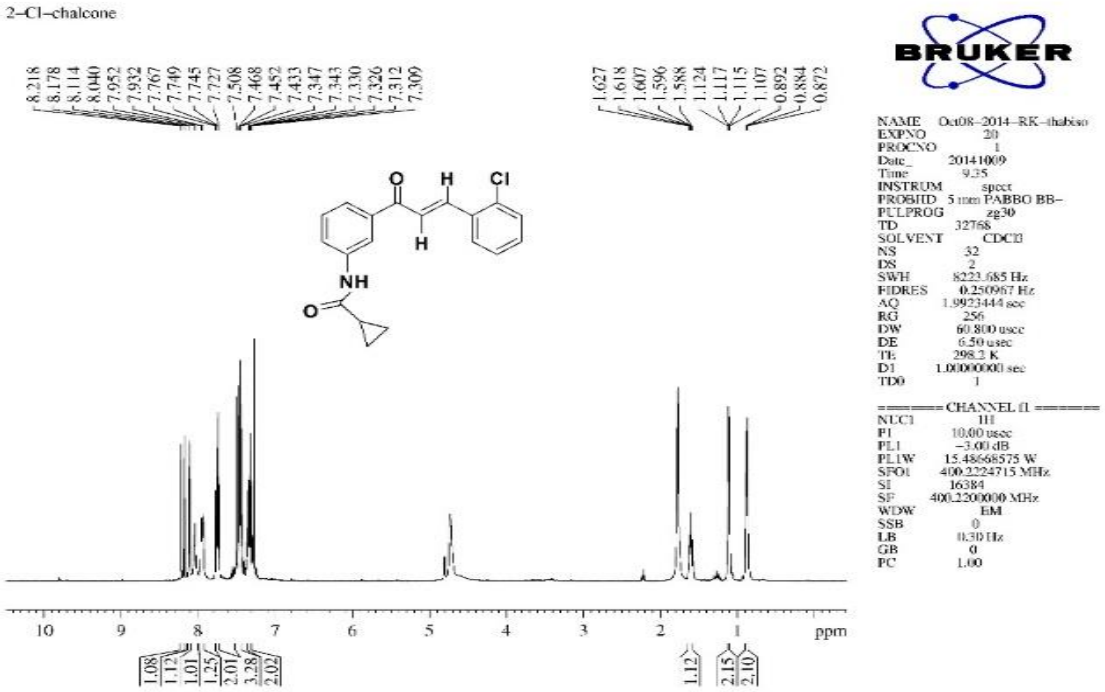


Figure 15. 5b-AMPK interaction (hydrophobic interactions shown in green).

Spectrum 1: C-NMR and 1H-NMR of compound 5b

A



B

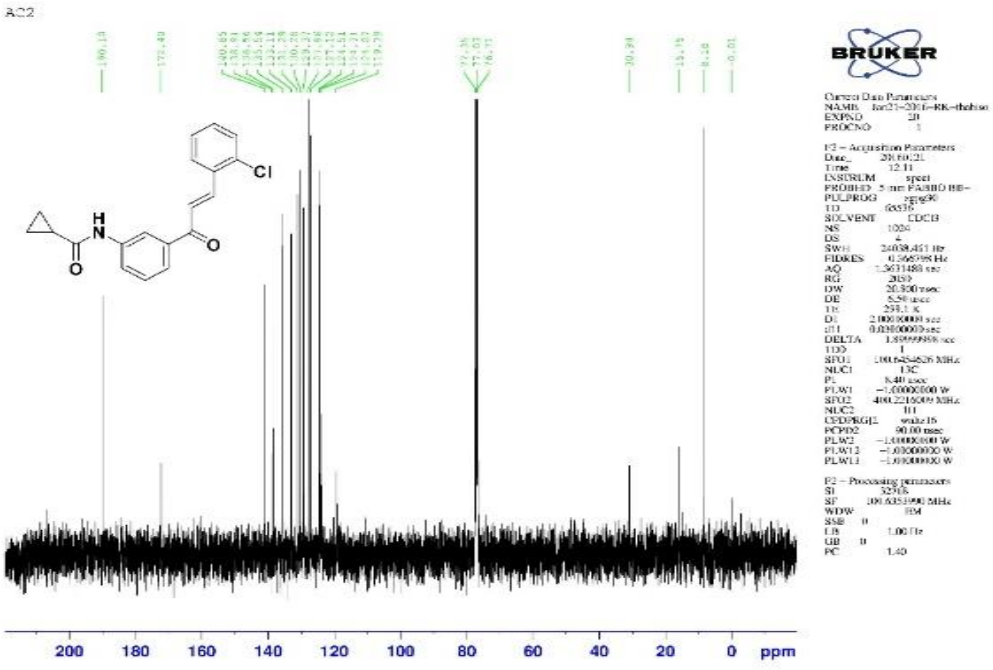
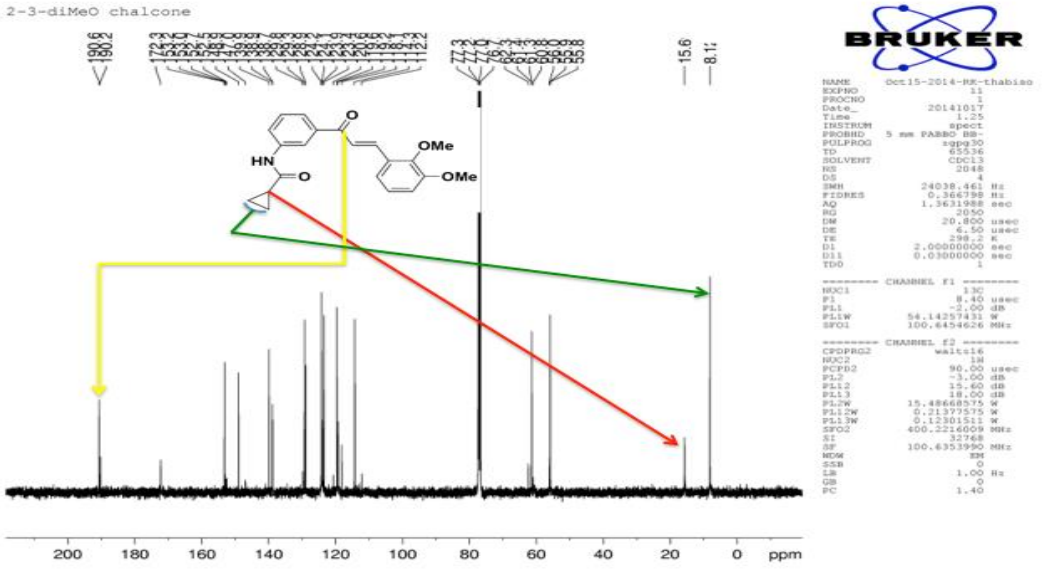


Figure 16. **A:** ^{13}C-NMR spectrum of compound 5b. There are certain distinct peaks on the spectra that identify carbon atoms on the compounds. The peak appearing at ≈ δ 8 ppm on the ^{13}C-NMR spectras indicates the presence of the secondary carbon (CH_2) of the cyclopropylcarbonyl amide. **B:** ^{1}H-NMR spectra of compound. The chemical shift for all the CH_2 protons on the cycloalkane resonated around δ 1.16 – 0.80 ppm as multiplets, while the other CH multiplet resonated around δ 1.62 – 1.58 ppm.

Spectrum 2: 1H-NMR C-NMR of compound 5k

A



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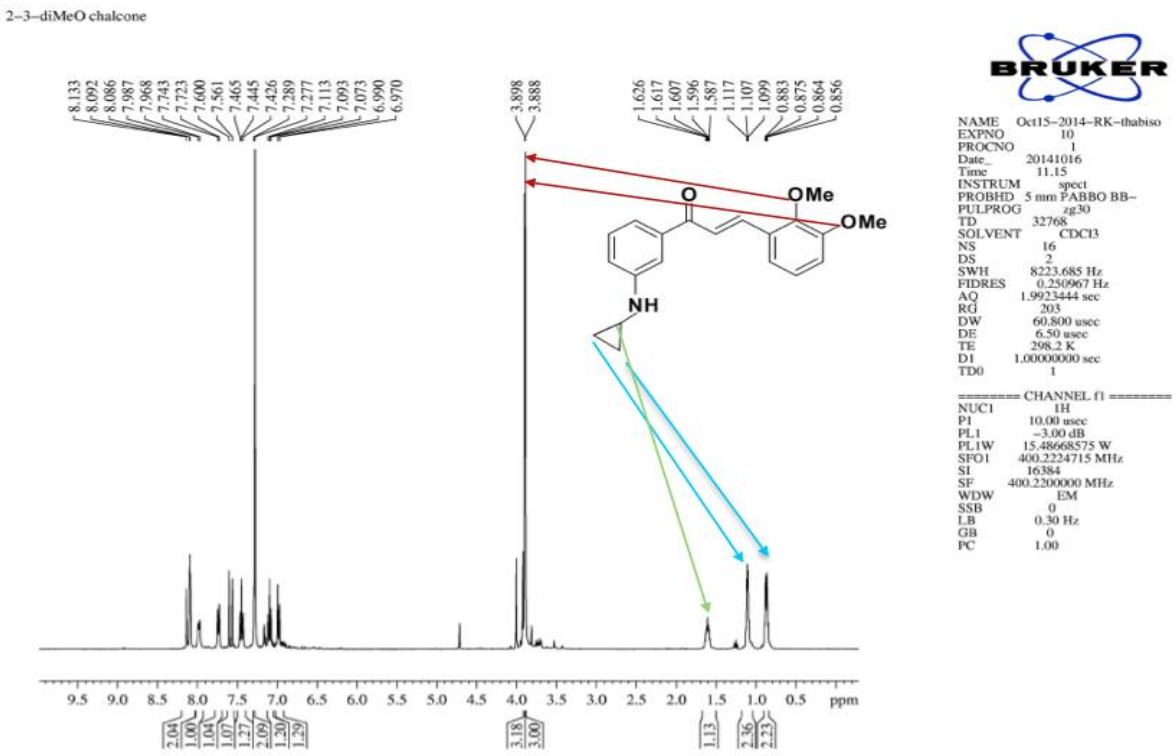
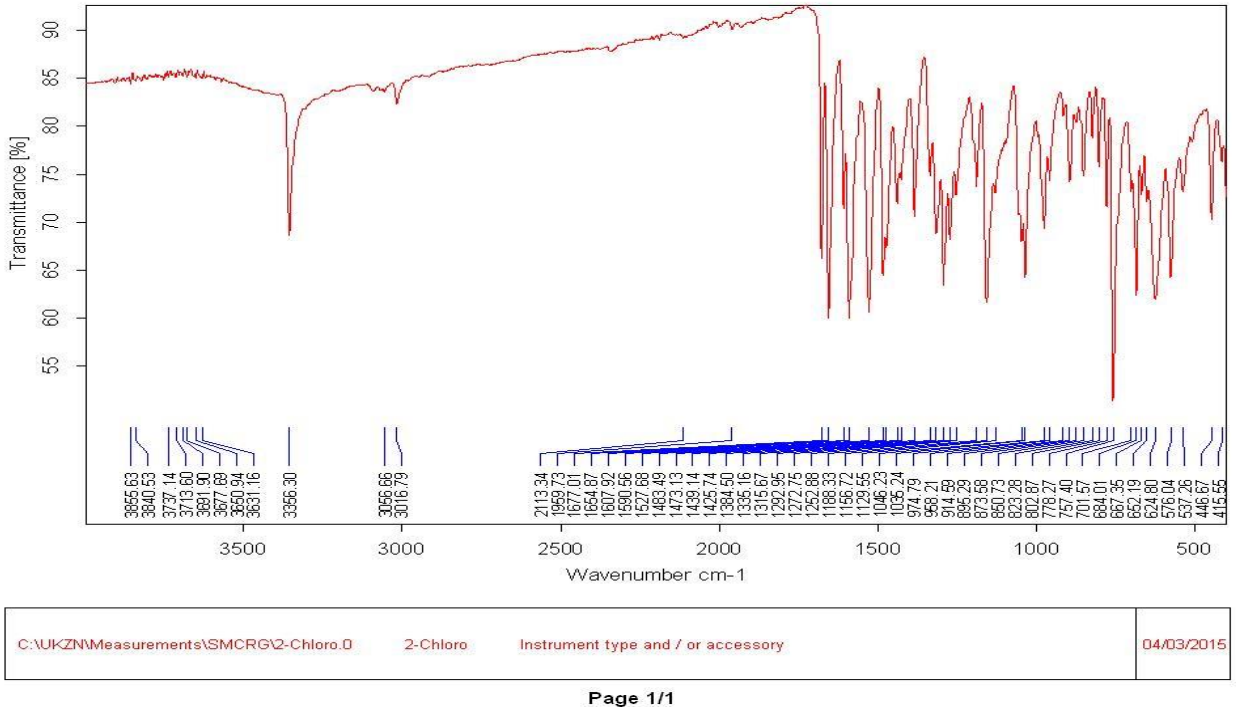


Figure 17. A: ^{13}C-NMR spectrum of compound 5k. Carbonyl group of the chalcone moiety shown by the yellow arrow, CH2 group of the cyclopropylcarbonyl amide shown by a green arrow and the CH group shown by a red arrow. **B:** ^{1}H-NMR spectra of compound 5k, two CH_2 groups of the cycloalkane represented by blue arrows, CH group represented by the green arrow and the two sets of 3H on the methoxy groups represented by the red arrow.

Spectrum 3: IR spectrum of compound 5b & 5k

A



B

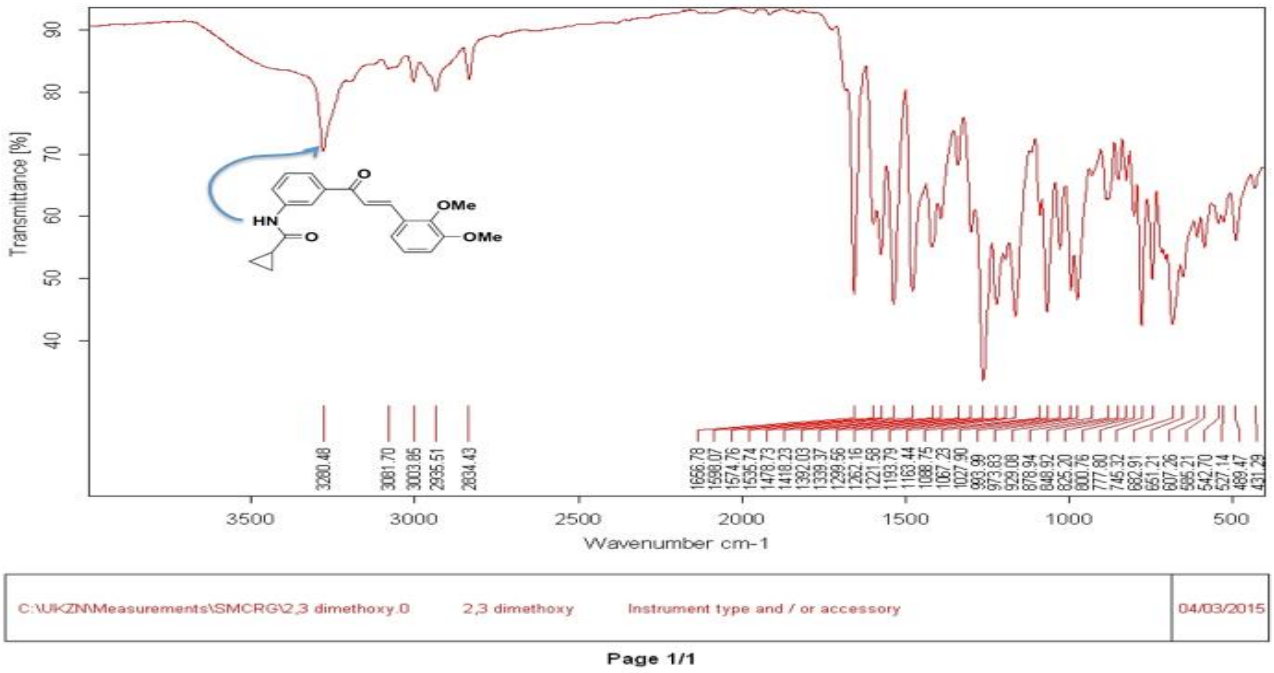


Figure 18. A: The IR spectra of compound 5b shows a consistent N-H stretch of the amide around wavelength number 3356.30 to 3249.67 cm^{-1}. **B:** The IR spectra of compound 5k shows a consistent N-H stretch of the amide around wavelength number 3356.30 to 3249.67 cm^{-1}.

3.2 **Biochemical assays**

3.2.1 **Effects of chalcones on cell viability (MTT Assay)**

To determine the potential effects on cell viability and proliferation of chalcones 5b and 5k in C2C12 and Chang cell lines, cells were exposed to a range of concentrations (10 – 750 µM) for 12, 24 and 48 hours. Calculated IC_{50} values are shown in Table 4. IC_{50} is defined as concentration necessary to reduce the absorbance of treated cells (chalcones) by 50% compared to the control (untreated cells) [333] (Table 3).

Cell viability of the compounds were inversely proportional to concentrations at three different time intervals (Fig.19). The IC_{50} values when C2C12 cells were exposed to treatment with Chalcone 5b at 12, 24 and 48 hours were 53.79, 13.97 and 32.83 µg/ml respectively.

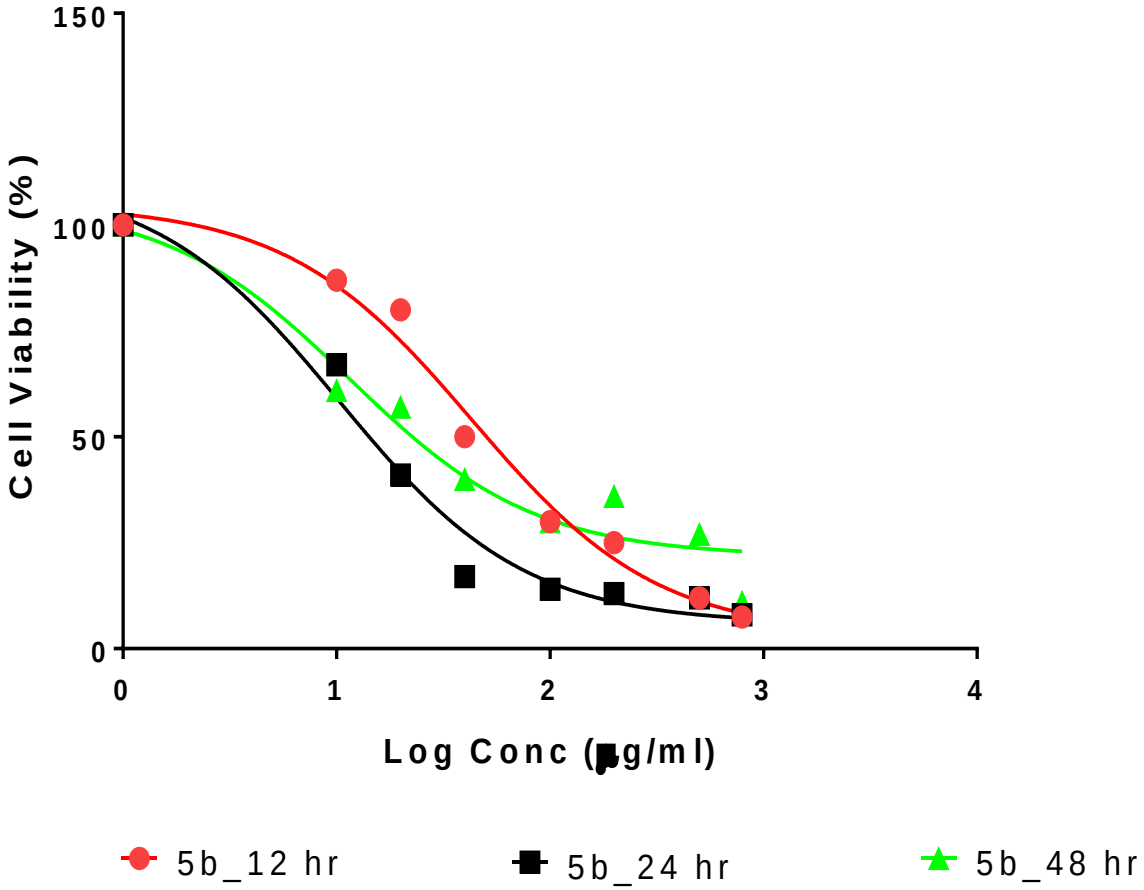


Figure 19. C2C12 cells viability measured by MTT assays, expressed as percentage of the controls following exposure to compound 5b (10-750 µM) in cell culture media for 12, 24 and 48 hours.

The IC_{50} values when C2C12 cells were treated with compound 5k at 12, 24 and 48 were 26.59, 8.44 and 20.99 µg/ml respectively (Fig. 20).

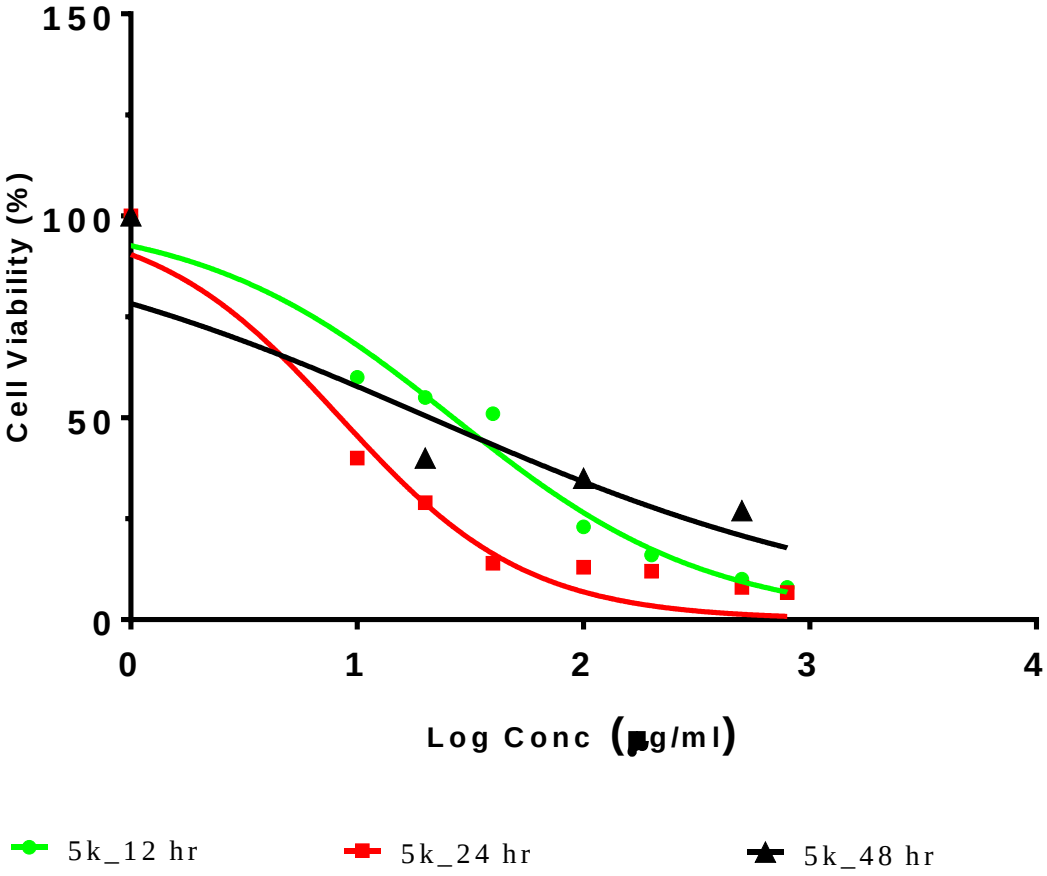


Figure 20. C2C12 cells viability was measured by MTT assays, expressed as percentage of the controls following exposure to compound 5k (10-750 µM) in cell culture media for 12, 24 and 48 hours.

Compound 5b when exposed to Chang cells at 12, 24 and 48 hours has shown IC_{50} values of 32.08, 34.97 and 50.07 µg/ml respectively to C2C12 treated cells (Fig. 21).

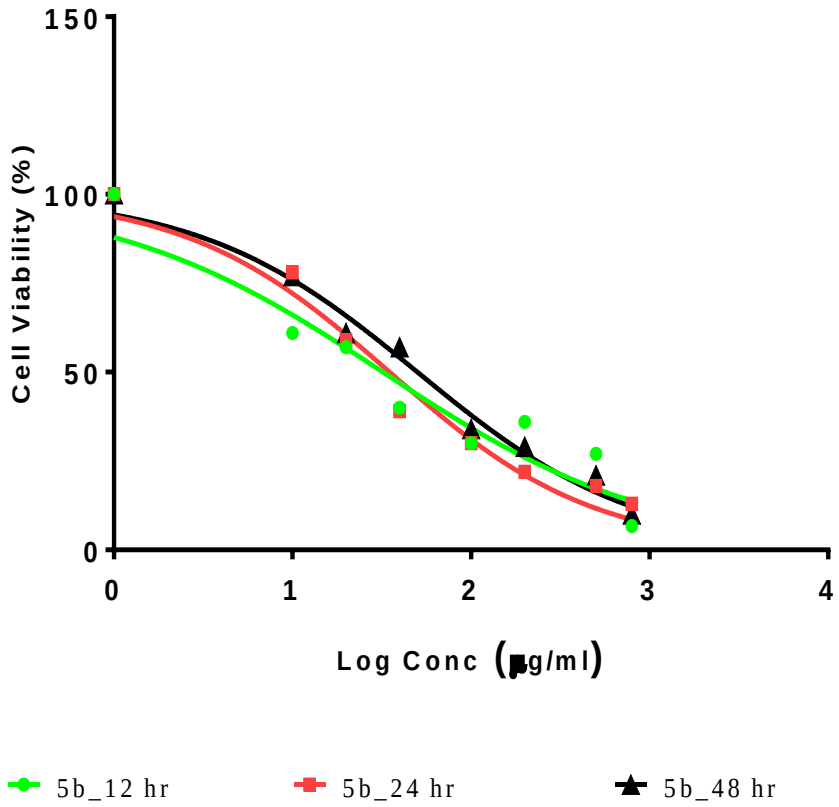


Figure 21. Chang cells viability was measured by MTT assays, expressed as percentage of the controls following exposure to compound 5b (10-750 µM) in cell culture media for 12, 24 and 48 hours.

The IC_{50} values when Chang cells were treated with compound 5k at 12, 24 and 48 were 25.38, 18.04 and 33.26 µg/ml respectively (Fig. 22).

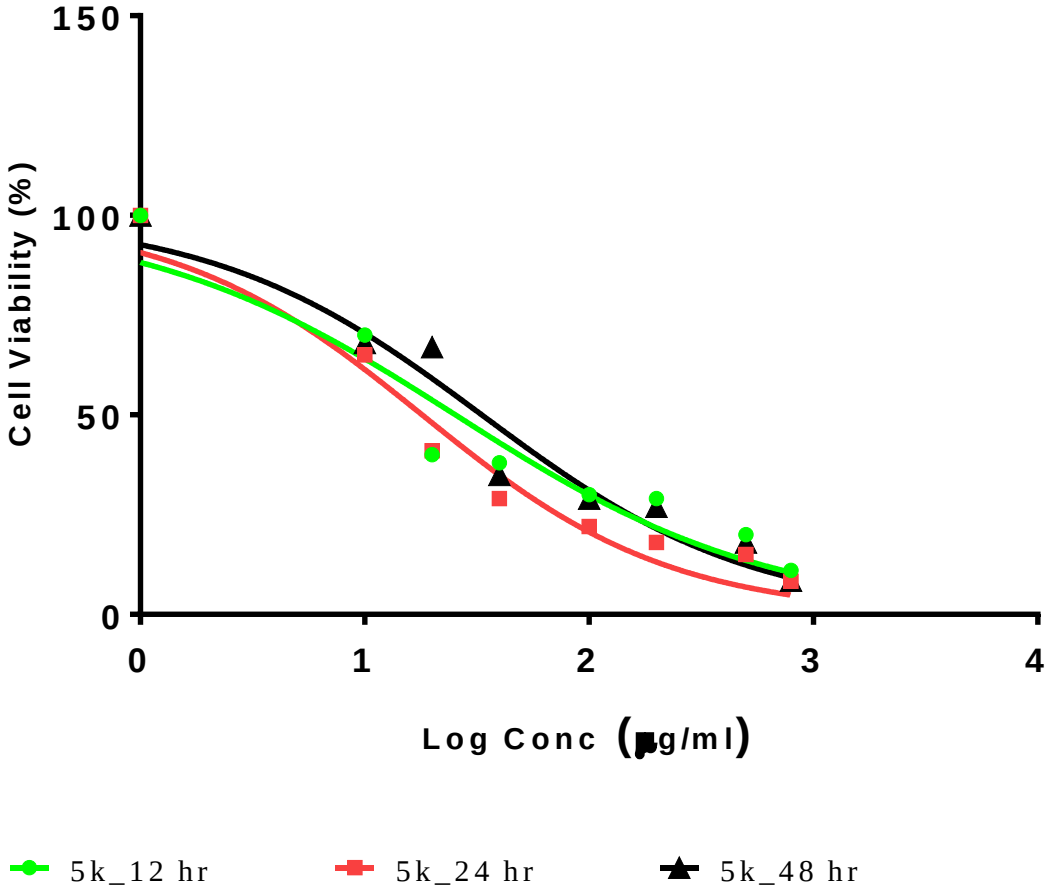


Figure 22. Chang cell viability was measured by MTT assays, expressed as percentages of the controls following exposure to compound 5k (10-750 µM) in cell culture media for 12, 24 and 48 hours.

Based on the findings from MTT assay, tested doses of cells that were incubated for 12 and 48 hours with chalcones had reduced cell viability and higher IC_{50} values, hence the concentrations at 48 hours were selected for further studies.

Table 3. Calculated IC_{50} values from MTT assays after the C2C12 cells were treated with 5b (2-chlorophenyl) and 5k (2, 3-dimethoxyphenyl) at three different time intervals.

Chalcones	Time (hours)	n	Mean concentrations (µM) ± SD of chalcones	IC_{50} (µg/ml)	R-Squared
C2C12- 5b	12	8	49.31 ± 0.05	53.79	0.9809
	24	8	32.75 ± 0.08	**13.97**	0.9429
	48	8	45.25 ± 0.12	32.83	0.9164

C2C12 - 5k	12	8	40.38 ± 0.06	26.59	0.9704
	24	8	27.84 ± 0.09	**8.436**	0.9538
	48	8	42.88 ± 0.22	20.99	0.7807
Chang-5b	12	8	50.14± 0.12	**32.08**	0.9127
	24	8	44.88 ± 0.06	34.97	0.9692
	48	8	53.5± 0.05	50.07	0.9827
Chang -5k	12	8	44.38 ± 0.12	25.38	0.9184
	24	8	42.63 ± 0.08	**18.04**	0.9571
	48	8	44.5± 0.08	33.26	0.9517

3.2.2 **Effects of chalcones on pAMPK activity**

Potential effects of chalcones 5b and 5k on AMPK were evaluated by analyzing the phosphorylated levels of the enzyme in total cell lysates by enzyme-linked immunosorbent assay. Interpolated concentrations of compounds were obtained from standard curves with corresponding absorbance values (Table 4). The magnitude of the absorbance, which was detected by development of the color, was directly proportional to the quantity of Phospho-AMPKα (Thr 172) protein. The total protein content in lysates was determined by Bradford assays and standardized.

Table 4: Calculated concentrations of pAMPKα (µg/ml) from standard curve

	Chang		**C2C12**	
Concentration	ABS (450 nm)	pAMPKα (µg/ml)	ABS (450 nm)	pAMPKα (µg/ml)
Controls (untreated cells)				
Controls	0.465	0.0810	0.359	0.0601
	0.497	0.0875	0.396	0.0671
	0.521	0.0925	0.468	0.0815
	0.547	0.0980	0.531	0.0947
Met (mM)				
2	0.812	0.1613	0.556	0.1000
5	0.825	0.1650	0.655	0.1221
10	0.847	0.1710	0.733	0.1410
12.5	0.901	0.1867	0.806	0.1597
NAR (µM)				

50	0.511	0.0904	0.501	0.0883
200	0.595	0.1084	0.556	0.1000
300	0.681	0.1283	0.588	0.1069
500	0.727	0.1393	0.648	0.1205
Chalcone 5b (µM) 10	0.461	0.0802	0.403	0.0685
50	0.504	0.0888	0.456	0.0792
100	0.548	0.0982	0.468	0.0815
200	0.574	0.1038	0.548	0.0983
500	0.643	0.1194	0.573	0.1036
Chalcone 5k (µM)				
10	0.484	0.0849	0.309	0.0507
50	0.503	0.0887	0.387	0.0655
100	0.508	0.0898	0.456	0.0792
200	0.540	0.0964	0.487	0.0855
500	0.553	0.0992	0.585	0.1062

The model of the standard data was fitted using a polynomial curve of second order, which was for both Chang and C2C12 at 24 hours interval. Unknown concentrations of pAMPK were interpolated from standard curve using optical densities (Fig. 23).

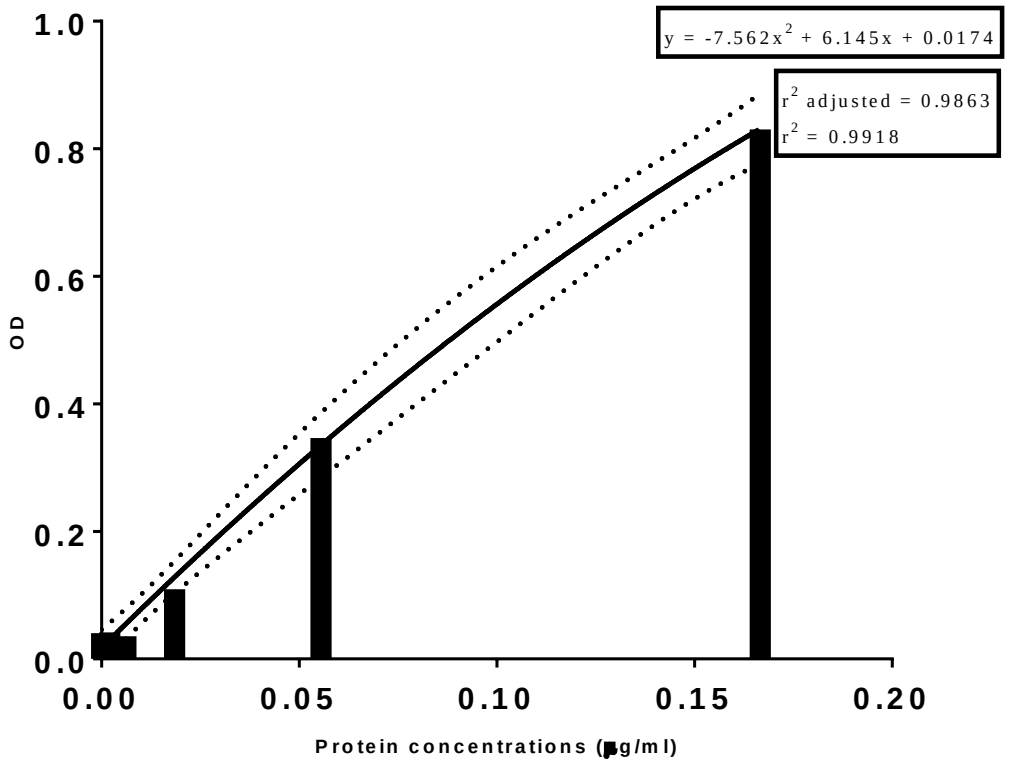


Figure 23. Standard curve showing interpolated concentrations of pAMPK using quadratic curve fit, (dotted lines showing the 95% CI asymptotic).

Metformin and naringenin significantly ($p < 0.01$ and $P < 0.05$, respectively) increased pAMPK levels compared to controls. However, cells exposed to co-treatment of chalcones with metformin and/or naringenin had significantly (Mt+5b, $p < 0.0001$; Mt+5k, $p < 0.01$; Nar+5b <0.05, $p < 0.05$ and Nar+5k, $p < 0.05$) increased pAMPK levels as compared to cells exposed to chalcones alone (Fig. 24). There were no significant differences in pAMPK levels between test compounds and the controls.

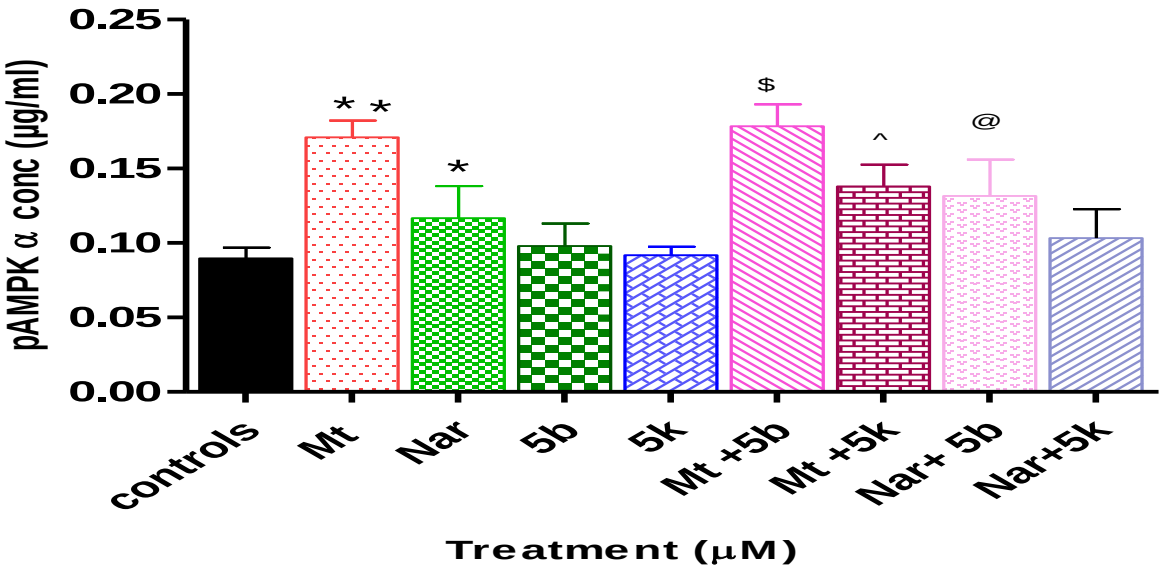


Figure 24. Expression of pAMPK after exposure to naringenin, metformin and chalcones on Chang cell lines after 48 hours incubation. Cells harvested, lysed and equal amount of proteins were used for ELISA with specific antibody recognizing phosphorylated Thr 172. **p < 0.01; *p < 0.05 compared to controls, $p < 0.0001 compared to 5b, ^p < 0.01compared to 5k, @p < 0.05 compared to 5b and #p < 0.05 compared to 5k, respectively. Metformin (Mt), naringenin (Nar), 2-chlophenyl (5b) and 2, 3-dimethoxyphenyl (5k), phosphorylated-AMPK (p-AMPK).

Metformin and or naringenin significantly (p < 0.01; P < 0.05), increased pAMPK levels compared to controls respectively on C2C12 cell lines. However, cells exposed to co-treatment of chalcones with metformin and/or naringenin had significantly (Mt+5b; p < 0.0001, Mt+5k; p < 0.01 and Nar+5b <0.05) increased pAMPK levels compared to cells exposed chalcones alone (Fig. 25). There was no significance increase in pAMPK activity in cells treated with naringenin plus compound 5k and compound 5k alone.

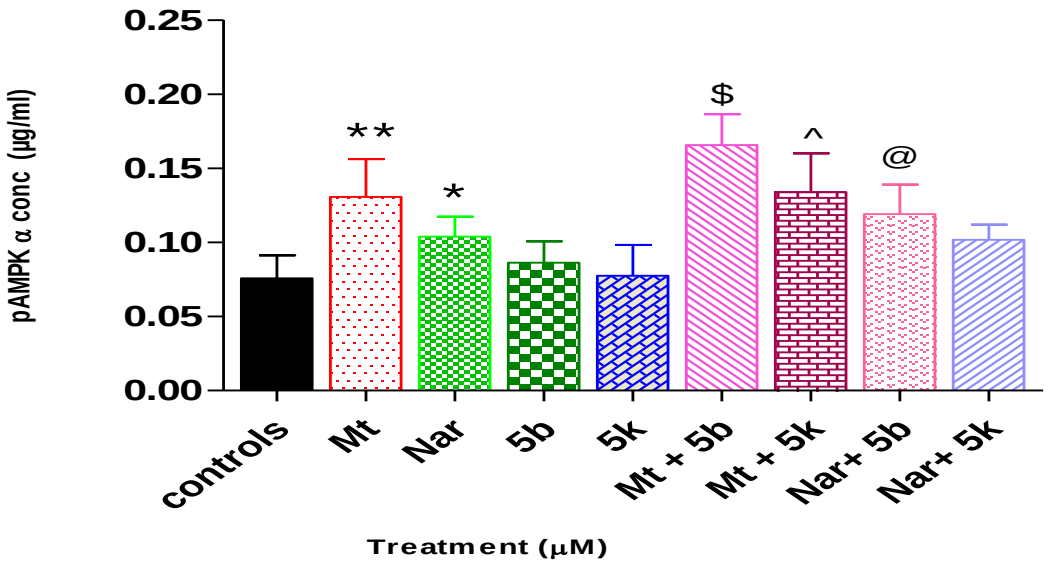
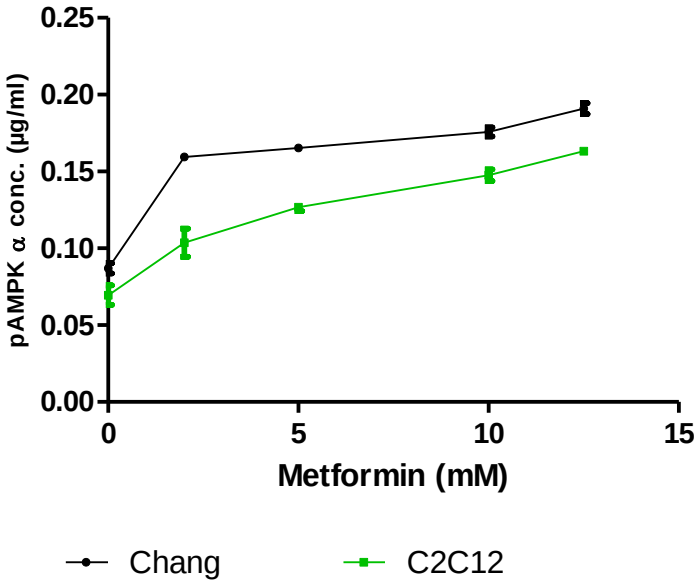


Figure 25. Expression of pAMPK after exposure to naringenin, metformin and/or chalcones on C2C12 cell lines after 48 hours incubation. Cells harvested, lysed and equal amount of proteins were used for ELISA with specific antibody recognizing phosphorylated Thr 172. **p < 0.01; *p < 0.05 compared to controls, $p < 0.0001 compared to 5b, ^p < 0.01compared to 5k and @p < 0.05 compared to 5b, respectively.

Concentration-dependent expression of p-AMPK following exposure to metformin, naringenin and chalcones (Fig. 26 A and 27 A). Chang liver cells were more responsive to phosphorylated AMPK effects compared to C2C12 cells (p<0.05 compared to Chang cells) (Fig. 26 B).

A


0.25
0.20
0.15
0.10
0.05
0.00
pAMPK α conc. (µg/ml)
0
5
10
15
Metformin (mM)
Chang
C2C12

B

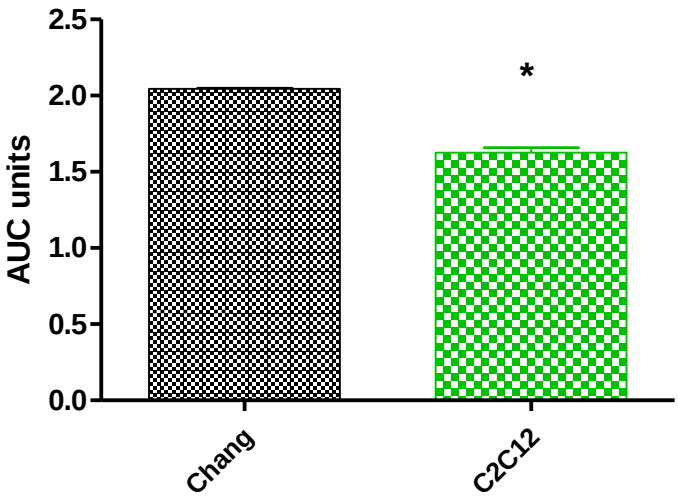
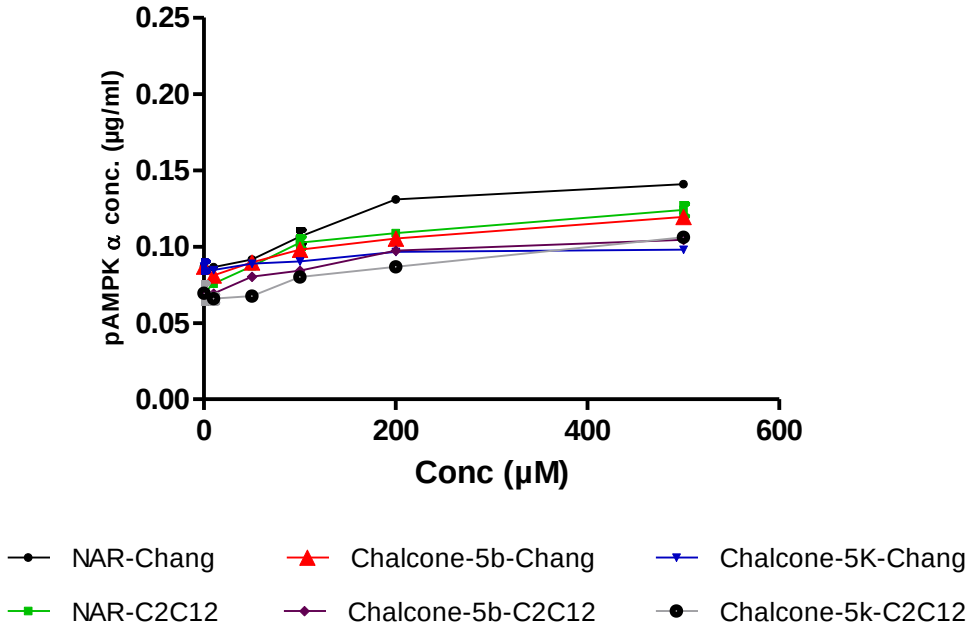

2.5
2.0
1.5
1.0
0.5
0.0
AUC units
*
Chang
C2C12

Figure 26. A: Metformin concentrations plotted against the activities of phosphorylated AMPK-α in Chang and C2C12 cell lines, respectively and **B**: calculated Area-Under-the Curve expressed as AUC units (µg/ml X mM). * p<0.05 compared to Chang cells.

Concentrations of chalcones were plotted against p-AMPK concentrations and concentrations of chalcones were directly proportional to concentrations of p-AMPK (Fig. 27. A). There was a significant difference in Chang-treated cells with Chalcones 5b and 5k compared to Chang-treated cells with naringenin, (P <0.05 compared to Chang cells; # p <0.05 compared to Nar-Chang cells). There was significant difference between C2C12-treated cells with chalcones compared to C2C12-treated cells with naringenin, (^ p<0.05 compared to NAR-C2C12 cells) (Fig. 27 B).

A



B

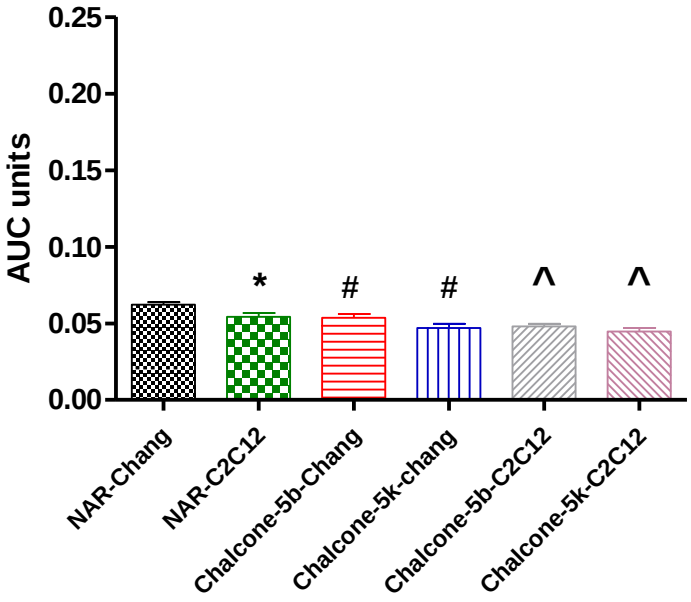


Figure 27. A: Concentrations of naringenin and its chalcones 5b and 5k plotted against the activities of phosphorylated AMPK in Chang and C2C12 cell lines, respectively and **B**: Calculated Area-Under-the Curve expressed as AUC units (µg/ml X mM). *p<0.05 compared to Chang cells; # p<0.05 compared to Nar-Chang cells; ^ p<0.05 compared to NAR-C2C12 cells.

Glycogen determination in Liver and Muscle cells

The model of the standard curve was fitted using a linear regression curve to determine unknown glycogen concentrations, for both Chang and C2C12 at 48 hours interval. Interpolations were conducted from the fitted curve for more results of glycogen concentration and the model fitted on the data has a variation measure (r^2 adjusted) of 99 07% (Fig. 28).

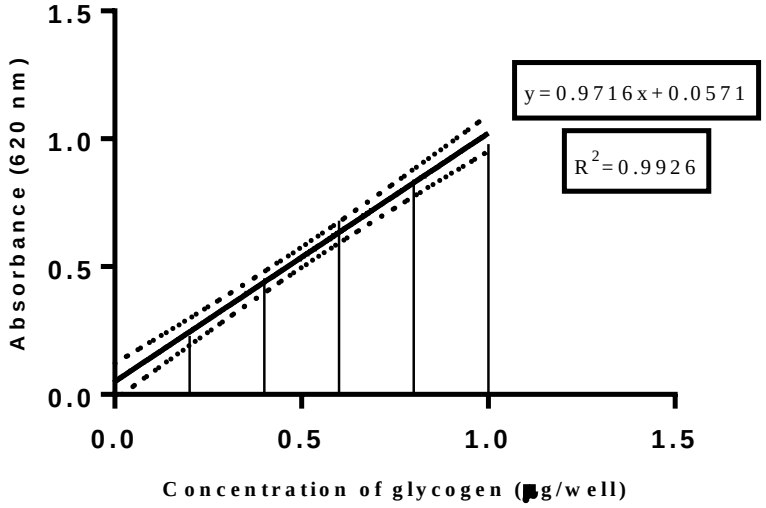


Figure 28. Glycogen standard calibration curve using fluorometric assay (dotted lines showing the 95% CI asymptotic).

The glycogen levels in metformin and naringenin exposure were significantly ($p < 0.05$) decreased compared to untreated controls. Glycogen content in cells exposed to chalcone 5b was reduced similarly to metformin and naringenin. However, there was no significance difference in glycogen synthesis in test compound 5k compared to naringenin and metformin (Fig. 29).

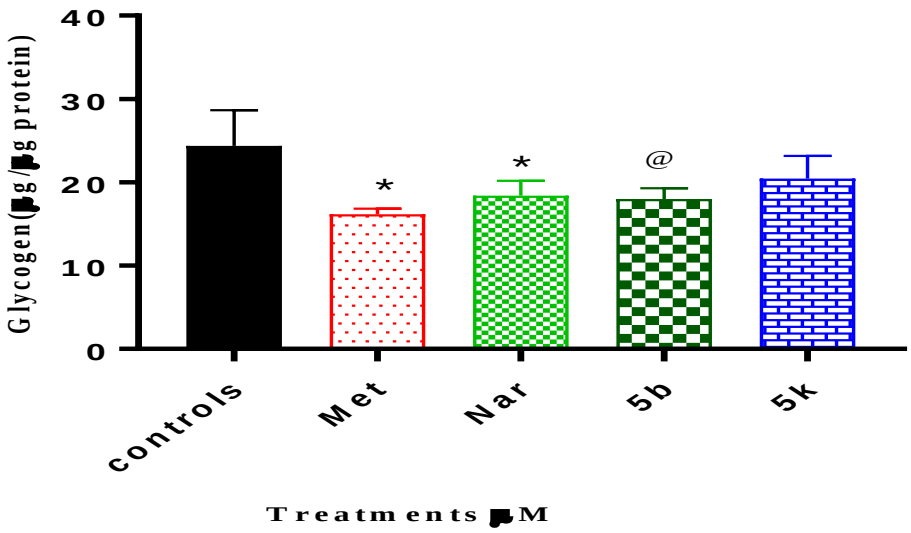


Figure 29. Glycogen synthesis in Chang liver cells that were treated for 24 h with metformin, naringenin, and chalcones respectively. Data presented as mean ± SEM, each carried out in triplicates and glycogen content was measured by spectrophotometer to obtain optical densities. *$p < 0.05$ compared to controls, $^{@}p < 0.05$ compared to control.

Metformin and naringenin significantly ($P< 0.05$) decreased glycogen synthesis compared to controls in C2C12 cell lines. However, there was no significant decreases in glycogen synthesis in cells exposed to chalcones compared to metformin or naringenin (Fig 30).

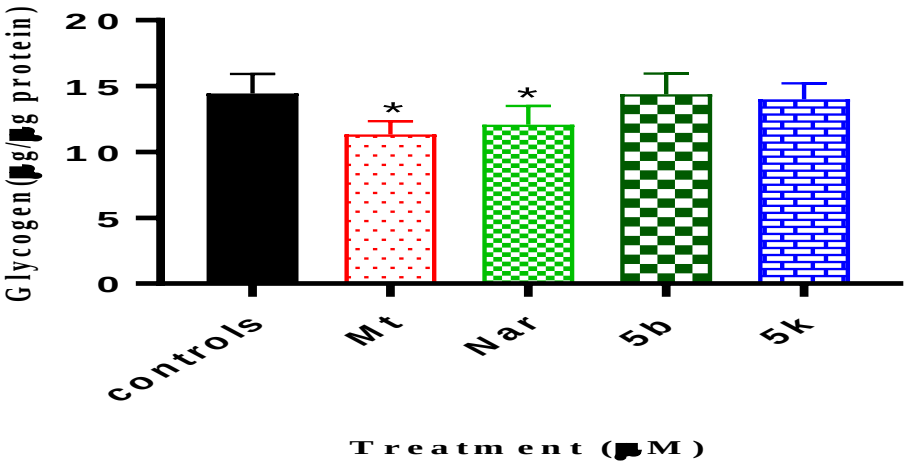


Figure 30. Glycogen synthesis in C2C12 cells that were treated for 24 h with metformin, naringenin, or chalcones, respectively. Data presented as mean ± SEM, each carried out in triplicates and glycogen content was measured by spectrophotometer to obtain optical densities. *$p < 0.05$ compared to controls.

Chapter Four

4.1 **DISCUSSION**

A number of patients fail to achieve optimum glycemic control with current medications hence there is an urgent need to continue working on the prevention and control of diabetes and its complications. In this regard, phytochemicals have recently attracted attention as source materials for the development of new potential therapeutic agents or alternative therapy for the management of diabetes and its related complications.

Naringenin has been reported to have antioxidant, antiatherogenic, anticancer and antidiabetic effects [335]. In the current study, we chose an *in vitro* model to determine the putative effects of chalco- naringenin analogs on C2C12 and Chang cells. C2C12 and Chang cells derived from the murine C3H muscle myoblast and human liver lines, respectively, were used in the study since they have a complete compliment of glycogen metabolism [336].

The Claisen-Schmidt condensation of acetophenones with aldehydes in the presence of a base or acid catalyst was the method used in the successful synthesis of 11 novel chalco-naringenin analogs. Although 11 chalcones were synthesized, only two analogs (compounds 5b & 5k) were used in the study because they were shown to fit very well in the hydrophobic binding pockets of AMPK as evidenced by docking results hence hypothesized to have metformin-like effects (Fig. 15). The binding free energies of ligands docked onto AMP suggested that the chalcones had comparable binding affinities to naringenin and endogenous AMP. Compound 5b had the highest biding affinity (-9.2 Kcal/mol) comparable to AMP and naringenin had a comparable binding affinity of -8.7 Kcal/mol.

These binding affinities were close or comparable to endogenous AMP (-11.8 Kcal/mol) which is a second messenger that directly triggers activation of AMPK in increased ratios.

The hydrophobic interactions may explain the less binding affinity observed for naringenin, which could be due to 3-OH groups present on the benzene ring thus makes naringenin less lipophilic with a reduced cell permeability. The pharmacophore of the test compounds is related to that of naringenin, which has been shown to bind to and activate AMPK hence suppression of blood glucose.

The compounds showed characteristic peaks observed on IR, ^{1}H-NMR and ^{13}C-NMR as shown by (Fig. 16-18) for 5b and 5k. On the ^{1}H NMR spectras, as expected, all compounds showed characteristic peaks for cycloalkane CH_2 protons at around δ 1.16 ppm as multiplets and the CH proton of the cycloalkane resonated around δ 1.62 ppm. The disappearance of signal of methyl protons and the appearance of new proton signals in the aromatic region confirms the

formation of chalcones (CH=CH). ^{13}C NMR also allowed characterization of the compounds, a distinct characteristic peak of the carbonyl group carbon found on the chalcone moiety, resonated around δ 190 ppm.

On the other hand, the other two characteristic peaks appeared on the cyclopropylcarbonyl amide substitution where the CH_2 group resonates around δ 8 ppm and the carbon of CH observed at around δ 15 ppm. Absence of methyl carbon signal, appearance of alkenic signals and aromatic carbons confirmed the product formation.

Docking of molecules on the active site of AMPK gives pharmacological insights on the potential *in vivo* and *in vitro* binding affinity of these molecules. Molecules with a high binding affinity *in silico* are more likely to bind to the target *in vivo*. The results from the docking scores suggest that compound 5b is more likely to bind to the active site of AMPK. This further indicates the likelihood of compound 5b as potential putative therapeutic agent in glycemic control that could be due to activation of AMPK, hence the aim of the present study.

AMPK is the regulator of diverse metabolic pathways and functions as a cellular gauge hence its actions on glucose metabolism make it an attractive target in the management of T2D [337]. It down-regulates hepatic glucogeonegenic enzymes and facilitates glucose transport into skeletal muscles. Attenuation of hyperglycemia is a major treatment goal for the management of diabetes. Given the hypothesized relation between anti-inflammatory, anti-dyslipidemia and the potential for naringenin to protect the body against free radicals and other pro-oxidative compounds, it is likely that consumption of naringenin may have a great potential in management of metabolic disorders.

Therefore, given the evidence from docking results, AMPK could be a potential target for synthetic chalco-naringenin analogs that could then result in therapeutic applications including management of metabolic disorders. Cells were exposed to different concentrations of chalcones (10-750 μM) and from these results cell viability was inversely proportional to concentrations at all-time intervals (Fig. 19-22). Cell viability of chalcones was compared to control (untreated cells). In this study, we endeavored to calculate IC_{50} values of newly synthesized chalco-naringenin analogs as potential agents for AMPK target on C2C12 and Chang cell lines.

Compound 5b when exposed to Chang cell lines showed higher IC_{50} value of 50.07 μg/ml at 48 hours pre-incubation, which could be because of less deleterious effects compared to 12 and 24 hours (Table 3).

Cell survival in low concentrations of chalcones 5b and 5k (10, 20 and 30 μM) indicated no significant decrease in cell viability compared to control (100%), but at higher concentrations

(200-750 μM) cell viability decreased sharply after 24 hours of incubation. Treatment of Chang and C2C12 cells for 12 and 48 hours with chalcone 5b and 5k increased cell viability, as determined by the MTT assay, indicating that the concentrations selected for the study had less deleterious effects during the pre-incubation (Table 3). However, at 24 hours incubation period, cell viability was dropping rapidly on C2C12 cells. This is also evidenced by IC_{50} values of 13.97 μg/ml and 8.43 μg/ml for compound 5b and 5k, respectively (Fig. 19 and 20).

The results demonstrated that there were significant differences in IC_{50} values of each compound against C2C12 and Chang cell lines (Tables 3). The estimated IC_{50} values suggested that chalcone 5b showed relatively low cytotoxic effects on both cell lines at three time intervals compared to 5k on both cell lines. However, both compounds did not evoke changes in cell viability, especially on Chang cells as compared to C2C12 cells (Fig. 19 and 21). From this results the conclusion can be made that the test compounds presented with higher cell viability and IC_{50} values on Chang cell lines as compared to C2C12 cell lines.

This was further evidenced by IC_{50} values estimated which were higher on Chang compared to control (Table 3). At 48 hours exposure, there was a significant increase in cell viability for both cell lines, which could be advocated to proliferative properties of cells and the preservation of the regular cell cycle. Hence it was suitable for further assays. Cell viability of compounds was reduced in a concentration dependent manner (Fig.19-22) and these trends were consistent with previous studies reported on naringenin [338].

Collectively, our results indicated that tested compounds (5b and 5k) showed remarkably lower toxic activity *in vitro* compared to controls (IC_{50} between 8.43- 53.79 μg/ml). The test compounds were exposed to cells for 48 hours for measurement of phosphorylated AMPK and glycogen synthesis. Metformin was used as a positive control in our study since we hypothesized that the synthetic analogs of naringenin exert similar effects to the well-known drug by AMPK mediated-pathways. Metformin has been shown to reduce glucose production either by decreasing gluconeogenesis and glycogenlysis through AMPK activation [231]. The postulated mechanisms of action to suppress gluconeogenesis include inhibition of uptake of gluconeogenic precursor lactate, inhibition of rate limiting enzymes (PEPCK and G-6Pase) and increased flux through pyruvate kinase secondary to decreased cellular ATP content [265]. There is substantial evidence suggesting that metformin suppresses hepatic gluconeogenesis by increasing AMPK activation [188, 229]. Therefore, compounds such as naringenin that activate AMPK may play a significant role in glucose homeostasis hence the study investigated the effects of chalco-naringenin analogs on the expression of p-AMPK *in vitro*.

The C2C12 and Chang cells were exposed to different concentrations of naringenin, metformin and chalcones for 24 hours. The protein expression of p-AMPK was significantly increased ($p< 0.01$) in metformin treated cells at concentrations (2-10 mM) as compared to control (Fig. 24 and 25). Previous studies that have shown that metformin activate AMPK by increasing the phosphorylation of AMPKα at Thr-172 in primary hepatocytes which could be through inhibition of the respiratory chain complex [233, 328, 342].

A subsequent study showed that hepatic knockout of LKB1, the upstream kinase for AMPKα phosphorylation at Thr172, abolished metformin suppression of hepatic glucose production that then suggests that activity of LKB1 is constitutive by promoting AMPK activation [343]. Although metformin is known to activate AMPK through LKB1 phosphorylation, it is unclear how the glucose-lowering effects are related to AMPK activation [344]. Inhibition of the respiratory chain complex 1 results in decreased cellular ATP synthesis thereby increasing cytosolic ADP: ATP ratio and consequently, an increase in AMPK activation, which in turn is associated with decreased gluconeogenesis [345].

Increased AMP levels lead to activation of AMPK, but this does not necessarily imply that AMPK mediates the therapeutic actions of metformin, as AMPK has multiple cellular targets. This study reports the potentiated effects of metformin plus test compounds on both cell lines evidenced by significantly elevated levels of pAMPK ($p<0.05$) compared to cells treated with chalcones alone (5b and 5k) only (Fig . 24 and 25).

The results show that metformin somehow enhances the effects of compounds 5b and 5k but the raised question is “how does metformin plus chalcones when exposed to cells in culture increase levels of phosphorylated AMPK?” Further studies could be carried out to clarify the phenomena and hence provide new insights into potential role of chalcones as putative therapeutic agents through AMPK mediated pathways. Treatment of Chang and C2C12 cell lines with naringenin also significantly increased phosphorylation of AMPK compared to control ($p <0.05$) (Fig. 24 and 25). The findings are in agreement with previously published studies by Zygmunt *et al*. (2010) who investigated direct effects of naringenin on skeletal muscle glucose uptake and the mechanism involved, further reported that antidiabetic effects of naringenin might be AMPK-mediated [337]. Hajiaghaalipour *et al*. (2015) also reported that antihyperglycemic effects of naringenin are mediated by downregulation of hepatic PEPCK and G6Pase respectively thus supporting findings of the present study [346]. Therefore, because of these beneficial effects of naringenin in attenuation of hyperglycemia and related complications, further investigations can be made for mechanisms at cellular and molecular

level. There was no significant increase in levels of pAMPK in cells treated with naringenin and chalcone 5b and 5b only (Fig. 26 and 25).

The findings are similar to that of metformin when compound 5b was added to it, therefore we can conclude that the effects which were seen were that of naringenin or metformin. These results suggest that the effects of chalcones did not enhance the effects of naringenin or metformin rendering opportunity to further explore chalcones *in-vivo iv-vitro* again and later in clinical trials. However, compound 5k when co-exposed to both cell lines with naringenin did not have any significant difference in phosphorylated AMPK (Fig. 24 and 25).

Additionally, there was no significant difference of phosphorylated AMPK in test compounds as compared to controls ($p>0.05$) (Fig. 24 and 25). Furthermore, metformin concentrations were plotted against the activities of phosphorylated AMPK-α in Chang and C2C12 cell lines to calculate the Area-Under-the Curve (AUC). Metformin had the larger AUC, which suggests that metformin caused activation and upregulation of p-AMPK more compared to naringenin and chalcones (Fig 26 and 27). The results also indicated that induction of AMPK activity was more pronounced in the Chang cell lines compared to C2C12 ($p <0.05$) (Fig. 26. B). Although metformin effects on AMPK have been shown to be more pronounced, naringenin and its synthetic analogs nonetheless had effects on AMPK even though they were significant to a lesser extent (Fig. 27 A and B). There was significant difference of p-AMPK in NAR-Chang cells than NAR-C2C12 cells suggesting that, there was more up-regulation of AMPK by naringenin in Chang cells than C2C12 cells ($p < 0.05$) (Fig 27 B).

Although the chalcones are analogs of naringenin, their putative therapeutic applications could as well be through non-AMPK mediated pathways. The pharmacophore of chalcones is similar to that of naringenin but that does not render chalcones mechanism of action to be similar to naringenin. Moreover, the decreased significant difference of chalcones as compared to control could be due to concentrations of test compounds used (10-500 µM) therefore, we anticipate that high concentrations and/or longer-term experiments might be required in order to see effects of chalcones on AMPK in cells or tissues.

Glucose uptake assays were not performed to compare the magnitude of suppression of glucose metabolism by naringenin and/or metformin to that of chalcones hence future studies using primary cell lines and *in-vivo* models may help to clarify the effects of chalco-naringenin analogs on glucose uptake.

It was reported that naringenin stimulated glucose uptake when exposed to myotubes by inhibiting the activity of PI3K, a key regulator of insulin-induced GLUT 4 translocation, as shown by impaired phosphorylation of the downstream signaling molecule Akt [287, 347].

Metformin has been shown to stimulate glucose uptake through AMPK activation, however, it has also been shown to reduce elevated levels of FFA that increases hepatic glucose production and peripheral insulin resistance through AMPK independent mechanisms [231]. Therefore, it is possible that AMPK-independent actions of metformin can reduce circulating plasma FFA levels and contribute to reduced rates of gluconeogenesis without AMPK hence we hypothesize that the chalcones can reduce hepatic glucose production by similar mechanisms.

Moreover, naringenin also significantly reduced plasma and hepatic lipids, normalized glucose tolerance and insulin sensitivity independent of AMPK, hence enhanced glucose uptake in skeletal muscles [348]. More *in vitro* studies could be carried out to firmly establish the role of AMPK in mediating effects of chalcones. Treated cells with chalcones could be co-incubated with AMPK inhibitors, the most commonly used compound C to exhibit significant inhibition of several structurally related kinases to AMPK including PKCθ, PKA and Janus kinase 3 (JAK 3).

Additional studies will be required to further elucidate potentiated effects of metformin and naringenin when co-treated with chalcones on inhibition of hepatic glucose production [188]. Thus, the observed association of increased AMPK activity when cells were treated with test compounds plus metformin and naringenin can suggest that AMPK could be a potential target for synthetic chalco-naringenin analogs.

Glycogen is the principal storage form of glucose in animal and human cells which is highly branched and made of α (1, 4) linkages with α (1, 6) linkages occurring every 8-10 glucose units along the backbone [349]. Glycogen is synthesized primarily by the liver and skeletal muscles, but it can also be made in other organs. Glycogen content can be significantly altered upon disruption of glucose homeostasis in metabolic syndromes, such as diabetes [349].

The liver plays a critical role in maintaining blood glucose concentrations both through its ability to supply glucose to the circulation via glycogenolysis and gluconeogenesis in the post-absorptive state and to remove glucose from the circulation after meal ingestion [350].

Glycogen determination was estimated using anthrone reagent as described by Seifter *et al*. (1949). The linear regression curve was used to calculate unknown glycogen concentrations from absorbance values obtained by a spectrophotometer.

Metformin significantly decreased glycogen synthesis as compared to control ($p < 0.05$) (Fig. 27 and 28). Previous studies have also demonstrated that metformin decreases glycogen stores in dose-dependent manner in cultured hepatocytes [349, 351]. Metformin is known to have

beneficial effects on body weight and body composition, although the precise mechanism is unclear.

Metformin enhances uptake of glucose without prompting gluconeogenesis, however AMPK activation channels ATP synthesis towards utilization not glucose storage hence decreased glycogen stores. These could then explain why metformin reduces body weight hence obesity in T2D. Metformin has been shown to cause accumulation of AMP in the liver, resulting in the inhibition of adenylate cyclase [351]. The reduction of cAMP levels and PKA activity suppresses glucagon-induced gluconeogenesis in the liver. In addition, naringenin and chalcone 5b significantly decreased glycogen synthesis in Chang cell lines compared to control ($p < 0.05$) (Fig. 27).

However, in C2C12 cell lines there was no significant decrease in glycogen synthesis when cells were treated with chalcones. Chen *et al*. (2015) reported that flavonoids increase the glucokinase level, thus promoting glycogen synthesis to reduce G6Pase and phosphoenolpyruvate carboxykinase gene expression, thus inhibiting gluconeogenesis or glycogenolysis or both [353]. Unlike metformin, which could reduce glycogen synthesis by glucose utilization, naringenin could enhance glucose uptake but in different molecular mechanism to metformin (Fig. 27). Mulvihill *et al*. (2009) had reported that naringenin reduces lipid accumulation and promotes glucose uptake, however it does not act directly in the skeletal muscle therefore reduced muscle lipid and improved glucose utilization could be due to reduced lipoprotein uptake. They further demonstrated that naringenin prevents hyperinsulinemia, stimulate lipogenesis, and activates hepatic fatty acid oxidation resulting in prevention of hepatic triglyceride accumulation and subsequently normalization of VLDL overproduction that leads to amelioration of dyslipidemia [348].

Although we hypothesize that chalcones could enhance glucose uptake, in the present study the test compounds did not significantly decrease glycogen synthesis as compared to metformin in the skeletal muscles cells. Collectively, these findings demonstrate that chalco-naringenin analogs especially 5b could have remarkably favorable effects on AMPK expression, although further studies could be carried out to determine their cellular and molecular mechanisms and their potential in glucose uptake.

Chapter Five

5.1 **CONCLUSION**

Emerging evidence from various, epidemiological, animal and *in vitro* studies have confirmed the beneficial effects of dietary flavonoids in the treatment and management of T2D and its related complications through AMPK activation. We have showed that chalco-naringenin analogs could have potential in expression of AMPK through computational chemistry technique. However, when using an *in-vitro* cell culture based model chalcone 5b showed more potential compared to 5k when added to metformin or naringenin. Chalcones did not have antagonizing effects when co-exposed with naringenin and metformin. MTT assay was also done to measure cell viability and proliferation of newly synthesized chalco-naringenin analogs and 5k had reduced cell viability compared to compound 5b on Chang and C2C12 cell lines. Calculated IC_{50} suggested low toxicity on compound 5b however, some toxicity studies can be done in future since the compounds are newly synthesized.

5.2 Future studies

The study recommends future work using primary hepatocytes and *in vivo*-studies to clarify the mechanisms and develop therapeutic targets for the effective management of diabetes and associated complications.

5.3 Limitations of study

In the present study, the analogs did not have a prominent impact on AMPK as shown by docking studies. The rationale could be because of low concentrations of chalcones used in experiments and or pipetting errors, which could have resulted to lower efficacy of compounds.

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