In-vivo and *in-vitro* study of mechanism of action of 4 hydroxyisoleucine as an amino acid derived from fenugreek seed with anti-diabetic and properties

A thesis submitted for the Degree of Doctor of Philosophy

By Hamidreza Khalatbari Limaki

London Metropolitan University

Faculty of Life Science and Computing

April 2014

Author's declaration

I attest that the work presented in this thesis entitled " *In-vivo* and *in-vitro* study of mechanism of action of 4 hydroxyisoleucine as an amino acid derived from fenugreek seed with anti-diabetic properties" has not been submitted in support of any qualification in this or other educational institutions in the UK or elsewhere.

Hamidreza Khalatbari Limaki

Date : April 2014

Table of contents

Acknowledgements

I am very grateful to Professor Christopher Branford-White and Dr Kenneth White for giving me the opportunity to work and carry out my research in Institute of Health and Policy (IHRP) at London Metropolitan University. Their scientific knowledge, enthusiasm, passion, countless support and precious advise were a great help in my research and scientific journey.

I would like to express my gratitude to Dr Mohammad Reza Haeri who brought the idea for my research and provided me the facility to run the animal study in his laboratory at the University of Qom, Medical School in Iran. He helped me to develop my research skills and without his support this work would not have been achievable.

I thank my colleague, Ravi Velga for his great help and excellent cooperation to run the experiments and overcome the problem with his great scientific knowledge.

Many thanks to the members of technical support staff for ensuring the smooth running of all activities in the science centre.

I show all my gratitude to my parents for their support and encouragement. They sponsored all my research expenses in this presented work and persistently supported me financially and emotionally in all the conditions and situations.

I am deeply grateful to my wife, Leila Razavi for her moral and emotional supports. Her encouragement and understanding have given

me confidence and motivation to continue my work and overcome the problems and obstacles. I believe that without her continuous help I could not have completed this work. Thank you with all my heart for always taking care of me and love me.

Abstract

Diabetes is a progressive multi-factorial metabolic syndrome with serious short and long term complications affecting many of organs with high increasing prevalence in the world. Using herbs and their derivatives for treating diabetes has a long history in many traditional cultures across the world. Molecules and compounds were isolated from herbs are the basis of many therapeutics which we are using in medicine for treating a variety of health conditions. The seeds of fenugreek, Trigonella foenum graecum, commonly used as a spice in Middle Eastern countries and widely used in South Asia and Europe, are known to have anti-diabetic properties. In 1979, Hardman identified an unusual amino acid (2S, 3R, 4S) 4-hydroxyisoleucine (4HO-Ile) in a fenugreek seed extract as an active compound in fenugreek seed. It was so far found only in fenugreek seed, which is responsible for its anti-diabetic properties. Studies on 4-hydroxyisoleucine effects on type 2 diabetes and insulin resistant animal models revealed that it has anti-diabetic properties of enhancing insulin secretion under hyperglycaemic conditions, and increasing insulin sensitivity. Unfortunately, the available published researches for 4-hydroxyisoleucine are limited and its mechanism of actions is not clear. Here we describe for the first time the anti-diabetic activity of 4-hydroxyisoleucine in a model of type 1 diabetes as all the previous works focused on 4-hydroxyisoleucine activity in type 2 diabetes and insulin resistant condition. Treatment of streptozotocin-treated type 1 diabetes rats, where levels of insulin are much reduced, by 65%, compared to normal animals, with daily doses of 4-hydroxyisoleucine at 50 mg/kg/day for four weeks could reduce plasma glucose in the diabetic group. Moreover the high levels of lipids (cholesterol, HDL,

LDL and triglyceride) and uric acid in the diabetic rats, could be restored to levels found in non-diabetic controls by the treatment with 4-hydroxyisoleucine. These results demonstrate that 4-hydroxyisoleucine has significant anti-diabetic activities in type 1 diabetes as well as previously studied type 2 diabetes and insulin resistance model that are independent of insulin. The findings suggest the potential of 4HO-Ile as an adjunct to diabetes treatment and for type 1 as well as type 2 diabetes.

To investigate the insulin-independent effects of 4-hydroxyisoleucine further, the cell based experiments were designed to assess the effect of 4-hydroxyisoleucine on cellular glucose uptake and ATP content after one day incubation. Isoleucine was added to the experiment as a positive control because firstly it has some level of anti-diabetic properties according to previously published studies and secondly it has similar molecular backbone as 4-hydroxyisoleucine. BRIN-BD 11, a functional and glucose responsive pancreatic beta cell, was selected as a cell model which is not insulin-responsive and dependent on the insulin signalling pathway for glucose uptake. Use of the model provides the opportunity to study the mechanisms of action of both 4-hydroxyisoleucine and isoleucine independently. We adopted a unique approach using inhibitors to target suggested pathway and molecules within the cell which may be involved in both 4-hydroxyisoleucine and isoleucine mechanism of actions. The results revealed that 4-hydroxyisoleucine and isoleucine could increase glucose uptake in BRIN-BD 11 cells, but as previously suggested, 4-hydroxyisoleucine activity is in direct correlation with glucose concentration. 4-hydroxyisoleucine has higher activity in higher concentrations of glucose. 4-hydroxyisoleucine increased the glucose uptake much greater than isoleucine at 11mM and 22mM concentration of glucose in

cell culture medium. Endpoint measurements of ATP content of the BRIN-BD11 cells did not show any significant changes between 4-hydroxyisoleucine and isoleucine groups and control as well as an insulin level measurement in culture medium after 24 hours. The results showed that there are substantial differences between isoleucine and 4-hydroxyisoleucine mechanisms of action unlike their similar glucose uptake stimulatory effect which is greater in 4-hydroxyisoleucine. 4-hydroxyisoleucine activity strongly dependent on new protein synthesis and GLUT 1 activity. GLUT 1 is widely available in most of the cells and it controls the basal glucose uptake independent of insulin. The connection between GLUT 1 and 4-hydroxyisoleucine effect in cellular level, supports the idea that 4-hydroxyisoleucine utilises the glucose basal consumption and uptake of cells. Mitochondrial calcium channel signalling inhibition affects 4-hydroxyisoleucine and isoleucine functionality as well as inhibition of mitochondria pyruvate carrier. Real time monitoring of cell metabolism by Seahorse XF-24 autoanalyser after 24 hours incubation with 4-hydroxyisoleucine and isoleucine revealed that both 4-hydroxyisoleucine and isoleucine activities are strongly dependent on mitochondrial respiration but 4-hydroxyisoleucine significantly up-regulates glycolysis which is not affected by isoleucine. The connection between mitochondria calcium signalling and contradictory behaviour of 4-hydroxyisoleucine and isoleucine support the very important role of mitochondria in their mechanisms of action.

Chapter I

Introduction

Diabetes mellitus refers to a group of metabolic diseases defined by hyperglycaemia (high blood sugar) and other classical symptoms such as polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). Diabetes is classified into four main types based on the pathogenic process which creates hyperglycaemia including Type 1, Type 2, Other Specific Types known as MODYs (Maturity onset diabetes of the young) and Gestational Diabetes **(Fig 1.1)**.

Type 1 diabetes is result of insulin deficiency which pancreatic cells can not produce and secrete insulin. Type 2 diabetes is a group of progressive disorders characterized by insulin resistance, impaired insulin secretion and increased gluconeogenesis (production of glucose). Diabetes can happen with other types of pathogenesis, such as exocrine disease of the pancreas, genetic defects in insulin secretion, mitochondrial abnormalities and mutation in the insulin receptor, which are categorized as Other Specific Type. Gestational Diabetes refers to glucose intolerance during pregnancy due to metabolic changes of pregnancy and increased insulin demands.

Diabetes is one of the main public health concerns as a major cause of mortality in most countries with a high prevalence rate, which has increased dramatically in the last two decades **(Fig 1.2)**.

Fig 1.1. **Spectrum of glucose homeostasis and diabetes mellitus (DM).** The spectrum from normal glucose tolerance to diabetes in type 1, type 2, other specific types of diabetes, and gestational diabetes is shown from left to right. In most types of diabetes, the individual traverses from normal glucose tolerance to impaired glucose tolerance to overt diabetes (these should be viewed not as abrupt categories but as a spectrum). Arrows indicate that changes in glucose tolerance may be bidirectional in some types of diabetes. For example, individuals with type 2 diabetes may return to the impaired glucose tolerance category with weight loss; in gestational diabetes, diabetes may revert to impaired glucose tolerance or even normal glucose tolerance after delivery. The fasting plasma glucose (FPG), the 2-h plasma glucose (PG) after a glucose challenge, and the A1C (Glycated hemoglobin or glycosylated hemoglobin or HbA1c) for the different categories of glucose tolerance are shown in the lower part of the figure. These values do not apply to the diagnosis of gestational diabetes. The World Health Organization uses an FPG of 110–125 mg/dL for the prediabetes category. Some types of diabetes may or may not require insulin for survival. *Some use the term "increased risk for diabetes" (ADA, American Diabetes Associassion) or "intermediate hyperglycaemia" (WHO) rather than "prediabetes." (Adapted from the American Diabetes Association, 2007.)

Fig 1.2. Worldwide prevalence of diabetes mellitus. Comparative prevalence (% of total population) and estimates numbers of diabetes patients (20–79 years), 2010. (Used with permission from IDF Diabetes Atlas, the International Diabetes Federation, 2000 Adopted from Harrison's Principles of internal medicine, 18th Edition)

Prevalence of Type 2 diabetes is increasing more rapidly due to increasing obesity, reducing physical activity and industrialisation. The increasing rate of diabetes will place a burden of large costs to societies which puts this disease at the centre of attention for all public health policy makers, researchers and medical groups. The number of people with diabetes in the UK has increased from 1.4 million in 1996 to 2.9 million and most of the cases is Type 2 diabetes (www.diabetes.org.uk). The figures announced by the WHO and the UK health authorities show that diabetes, particularly Type 2 diabetes, is one of the biggest health challenges in the world today. The seriousness and importance of this issue make diabetes research more in demand in order to find new solutions to reduce the rate of diabetes prevalence and new treatments to control diabetes and its complications.

1.1 Type 1 Diabetes

Type 1 diabetes is characterised by total insulin deficiency as a result of destruction of pancreatic beta cells by interactions of genetic, environmental and immunological (autoimmune pancreatic cell destruction) factors (Harrison's Principles of internal medicine, 18th Edition). The main pathophysiology of type 1 diabetes is the activation of an innate immune system response towards pancreatic beta cells, involving expansion of auto-reactive immune system cells such as CD4 , CD8 and T-Helper cells and producing auto-antibodies against beta cells (Bluestone *et al*. 2010). Multiple genes are involved in susceptibility to type 1 diabetes. The major susceptibility gene for type 1 diabetes is located in the HLA region on chromosome 6 which account for 40–50% of the genetic risk of developing type 1 diabetes (Bluestone *et al*. 2010). In addition to MHC class II associations, genome association studies have identified at least 20 different genetic loci that contribute susceptibility to type 1 diabetes, such as polymorphisms in the promoter region of the insulin gene, the CTLA-4 gene, interleukin-2 receptor, *CTLA4*, and PTPN22, etc. (Grant *et al*. 2009).

Type 1 diabetes is fatal and should be treated by injecting appropriate amounts of insulin to control high blood sugar levels. Type 1 diabetes can be distinguished from type 2 diabetes by measuring endogenous insulin production using the C-Peptide assay. Diabetic ketoacidosis (DKA) is the most important acute complication of untreated or poorly controlled type 1 diabetes and which needs special medical attention. DKA develops when the ratio of insulin to glucagon becomes decreased in a severe insulin deficiency condition, leading to ketone body formation in the liver and create acidosis, electrolyte imbalance and

metabolic complications (Harrison's Principles of internal medicine, 18th Edition).

1.2 Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes and has genetic predisposing factors. It is characterized by insulin resistance, impaired insulin secretion, obesity and abnormal fat metabolism (Harrison's Principles of internal medicine, 18th Edition). Insulin resistance is the main underlying pathogenesis of type 2 diabetes, and in turn leads to impaired insulin secretion and other metabolic disturbances. A glucose tolerance test is normal or near normal in the early stage of disease because pancreatic beta cells compensate insulin resistance by increasing insulin secretion (Harrison's Principles of internal medicine, 18th Edition).

It is expected that the level of serum insulin increases (hyperinsulinemia) in the early stages of type 2 diabetes as a response to impaired insulin action (Stefano Del Prato *et al*. 2002). Continuous stimulation of beta pancreatic cells to keep the compensatory output of insulin high leads to impaired insulin secretion as a result of exhaustion of the cells and failure to produce insulin **(Fig 1.3)**.

Type 2 diabetes risk increases with a positive familial history due to the strong genetic component with concordance between 70% and 90% in identical twins (Harrison's Principles of internal medicine, 18th Edition). The risk of type 2 diabetes can increase up to 40% if both parents are diabetic. Apart from genetic factors, environmental factors such as food intake, and exercise play an important role in developing diabetes. Studies show that there is a connection between consumption of sugar

sweetened drinks and risk of type 2 diabetes (Malik *et al*. 2010). Lifestyle and diet modification, are considered as a very effective preventive approach, especially for those people with higher risk, including people with obesity and a positive familial history for diabetes. NICE guide line recommends bariatric surgery for people with type 2 diabetes with BMI higher than 35 kg/m^2 as effect intervention to control obesity and diabetes. A recent study showed that bariatric surgery can significantly improve type 2diabetes and other metabolic risk factors in severely obese patients (Brethauer *et al*. 2013).

Fig 1.3. Metabolic changes during the development of type 2 diabetes mellitus (DM). Insulin secretion and insulin sensitivity are related, and as an individual becomes more insulin resistant (by moving from point A to point B), insulin secretion increases. A failure to compensate by increasing the insulin secretion results initially in impaired glucose tolerance (IGT; point C) and ultimately in type 2 diabetes (point D). (Adapted from SE Kahn: J Clin Endocrinol Metab 86:4047, 2001; RN Bergman, M Ader: Trends Endocrinol Metab 11:351, 2000) M Value is a score developed by McCauley et al in 2001 for measuring the Insulin Sensitivity Index (ISI) by the euglycemic-hyperinsulinemic glucose clamp technique. The ISI is calculated for fat-free body mass by dividing the whole body glucose disposal rate (M – mg/min or μmol/min per Kg) by the average plasma insulin concentration over the final 60 minutes of the 120 minute test (McAuley *et al*. 2001).

Many genes are involved in diabetes with subtle variation known as single nucleotide polymorphism (SNPs) which increase the risk of developing diabetes. Early whole genome linkage studies identified calpain 10 (CAPN10) and hepatocyte nuclear factor 4 alpha (HNF4A) as directly linked to diabetes (Dean *et al*. 2004). More than 36 genes have been identified in relation to the risk of type 2 diabetes (Herder *et al*. 2011). Genome-wide association studies (GWAS) have identified novel pathways linked to fundamental biology, which confirms previous epidemiological observations, showed the role of β-cell dysfunction in type 2 diabetes, explained about 10% of disease heritability, and determined possible targets for pharmacotherapy (Billings *et al*. 2010).

GWAS has discovered the variant rs13266634, which encodes a R→W change at position 325 in the beta cell zinc transporter ZnT-8 which encoded by SLC30A8 gene with strong associations with type 2 diabetes (Scott *et al*. 2007). The GWAS method provided potential genetic links between lipid dysregulation and glycemia (FADS1, GCKR, HNF1A), circadian rhythmicity and metabolic derangements (MTNR1B, CRY2), and low birth weight with subsequent type 2 diabetes risk (ADCY5) (Billings *et al*. 2010). The genomic study revealed that the risk of developing diabetes, especially type 2 diabetes is a combination of genetic risk for pancreatic beta cell dysfunction superimposed on genetic and environmental factors (e.g. obesity, Western diet, sedentary lifestyle) that promote insulin resistance.

1.3 Diabetes Complications

Chronic long term complications can develop in both type 1 and type 2 diabetes. The main chronic complications of diabetes include:

- Diabetic cardiomyopathy and diabetic vasculopathy
- Diabetic nephropathy
- Diabetic retinopathy
- Diabetic neuropathy

Studies show that type 1 and type 2 diabetes complications arise from a change in the balance of metabolites such as lipids, carbohydrates and blood coagulation factors (Mard-Soltani *et al*. 2011). Chronic complications may occur despite control of blood glucose. Studies show that rapid tight control of blood sugar levels worsens diabetes complication rather than improving the conditions (Taubes. 2008). Other studies showed that the improved glucose control after 41 months did not improve complications (Brinchmann-Hansen *et al*. 1988).

Hyperglycaemia is an important aetiological factor for diabetes complications but studies show there are many other unknown factors that may be involved based on the observation that complications develop despite control of glucose. Type 2 diabetes is a chronic disease with higher possibilities to develop chronic complications compared to type 1 diabetes with a ten year shorter life expectancy.

The risk of cardiovascular complications including ischaemic heart disease and stroke is increased by two to four times in type 2 diabetes and there is a 20 fold increase in lower limb amputations (Williams textbook of endocrinology. 2012 (12th ed,). Philadelphia: Elsevier/Saunders. pp. 1371–1435.ISBN 978-1-4377-0324-5).

Type 2 diabetes is associated with the most frequent cause of non-traumatic blindness and kidney failure. Other complications such as sexual dysfunction and risk of infection are increased in type 2 diabetes (Ripsin *et al*. 2009). Abnormal fat metabolism due to a lack of or impaired insulin secretion and insulin resistance lead to hyperlipidemia in diabetic patients. Increased levels of cholesterol and triglyceride are metabolic complications of diabetes and increase the risk of organ disease, particularly cardiovascular disorders. Elevated serum low density lipoprotein (LDL) is one of the most important side effect of diabetes as a result of fat metabolism abnormalities and it is a target for diabetes management to achieve an LDL level lower than 100mg/dl (2.6 mmol/l) (ADA, Standards of Medical Care in Diabetes - 2010. Diabetes Care, 2010). Abnormal lipid metabolism, increased circulatory lipid concentration and elevated deposition of lipids in the skeletal muscle are widely displayed in patients with type 2 diabetes and insulin resistance (McGarry, Banting lecture. Dysregulation of fatty acid metabolism in the aetiology of type 2 diabetes. Diabetes 2001; 51: 7-18).

Hypertension is another complication of diabetes which affects approximately 70% of diabetic patients (Rodrigo *et al*. 2007) and directly increases the risk of cardiovascular and kidney complications which need special attention and management.

Type 2 diabetes, insulin resistance, and untreated lack of insulin are associated with high levels of serum uric acid (Cappuccio *et al*. 1993). It has been shown that high serum uric acid increases the risk of Type 2 diabetes independent of the other risk factors such as obesity and dyslipidemia (Dehghan *et al*. 2008). High serum uric acid can occur in Type 1 diabetes and it is directly linked to renal damage, causes increased morbidity and mortality (Rosolowsky *et al*. 2008).

1.4 Diagnosis Criteria

Based on WHO guidelines, diabetes is defined by recurrent or persistent hyperglycaemia and is diagnosed by demonstrating one of the following criteria: ("Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications" (PDF). World Health Organisation. 1999)

- Fasting Plasma Glucose level in greater than 125 mg/dl (7.0 mmol/l) from at least two independent measurements.
- Two-hour plasma glucose greater than 200 mg/dl (11.1 mmol/l) after 75g oral glucose load (Oral Glucose Tolerance Test).
- Random blood glucose level greater than 200 mg/dl (11.1 mmol/l) in presence of diabetes symptom including polydipsia, polyuria and polyphagia.
- Haemoglobin A1C[1] (Glycated Haemoglobin) greater than 6.5%.

Fasting blood glucose levels from 110 mg/dl to 125 mg/dl (6.1 to 6.9 mmol/l) and oral glucose tolerance test above 140 mg/dl (7.8 mmol/l) but less than 200 mg/dl (11.1 mmol/l) are considered as impaired glucose homeostasis and are indicative of pre-diabetic status.

---

1 **Glycated hemoglobin** or **glycosylated hemoglobin** (hemoglobin A1c, HbA1c, A1C, or Hb1c; sometimes also **HbA1c**) is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymaticglycation pathway by hemoglobin's exposure to plasma glucose. Normal levels of glucose produce a normal amount of glycated hemoglobin. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin increases in a predictable way. This serves as a marker for average blood glucose levels over the previous months prior to the measurement. (Adapted from www.wikipedia.org , http://en.wikipedia.org/wiki/Glycated_hemoglobin)

The people at this stage are at a high risk to progress to full-blown diabetes mellitus and diabetic complication including cardiovascular diseases ("Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications" (PDF). World Health Organisation. 1999).

1.5 Insulin

1.5.1 Structure

Insulin is a polypeptide hormone with a molecular weight of 5808 Da synthesized in the beta cells of the Islets of Langerhans in the pancreas comprising two polypeptide chains, A with 21 amino acid residues, and B with 30amino acid residues, which are linked by disulphide bridges. Initially insulin is synthesised as pre-proinsulin with 110 amino acid residues which is processed in the endoplasmic reticulum to proinsulin containing 82 amino acid residues (Brunton LL, Chabner BA, Knollmann BC: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011). Proinsulin is comprised of chains A, B and the C-peptide. The C-peptide domain has 31 amino acid residues and keeps the insulin molecule inactive. Proinsulin will be transformed into active insulin by cleavage of C-peptide via endopeptidases which cleave the bonds between lysine 64 and arginine 65, and between arginine 31 and 32 **(Fig 1.4)**.

Conversion of proinsulin to active insulin is called maturation and is carried out in cytoplasmic granules by prohormone convertases 2 and 3 and carboxy-peptidase H (Hutton 1994). Insulin has a variety of biological effects in the body apart from maintaining glucose homeostasis. Insulin is one of the potent anabolic hormone which stimulates protein synthesis, lipogenesis, mitosis and cellular growth (Tamas Fulop *et al*. 2003).

Fig 1.4. Synthesis and processing of insulin. The initial peptide, preproinsulin (110 amino acids) consists of a signal peptide (SP), B chain, C peptide, and A chain. The SP is cleaved and S-S bonds form as the proinsulin folds. Two prohormone convertases, PC1 and PC2, cleave proinsulin into insulin, and C peptide. Insulin and C peptide are stored in granules and co-secreted in equimolar quantities. (Adopted from: Brunton LL, Chabner BA, Knollmann BC: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011)

1.5.2 Secretion

Insulin secretion from beta cells happens in two phases, rapid triggered and a slow release phase. The rapid triggered phase happens in response to various stimulator including glucose, arginine, leucin, acetylcholin, cholecyctokinin (CCK) (Cawston *et al*. 2010), glucagon-like peptide-1 (GLP-1), glucose-independent insulinotropic peptide (GIP) and epinephrine via ß2 receptors (Layden *et al*. 2010). Glucose is the major

physiological stimulus of insulin release, entering beta pancreatic cells via GLUT 2 transporters. Glucose is phosphorylated inside the cells by glucokinase (hexokinase IV) to glucose-6 phosphate (G6P) which is a rate-limiting step that controls glucose-regulated insulin secretion. G6P enters the glycolytic pathway in cytosol, converted to pyruvate. Pyruvate enters mitochondria to generate ATP through the citric acid cycle (Krebs Cycle). Increased the ATP/ADP ratio and elevated ATP inhibit ATP-sensitive potassium channels (Kir6.2, known as the sulfonylurea receptor) on the membranes of beta cells. Reduced potassium efflux leads to cell membrane depolarization which opens voltage- dependent calcium channels and an influx of calcium into the cell. Increased amounts of calcium stimulate exocytosis of insulin previously synthesized granules via activation of phospholipase C **(Fig 1.5)** (Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011 and Harrison's Principles of internal medicine, 18th Edition, 2012).

Glucokinase acts as the glucose sensor in pancreatic beta cells, and has unique features which distinguish it from other hexokinases and make it function as a glucose sensor. It has lower affinity for glucose compared to other hexokinases (higher Km of 10 mM, 100 times higher than other hexokinases with Km of 0.1 mM), such that the activity increases with rising concentrations of glucose and remains a half-maximal concentration of glucose at 144 mg/dl (8 mmol/l). Other hexokinases are inhibited by their products but glucokinase is not inhibited by G6P and remains active to stimulate insulin release amid significant amounts of its products (Matschinsky. 1996).

Fig 1.5. Mechanisms of glucose-stimulated insulin secretion and abnormalities in diabetes. Glucose and other nutrients regulate insulin secretion by the pancreatic beta cell. Glucose is transported by a glucose transporter GLUT 2; subsequent glucose metabolism by the beta cell alters ion channel activity, leading to insulin secretion. The SUR receptor is the binding site for some drugs that act as insulin secretagogues. SUR, sulfonylurea receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate, cAMP, cyclic adenosine monophosphate. IAPP, islet amyloid polypeptide or amylin. (Adopted from: Harrison's Principles of internal medicine, 18th Edition)

1.5.3 Mechanism of Action of Insulin

Insulin elicits its biological effect through binding to insulin receptors on cell plasma membranes expressed in all types of cells in the body. The insulin receptor is a member of the tyrosine kinase receptor family and has functional similarity to the insulin-like growth factor-1 (IGF-1) receptor (Taniguchi *et al*. 2006). The insulin receptor is a transmembrane

receptor comprising two extracellular alpha subunits and two membrane-spanning beta subunits attached by a disulfide link shaped a heterotetramer with approximately molecular weight of 320 Da. Insulin receptor subunits are encoded by INSR genes on chromosome 19 (Belfiore *et al*. 2009).

Alpha subunits act as ligand binding sites and beta subunits have inherent tyrosine kinase activity. In the normal status of no ligand binding, the alpha subunits inhibit the tyrosine kinase activity of beta subunits. Binding of insulin to the alpha subunits changes the conformation of the receptor and releases inhibition of alpha subunits, leading to activation of the beta subunit tyrosine kinase. One beta subunit transphosphorylates the other one, resulting in autophosphorylation. Beta subunit activation initiates signalling cascade via phosphorylation of intracellular proteins including insulin receptor substrate (IRS) and Src-homology-2-containing protein (Shc) (Brunton LL, Chabner BA, Knollmann BC: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011). Phosphorylated IRS-1 protein binds to P85 regulatory subunit of phosphatidylinositol 3-kinases (PI3Kinase) leading to activation of the P110 catalytic subunit of the enzyme. Activated PI3Kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane to phosphatidylinositol (3,4,5)-triphosphate (PIP3).

PIP3 activates protein kinase B (PKB or Akt) which facilitate GLUT 4 (glucose transporter 4) vesicle fusion to the plasma membrane resulting in increased glucose uptake (Edward et al. 2005).

Activated protein kinase B inhibits glycogen synthesis by inhibiting glycogen synthase kinase (GSK) through phosphorylation which leads to inactivation of glycogen synthase (Xianjun Fang *et al*. 2000).

PI3Kinase activation triggers several downstream kinases via producing

PIP3 including Akt 2 (PKB), mammalian target of rapamycin (mTOR) and protein kinase C (PKC) *(Huang S and Czech MP. 2007).* The PI3Kinase I/Akt2 activation plays a major role in mediating insulin action for glucose transport in skeletal muscle and adipose tissue and glucose production regulation and liver cells (Zaid H *et al*. 2008).

Phosphorylation of beta subunits of insulin receptors activates Src-homology-2-containing protein (Shc) and Growth factor receptor-bound protein 2 (Grb 2) which leads to initiation of a protein kinase cascade involving mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinases (ERKs) that mediate insulin actions for gene transcription and cell growth **(Fig 1.6)** (Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011).

MAPK cascade activation induces gene expression regulation, cell growth and differentiation actions of insulin (Louise *et al.* 2008). MAPK inhibitors inhibit insulin-stimulated glucose uptake without any detectable effect on GLUT4 translocation (Sweeney *et al.* 1999). The MAPK pathway is involved in intrinsic GLUT4 regulation, the mechanism is still not well understood.

Activation of small GTP-binding proteins like TC-10 and Rac, independent of PI3Kinase activation, is important for insulin action for increasing glucose transport in peripheral cells as these proteins are critical for vesicle trafficking (Kjoller *et al*. 1999 , Chiang *et al*. 2001).

Insulin signalling is terminated by degradation of receptor bound to insulin and decreases the amount of insulin receptor on the plasma membrane (Najjar. 2001). Dephosphorylation of tyrosine residues by tyrosine phosphatases is another mechanism that leads to termination of insulin signalling within the cells.

Fig 1.6. Pathways of insulin signalling. The binding of insulin to its plasma membrane receptor activates a cascade of downstream signalling events. Insulin binding activates the intrinsic tyrosine kinase activity of the receptor dimer, resulting in the tyrosine phosphorylation (Y-P) of the receptor's beta subunits and a small number of specific substrates (yellow shapes): the Insulin Receptor Substrate (IRS) proteins, Gab-1 and SHC; within the membrane, a caveolar pool of insulin receptor phosphorylates caveolin (Cav), APS, and Cbl. These tyrosine-phosphorylated proteins interact with signalling cascades via SH2 and SH3 domains to mediate the effects of insulin, with specific effects resulting from each pathway. In target tissues such as skeletal muscle and adipocytes, a key event is the translocation of the Glut4 glucose transporter from intracellular vesicles to the plasma membrane; this translocation is stimulated by both the caveolar and non-caveolar pathways. In the non-caveolar pathway, the activation of PI3K is crucial, and PKB/Akt (anchored at the membrane by PIP3) and/or an atypical form of PKC is involved. In the caveolar pathway, caveolar protein flotillin localizes the signalling complex to the caveola; the signalling pathway involves series of SH2 domain interactions that add the adaptor protein CrkII, the guanine nucleotide exchange protein C3G, and small GTP-binding protein, TC10. The pathways are inactivated by specific phosphoprotein phosphatases (eg, PTB1B). In addition to the actions shown, insulin also stimulates

the plasma membrane Na^+,K^+-ATPase by a mechanism that is still being elucidated; the result is an increase in pump activity and a net accumulation of K^+ in the cell. Abbreviations: APS, adaptor protein with PH and SH2 domains; CAP, Cbl associated protein; CrkII, chicken tumor virus regulator of kinase II; GLUT4, glucose transporter 4; Gab-1, Grb-2 associated binder; MAP kinase, mitogen-activated protein kinase; PDK, phosphoinositide-dependent kinase; PI3kinase, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol trisphosphate; PKB, protein kinase B (also called Akt); aPKC, atypical isoform of protein kinase C; Y, tyrosine residue; Y-P, phosphorylated tyrosine residue. (Adapted from: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011)

1.6 Glucose Transporters

Glucose is a vital source of energy and important metabolic substrate for mammalian cells. Glucose transporters are a broad group of membrane proteins which are responsible for glucose uptake and transport through the plasma membrane of cells. In general glucose transporters comprise two groups based on their function and structure including Na-dependent glucose transporters (SGLT) (Wright. 2001) and facilitative Na - independent sugar transporters (GLUT family) (Mueckler . 1994).

SGLTs are Na+/K+ ATPase pumps that facilitate glucose transport in the small intestine (SGLT-1) and the proximal tubule of nephrons in kidney (SGLT-1 and SGLT-2). SGLT-1 is a high affinity and SGLT-2 is a low affinity Na-dependent glucose transporters (I. Stuart Wood *et al*. 2003).

GLUTs are tarnsmembrane proteins with 12 membrane-spanning helices which facilitate diffusion of glucose and other sugars across the plasma membrane. Binding of glucose to the extra-membrane site of GLUT provokes a conformational change of the transporter and leads to release of glucose from the other intra-membrane side of the transporter (Hruz *et al*. 2001).

The GLUT family consists of three subclasses (Class I,II,III) with 13 members (Hruz *et al*. 2001, Joost *et al*. 2001). Each GLUT isoform plays specific role in glucose uptake in different tissues and physiological conditions. Substrate specificity and transporter kinetics are different in each isoform which creates a specific role for each GLUT isoform in sugar metabolism (Thorens. 1996). Class I GLUTs are well defined and comprise GLUT 1 to GLUT4 **(Table 1.1).**

GLUT1 is a high affinity glucose transporter responsible for low-level basal glucose uptake when the glucose concentration is low and is widely expressed in erythrocytes and endothelial cells of vascular tissues specially barrier tissues like the blood brain barrier (BBR). It is also responsible for vitamin C uptake (Nelson *et al*. 2008).

GLUT2 is a very efficient glucose transporter with high capacity and low affinity (Higher Km $\geq$ 10 mM), acts as a glucose sensor in pancreatic beta cells and also carries glucosamine as well as glucose (Efrat. 1997). Glut2 is also expressed in small intestine cells, proximal renal tubules and hepatocytes. It plays an important role in transporting glucose in fed-state from the intestinal lumen (Bell *et al*. 1990).

GLUT3 is a glucose transporter with very high affinity, five times greater than other class I GLUTs, and is mainly expressed in neurons (Simpson *et al*. 2008, Vannucci *et al*. 1997). GLUT1 transporter in the brain blood barrier and GLUT3 with high affinity for glucose play a central role in glucose uptake and metabolism in central nervous system at low glucose concentration.

GLUT4 is the insulin-responsive glucose transporter found in adipose tissue, skeletal muscle and heart which is responsible for the reduction of postprandial plasma glucose rise in response to insulin (Rayner *et al*. 1994). Insulin increases glucose uptake immediately, 10-20 fold by stimulating GLUT4 translocation in adipose tissue and skeletal muscle (Shepherd *et al*. 1999). GLUT4 translocation in skeletal muscle can also be increased by exercise, independent of insulin (Ploug *et al*. 1998).

Name	Distribution	Notes
GLUT1	Widely distributed in fetal tissues. In the adult, it is expressed at highest levels in erythrocytes and also in the endothelial cells of barrier tissues such as the blood–brain barrier. However, it is responsible for the low-level of basal glucose uptake required to sustain respiration in all cells.	Levels in cell membranes are increased by reduced glucose levels and decreased by increased glucose levels.
GLUT2	A bidirectional transporter, allowing glucose to flow in 2 directions. Is expressed by renal tubular cells, small intestinal epithelial cells, liver cells and pancreatic beta cells. Bidirectionality is required in liver cells to take up glucose for glycolysis, and release glucose during gluconeogenesis. In pancreatic beta cells, free flowing glucose is required so that the intracellular environment of these cells can accurately gauge the serum glucose levels. All three monosaccharides (glucose, galactose and fructose) are transported from the intestinal mucosal cell into the portal circulation by GLUT2	Is a high-capacity and low-affinity isoform. There is some evidence that GLUT 1 and 3 are actually the functional transporters in beta cells.
GLUT3	Expressed mostly in neurons (where it is believed to be the main glucose transporter isoform), and in the placenta.	Is a high-affinity isoform, allowing it to transport even in times of low glucose concentrations.
GLUT4	Found in adipose tissues and striated muscle (skeletal muscle and cardiac muscle).	Is the insulin-regulated glucose transporter. Responsible for insulin-regulated glucose storage.

Table 1. Class I glucose transporters GLUT1-GLUT4. (Bell et al. 1990 and Thorens et al. 2010)

As GLUT4 translocation in response to insulin is strictly dependent on PI3Kinase activation and PI3Kinase inhibition completely prevents the insulin activity from increasing glucose uptake via GLUT4, previous studies show that inhibition of PI3Kinase by wortmannin has no effect on GLUT4 translocation in skeletal muscle in response to exercise (Brozinick *et al*. 1998).

GLUT5 is a specific transporter for fructose widely expressed at the apical side of small intestine cells, and is responsible for transport of dietary fructose (Burant *et al*. 1992). GLUT 5 is also found in many other types of cells, such as skeletal muscle, adipose, brain and kidney. GLUT 5 drew the attention in recent years researches because fructose is an important component of human diets. Increasing of fructose consumption in human diets in last decades raised a concern about the contribution of fructose to development of metabolic disorders such insulin resistance, diabetes and obesity as it was shown in many literatures that fructose-fed rodents could develop some degree of insulin resistance and diabetes. The peripheral cells uptake the fructose mainly via GLUT5 and with less degree with other GLUTs and it will go into the metabolic pathway of ATP production same as glucose but with an important difference. Fructose unlike the glucose which converted to G6P via hexokinase, converts to fructose 1 phosphate via fructokinase and end up to glyceraldehyde. It bypasses the rate controlled glycolysis process which is controlled by hexokinase negative feedback. Increasing G6P inhibits the hexokinase and controls the rate of glycolysis and glucose metabolism within the cell but conversion of fructose to fructose 1 phosphate does not follow the same path. On the other hand, fructose could not increase the insulin secretion from pancreatic beta cell same as glucose (Eliott *et al*. 2002).

Glucose transporters play a major role in glucose metabolism, particularly GLUT4 as an insulin-stimulated rate limiting transporter. Animal studies showed that suppression of GLUT4 expression leads to insulin resistance and type 2diabetes (Stenbit *et al*. 1997).

1.7 Insulin Resistance

Insulin resistance refers to the condition in which responsiveness of cells to insulin is decreased and leads to higher concentrations of insulin in the blood required to maintain normoglycemic status. Insulin resistance is one of the main pathogenesis pathways of type 2 diabetes and results in defects in glucose uptake and metabolism. Insulin resistance creates an imbalance in the insulin/glucagon ratio which leads to disruption of cellular energy production via decrease of glycolysis. It also increases liver glyconeogenesis which helps plasma glucose levels rise and worsens the situation. In adipose tissue insulin malfunction increases triglyceride hydrolysis (lipolysis) which leads to elevated free fatty acid concentration in blood (Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011).

Insulin resistance pathology is still not well characterised but previous studies revealed some risk factors including obesity, race,family history, hypertension, low level of high density lipoprotein (HDL), gestational diabetes and birth weight more than 4 kg, are associated with the insulin resistance condition (Jennifer Mayfield. 1998 and Type 2 diabetes: Risk factors. Mayo Clinic. Retrieved 21 December 2011).

Among the risk factors, obesity and high fat intake especially saturated fat have significant positive correlation with insulin resistance (J Lovejoy *et al*. 1992, LH Storlien *et al*. 1991). The high level of free fatty acid and triglyceride play an important role in the development of insulin resistance (Schinner *et al*. 2005). Some studies suggested that fructose contributes to insulin resistance development as it is metabolised to triglyceride in the liver, which increases triglyceride and free fatty acid levels in the blood (Heather *et al*. 2005).

It is well established that feeding rats with fructose creates insulin resistance and type 2 diabetes, which is used as a model for type 2 diabetes animal studies (Thorburn. 1989).

Insulin resistance increases insulin levels as a push to restore the normoglycemic status but excess insulin stimulates fat storage and new fat tissue formation which accelerates weight gain. This defective cycle makes the condition worse. Unhealthy diet like fast food with high fat and fructose content has been shown to play a fundamental role in insulin resistance and metabolic syndrome conditions (Elvira *et al*. 2005).

It is well established that elevated plasma free fatty acid concentration reduces insulin-stimulated glucose uptake, and vice versa, reduction in plasma lipid concentration improves insulin activity and glucose uptake in adipose tissue, skeletal muscle and liver (Moller. 2001). Elevated lipid can create functional defects in insulin receptors and insulin receptor substrates (IRS) which change the binding of ligands, insulin receptor expression, the phosphorylation state of insulin receptor kinase domain and the activity of receptor tyrosine kinases. High free fatty acid concentrations also affect insulin signalling downstream via inhibition of IRS-1 associated PI3Kinase activation in muscle and IRS-1 serine phosphorylation which decreases tyrosine phosphorylation of IRS-1 and leads to impaired IRS-1 activation (Yu C *et al*. 2002). It also inhibits AKT (PKB) activation by increasing the amount of ceramide and diacylglyerol in skeletal muscle (Chavez *et al*. 2003).

Elevated free fatty acid (FFA) concentration decreases insulin receptor activity via activation and over-expression of protein kinase C (PKC) (Greene *et al*. 2004). It has been demonstrated that activation and over-expression of PKC lead to decrease of Akt (PKB) activity and insulin receptor down-regulation which are the mechanisms involve in FFA-

induced insulin resistance (Ikeda *et al*. 2001). The insulin receptor gene expression is regulated positively by binding HMGA1 (High-mobility group protein 1) to its AT-rich sequences (C2 and E3) within the promoter region. Transfection of cells with HMGA1 increases insulin receptor expression which indicates the important role of HMGA1 in insulin receptor regulation (Brunetti *et al* 2001). There is some evidence that FFA-induced PKC activation leads to phosphorylation of HMGA1 which reduces its mobility and binding capability and result in insulin receptor down regulation and insulin resistance (Samir *et al*. 2007).

There is strong evidence for connections between malonyl-CoA, acetyl-CoA carboxylase (ACC1& ACC2) and insulin resistance. Injection of ACC1 and ACC2 anti-sense inhibitors restored diet-induced hepatic insulin resistance in rat (Savage *et al*. 2006). Acetyl-CoA carboxylase (ACC) is an enzyme with two isoforms that converts acetyl-CoA to malonyl-CoA and plays an important role in fatty acid synthesis (Tong L. 2005). ACC2 concentrations are much higher than ACC1 in oxidative tissues like skeletal muscle and heart. Both ACC1 and ACC2 are widely expressed in hepatic cells where both fatty acid oxidation and synthesis are important. ACCs are regulated by allosteric activators such as citrate, glutamate and dicarboxylic acids (Martin *et al*. 1962 and Boone AN et al. 2000) and phosphorylating inhibitor, AMP-activated protein kinase (AMPK) which leads to deactivation of ACCs (Park *et al*. 2002).
Activation of AMPK in muscle inhibits ACC2 and increases fatty acid and glucose oxidation and decreases levels of malonyl-CoA. It also increases the GLUT4 content in membrane and up-regulates the GLUT 4 expression (Koistinen *et al*. 2003). AMPK as a negative regulator of ACC activity is a major target for treatment of type 2 diabetes and insulin resistance (Ruderman *et al*. 2004).

Glucagon and catecholamines inhibit ACC via AMPK activation whereas glucose and insulin increase ACC activity by dephosphorylation via phosphatase activation (Kim *et al*. 1997, Witters *et al*. 1988).
Regulation of malonyl- CoA and fatty acid metabolism are playing an important role in beta pancreas cells and insulin secretion as well as adipose tissue and skeletal muscle. There is a notion that changes in malonyl-CoA, fatty acid synthesis and oxidation regulation could be involved in obesity and insulin resistance as the fundamental pathogenesis of type 2 diabetes (Prentki *et al*. 1996).

Abnormalities in energy homeostasis regulation is a key point in metabolic syndrome that leads to a variety of conditions and diseases like diabetes, insulin resistance, cancer and cardiovascular diseases. The insulin/IGF (insulin-like growth factor) pathway is a major regulator of energy homeostasis by controlling glucose and lipid metabolism and regulation of their homeostasis (Biddinger *et al*. 2006).

Forkhead transcription factor (FOXO) is an important mediator for the metabolic effects of the insulin/IGF pathway. FOXO family has three main variants encoded by three genes (FOXO1,3 and 6). FOXO1 is the main controller of glucose homeostasis in peripheral tissues and pancreatic beta cells (Accili *et al*. 2004).
FOXO1 is a critical mediator of insulin signalling as over activation and expression of FOXO1 in hepatic and pancreatic beta cells causes hepatic insulin resistance and apoptosis of pancreatic beta cells (beta cell loss) which are reversed by reduction of FOXO1 function (Kitamura *et al*. 2002, Nakae *et al*. 2002).

The mTOR (mammalian target of rapamycin) pathway is another

important mediator of insulin/IGF pathway, it directly controls Akt (Protein Kinase B) function via its phosphorylation. It also suggests dysregulation of mTOR signalling may have a role in pathogenesis of metabolic syndrome and insulin resistance (Sarbassov *et al*. 2005).

PPAR-γ (Peroxisome Proliferator-Activated Receptors Gamma) is a nuclear protein and plays an important role in insulin sensitivity and glucose homeostasis (Picard *et al*. 2002). Ligand binding to PPAR-γ creates conformational changes and stabilizes its interaction with RXR (Retinoid X Receptor). PPAR-γ/RXR interaction stimulates gene expression by recruiting a set of coactivators to the promoter region of the targeted gene (Berger *et al*. 2002). In-vivo and in-vitro studies showed that PPAR-γ activation increases insulin sensitivity and enhances insulin-induced glucose uptake in adipocytes (Norris *et al*. 2003 and Nugent *et al*. 2001). It also has been shown that PPARγ co-activator-1 (PGC-1) has a crucial role in glucose homeostasis via regulation of GLUT4 gene expression in which PGC-1 strongly induces endogenous GLUT4 gene expression (Michael *et al*. 2001).

Chronic low-grade inflammatory responses are associated with insulin resistance in adipose tissue (Ruan *et al*. 2003, Hotamisligil *et al*. 2003). It was well demonstrated that macrophage content and pro-inflammatory gene activation are increased in adipose tissue in obesity and insulin resistance status. There is strong evidence that activation of the TNF-α pathway, as an inflammatory cytokine response desensitizes insulin signalling and creates insulin resistance (Hotamisligil *et al*. 1995, Hotamisligil *et al*. 1994, Hotamisligil *et al*. 1993, Uysal *et al*. 1998). TNF-α pathway activation down-regulates GLUT-4 expressions by phosphorylation of serine residue IRS-1 which inactivate IRS-1 and

insulin signalling (Uysal *et al*. 1997). Several studies have shown that PPARγ agonists inhibit secretion of TNF- α, macrophage activation and also reduce the TNF- α -induced inhibition of insulin signalling in adipose tissue (Ricote *et al*. 1998, Peraldi *et al*. 1997, Ruan *et al*. 2003).

Mitochondrial dysfunction can cause insulin resistance and diabetes, since mitochondria are the main organelle in cells which maintain energy homeostasis. Mitochondria density is reduced by 38% in the muscle of young insulin-resistant offspring of type 2 diabetes parents (Morino *et al*. 2005).

Other regulatory hormones secreted from adipocytes, known as adipokines, are involved in glucose homeostasis modulation including adiponectin, resistin and leptin. Leptin is a 16 KDa protein, which regulates appetite and energy intake (Brennan *et al*. 2006). Leptin has both central and peripheral mode of actions. It was shown that interacts with six types receptors encoded by LEPR (Wang *et al*. 1996). There is a strong connection between leptin and obesity. Leptin inhibits the neuropeptide Y secretes neurons in arcuate nucleus in the brain which controls the satiety feeling (Baicy *et al*. 2007). Apart from leptin central activity in connection with hypothalamus, leptin's receptors are expressed in wide types of peripheral cells. It has been shown that leptin plays a role in cellular energy expenditure modulation via interacting with metabolism regulatory hormones and other energy regulation mechanisms (Margetic et al. 2002). People with leptin receptor mutations or leptin deficiency suffer from obesity (Montague *et al*. 1997, Farooqi *et al*. 1999). As obesity is a well established risk of insulin resistance, leptin may be involved in insulin resistance.

Adiponectin is another important modulator which increases insulin sensitivity and glucose uptake in both adipose tissue and muscles and

reduces gluconeogenesis and glucose output in hepatic cells. It was also shown that expression of adiponectin decreases in obese humans (Stumvoll *et al*. 2002). Opposite to adiponectin, resistin increases gluconeogenesis and hepatic glucose output and decreases insulin sensitivity and insulin-induced glucose uptake in both adipose and muscule tissues (Moon *et al*. 2003, Pravenec *et al*. 2003). There is a strong belief that adipokines may contribute to insulin resistance and pathogenesis of type 2 diabetes. They also attracted much research interest in recent years, not only for understanding the molecular pathology of insulin resistance, but also for new therapeutic approaches.

Other genomic regulators in insulin secretion and signalling are microRNAs (miRNA) which are recently attracting many researches in order to identify their role in pathogenesis of diabetes. MicroRNAs are short single-stranded non protein coding gene products with length of 19-23 nucleotides which post-transcriptionally regulate the expression of genes by interacting with specific mRNAs (Bartel. 2004, Lagos-Quintana *et al*. 2001, Fabian *et al*. 2010). MicroRNAs interaction with targeted mRNAs lead to mRNA degradation or inhibition of mRNA translation which results in negative regulation (Tranzer *et al*. 2006, Ying *et al*. 2006). It was established that microRNAs are directly involved in glucose homeostasis, insulin secretion and insulin sensitivity, as shown in **Table 1.2**. A recent study showed that a plasma signature of five miRNAs (miR-15a,miR-29b, miR-126, miR-223, and miR-28–3p) is closely connected with a high likelihood of developing type 2 diabetes (Zampetaki *et al*. 2010). These miRNAs are indicators of early type 2 diabetes and responsible for its progression (Regazzi. 2010). There is a hypothesis that miRNA may transmit from one tissue to another and act at a distance from their sites of biogenesis (Dinger *et al*. 2008). There is

supportive evidence for this hypothesis as it was shown that miR-150 is taken up by target endothelial cells from the bloodstream (Zhang *et al*. 2010). It suggests that microRNAs may be involved in insulin resistance and diabetes pathogenesis at the genomic level, which needs further detailed investigation. miRNAs could be targets for therapeutic purposes and developing new medications.

Biological Process	**Specific MicroRNAs**
ngn3-independent endocrine pancreas regeneration	miR-15a, miR-15b, miR-16 and miR-195
Regulates insulin expression	miR-30d, miR-375, miR-124a2
Regulates insulin secretion	miR-375, miR-9, miR-124a2
Regulates glucose-stimulated insulin secretion (GSIS)	miR-369-5p, miR-130a, miR-27a, miR-410, miR-200a, miR-337, miR-532, miR-320, miR-192 and miR-379, miR-375, miR-124a2
Regulates adipocyte differentiation	miR-143, miR-27b, miR-130, miR-519d
Regulates insulin sensitivity	miR-103, miR-107, miR-29, miR-320, mmu-mir-183-96-182 (cluster)

Table 1.2. Summary of key biological processes and miRNAs (Adopted from: Michael D.Williams *et al*. 2012)

There is a strong genetic links between diabetes type 1 and many genes. It was shown that there is a strong link between histocompatibility gene loci on chromosome 6 which encodes MHC class II (HLA) proteins and diabetes type I (Bluestone *et al*. 2010). It was shown in the same study that some variant of HLA gene including DRB1 (0401, 0402, 0405), DQA 0301 and DQB1(0302 and 0201) increase the risk of type 1 diabetes. However, these variants are also found in the general population, and only about 5 percent of individuals with the gene variants develop type 1 diabetes. HLA variations account for approximately 40 percent of the genetic risk for the condition. Other HLA variations appear to be protective against the disease.

1.8 Diabetes management and available therapeutics

Diabetes management has two main categories, insulin therapy and anti-diabetic medications. Insulin replacement is the main treatment of type 1 diabetes in which the insulin producing capability of beta cells has been lost. Anti-diabetic medication has got no, or a minimal place in the treatment of type 1 diabetes. Insulin therapy in type 1 diabetes is necessary in order to maintain normoglycemic status in the long term, but increases the risk of small blood vessel disease and may promote cardiovascular complications of diabetes (Sugimoto *et al*. 2003, Mudaliar . 2009). Insulin therapy remains as a last resort for type 2 diabetes particularly when other therapies with anti-diabetic medications fail to control blood glucose level.

The goals for treatment of diabetes based on the American Diabetes Association (ADA) recommendations are:

- HbA1c lower than 7%
- Peripheral plasma glucose level between 70 to 130 mg/dl (3.9 to 7.2 mmol/l)
- Peak postprandial capillary plasma glucose (OGTT) lower than 180 mg/dl(10 mmol/l)
- Low density lipoprotein (LDL) lower than 100 mg/dl (2.6 mmol/l)
- High density lipoprotein (HDL) greater than 40mg/dl (1 mmol/l) in men and 50 mg/dl (1.3 mmol/l) in women
- Triglyceride lower than 150 mg/dl (1.7 mmol/l)

The aims of treatment for both type 1 and 2 diabetes are to allow patients

to achieve a normal lifestyle, to eliminate hyperglycaemic symptoms, and to reduce long term complications of diabetes, especially cardiovascular complication.

Anti-diabetic medications are divided into four main categories as sensitizers, secretagogues, alpha glucosidase inhibitors and peptide analogues.

Sensitizers: Biguanides, like metformin exert their anti-hyperglycaemic action by suppressing gluconeogenesis in liver and enhance glucose uptake via AMP-activated protein kinase (AMPK) activation (Kirpichnikov *et al*. 2002, Musi *et al*. 2002). Thiazolidinediones like troglitazone, pioglitazone and rosiglitazone are another member of this category. Thiazolidinediones are PPAR-γ agonists, which increase insulin sensitivity.

Secretagogues: Sulfonylureas like tolbutamide, glipizide, gliclazide, glibenclamide and Meglitinides know as non-sulfonylurea secretagogus or short-acting secretagogus like repaglinide and nateglinide are members of this family. This family increases insulin secretion by inhibiting ATP-sensitive potassium channels in beta cells which leads to beta cell membrane depolarization and an influx of calcium and exoytosis of insulin vesicles.

Alpha Glucosidase Inhibitors: Acarbose and miglitol are members of this group, which reduce glucose absorption by slowing down starch digestion. This group technically is not categorized as hypoglycaemic agents but they help glucose to enter the blood stream from the intestine slowly, which can be matched effectively with an impaired insulin response.

Peptide Analogues: Incretins including glucagon-like peptide1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are secreted after meals and stimulate insulin secretion. They have a very short half life up to few minutes. GLP-1 is effective in type 2diabetes for stimulating insulin secretion whereas GIP is not. GLP-1 agonists including exenatide and liraglutide are effective in type 2 diabetes patients after a meal by stimulating insulin release, inhibiting glucagon and delaying gastric emptying *(*Brunton LL, Chabner BA, Knollmann BC: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011). Incretins are rapidly inactivated by dipeptidyl peptidase-4 (DPP-4). Recently a new category of anti-diabetic medication has been developed as DPP-4 inhibitors like sitagliptin and linagliptin. DPP-4 inhibitors reduce HbA1C **(Fig 1.7)** (Amori *et al*. 2007). All the available medications as a treatment regiment for diabetes work on utilising insulin secretion or increasing its effect on peripheral cells to reduce the blood glucose level but not targeting the fundamental metabolic disorder of diabetes.

Fig 1.7. Incretins activity and action of DPP-4 inhibitors. DPP-4 normally inactivates GLP-1. DPP-4 inhibitors block DPP-4 which in turn leaves GLP-1 active.

1.9 Fenugreek (4-Hydroxyisoleucine)

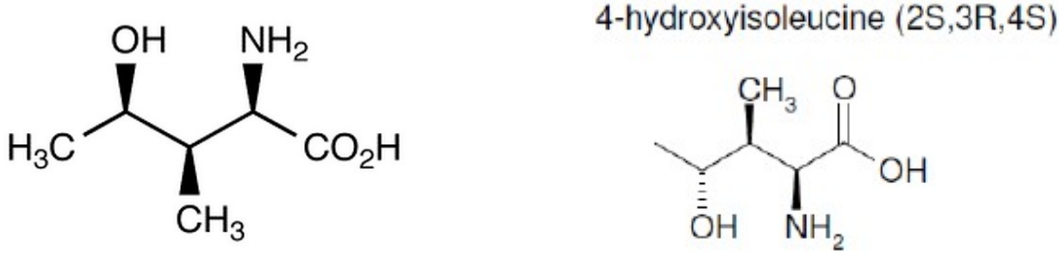
Trigonella foenum-graecum L. (Legominosae) known as Fenugreek **(Fig 1.8)** is one of the oldest medicinal plants and is native to southeastern Europe, northern Africa and western Asia. Fenugreek seeds often have a spicy and pungent aroma and bitter taste. Fenugreek seeds contain many active compounds such as iron, vitamin A, B, C, phosphates, flavonoids, saponins, alkaloids such as trigonelline and amino acids (Fenugreek seed bioactive compositions and methods for extracting same. United States Patent 7338675). The leaves and seeds have a long record of historical usage in many countries where it was grown for medicinal purposes. Applications of fenugreek were documented in ancient Egypt as incense for embalming mummies. Fenugreek is used as a supplement in wheat and maize flour for bread-making in modern Egypt (Morcos *et al*. 1981). In traditional Chinese medicine, fenugreek seeds are consumed as a tonic, as well as a remedy for weakness and oedema of legs (Basch *et al*. 2003). Fenugreek is commonly used as a condiment in India and used as a lactation stimulant as well (Yoshikawa *et al*. 1997). In Iranian traditional medicine the seeds have been used as a blood sugar lowering herb (Amin Gh. Popular medicinal plants of Iran. Tehran: Vice-choncellor of Research of Tehran University of Medical Sciences, 1991, pp: 101 – 2).

The possible hypoglycemic properties of oral fenugreek seed powder have been suggested by the results of preliminary animal and human trials. The studies showed that defatted seeds are associated with the hypoglycemic effects of fenugreek seeds. These effects have not been observed in studies of lipid extracts (Basch *et al*. 2003).

Fig 1.8. Trigonella foenum-graecum (Fenugreek) (Prof. Dr. Otto Wilhelm Thomé Flora von Deutschland, Österreich und der Schweiz 1885, Gera, Germany)

Administration of 100 g of defatted fenugreek seed powder to people with type 1 diabetes in 10 days showed significant amelioration in diabetes associated symptoms (Sharma *et al*. 1990). Another study on patients with type 2 diabetes showed that fenugreek seed powder administration for 6 weeks reduced blood glucose and cholesterols (Abu Saleh *et al*. 2006). Some studies conducted on fenugreek have focused on investigating the effect of a specific sub-fractions of the fenugreek seeds containing a unique amino acid known as 4-hydroxyisoleucine in animals and humans with diabetes or lipid disorder (Fenugreek seed bioactive compositions and methods for extracting same. United States Patent 7338675).

The previous works on 4-hydroxyisoleucine proved that it acts as a potent blood sugar lowering and anti-dyslipidemic agent (Broca *et al*. 2004, Sauvaire *et al*. 1998, Al-Habori *et al*. 1998). 4-hydroxyisoleucine is a branch amino acid which has been extracted from fenugreek seeds and it has not been found in any mammalian tissues **(Fig 1.9 &Table 1.3)**, or in other plants.



A **B**

Fig 1.9. **A**: (2R,3R,4S) 4-Hydroxyisoleucine. **B**: (2S,3R,4S) 4-Hydroxyisoleucine. (Adapted from Toronto Research Chemicals Inc, http://www.trc-canada.com)

CAS Number	**781658-23-9**
IUPAC Name	**2-amino-4-hydroxy-3-methylpentanoic acid**
Synonym	**2-Amino-2,3,5-trideoxy-3-methyl-D-xylonic Acid**
Major Isomer	**(2S,3R,4S)-4-Hydroxyisoleucine**
Minor Isomer	**(2R,3R,4S)-4-Hydroxyisoleucine**
Molecular Formula	**C6H13NO3**
Molecular Weight	**147.17g/mol**
Melting Point	**>203°C**

Table 1.3. 4-Hydroxyisoleucine chemical specifications.

The studies have confirmed the presence of 4-hydroxyisoleucine in fenugreek seeds in two diastero-isomers: the major one is the (2S, 3R, 4S) configuration, representing about 90% of the total content of 4-hydroxyisoleucine in the fenugreek seeds extract, and the minor one is the (2R, 3R, 4S) configuration (Alcock *et al*. 1989). The major isomer is presently interesting with respect to experimental evidence indicating its ability to stimulate glucose-induced insulin secretion in micromolar

concentrations (Sauvaire *et al*. 1998).

Some studies claimed that the natural analogue of 4- hydroxyisoleucine is more effective as an anti-diabetic agent than a synthetic version. There is, therefore, a suggestion that the therapeutic effects of 4-hydroxyisoleucine are best obtained from extracts of the fenugreek seeds (Fenugreek seed bioactive compositions and methods for extracting same. United States Patent 7338675). But another study has shown synthetic and natural 4-hydroxyisoleucine are same in hypoglycemic properties. (Rolland-Fulcrand *et al*. 2004) 4-hydroxyisoleucine is safe to be consumed by human without geneotoxicity as tested based on FDA (US Food and Drug Administration) protocols and recommended 4-hydroxyisoleucine for food ingredients (Flammang *et al*. 2004).

1.10 Isoleucine

Isoleucine (Ile) is an essential α-amino acid that cannot be synthesized by humans. It is hydrophobic due its hydrocarbon side chains. Isoleucine can have four possible stereoisomers but in nature it exists only as one enantiomeric form, (2S,3S)-2-amino-3-methylpentanoic acid. Isoleucine is a glucogenic amino acid involved in the metabolism of carbohydrate which can be converted to succinyl-CoA and acetyl-CoA and fed to the TCA cycle (Tricarboxylic Acid Cycle) **(Fig 1.10) (Table 1.4)** (Tappy *et al*. 1992).

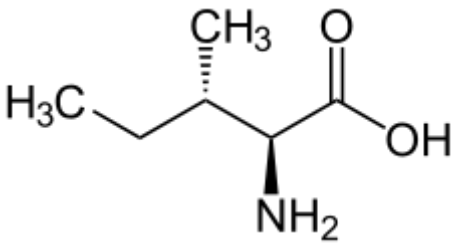


Fig 1.10. L-Isoleucine. (Adapted from Sigma Aldrich, http://www.sigmaaldrich.com)

CAS Number	**73325**
IUPAC Name	**(2S,3S)-2-Amino-3-methylpentanoic acid**
Molecular Formula	**C6H13NO2**
Molecular Weight	**131.17g/mol**

Table4. L-Isoleucine chemical specification.

In-vivo study shows that isoleucine has hypoglycaemic properties and can lower the blood glucose level and its hypoglycaemic effect reduces when its blood level concentration reaches saturation (Doi *et al*. 2007). Both Isoleucine and 4-hydroxyisoleucine with very close molecular structure has got anti-diabetic effects (Ikehara *et al*. 2008) but isoleucine's hypoglycaemic effect is not dependent on the concentration of glucose as 4-hydroxyisoleucine glucose lowering effect is only observed in high glucose concentration. It seems the extra OH group in 4-hydroxyisoleucine creates some unique anti-diabetic properties which are not seen in isoleucine.

1.11 Background (4-Hydroxyisoleucine)

Diabetes is a progressive multi-factorial metabolic disease with serious short and long term complications affecting different organs in the body with high increasing prevalence in the world. Understanding the basis of pathogenesis of diabetes specially type 2 diabetes which includes more than 90% of diabetes cases worldwide and finding effective remedies are very important as perspective of public health. It puts any potential substances with anti-diabetic effect in the centre of attention in point of view of researchers in relative fields. Fenugreek seeds as mentioned in the last chapter has got a strong historical track in traditional medicine of several cultures in Asia and middle-east for treatment of people with diabetes symptoms.

Sharma in 1990 fed type 1 diabetes patients with 100 g of defatted fenugreek seed powder per day divided in two doses for 10 days and results showed a significant decrease in fasting blood glucose level, 54% reduction in 24 hour urinary glucose excretion and improved glucose tolerance test. It also indicated that serum total cholesterol, LDL, VLDL and triglyceride have been significantly modified after 10 days without any changes in HDL level (Sharma *et al*. 1990).

Another study of using 25 g twice a day of fenugreek seed powder in 30 type 2 diabetes patients with hyperlipidemia for 6 weeks showed that serum total cholesterol, LDL and triglyceride have been reduced significantly compared to control group but the HDL level remained unchanged (Abu Saleh *et al*. 2006).

Gupta et al tested the effect of 1 gm/day hydroalcoholic extract of

fenugreek seeds on 25 newly diagnosed type 2 patients which showed a reduction in blood glucose level, an increase in insulin sensitivity using HOMA-model[1] insulin resistance and decrease in triglyceride after two months treatment. In this study they observed an increase in HDL level in the treatment group as well (Gupta *et al*. 2001).

Analava et al showed that anti-diabetic and anti-dysplipidemic effect of fenugreek seed powder is dose dependent and consuming more than 75 g/day can not produce significant differences in its effect on blood glucose level. The blood glucose lowering effect between 75 g and 100 g a day remained unchanged (Analava *et al*. 2006).

It was important question to determine what is responsible for anti-diabetic and anti-dyslipidemic effect of fenugreek seed powder or extract which well established in several studies. In 1979, Hardman detected 4-hydroxyisoleucine and its isomers in fenugreek seed extract (Hardman *et al*. 1979). This unique amino acid which only existed in fenugreek seed and has been never found in any mammalian tissues or other plants up to now, drew the attention of researchers. It could be responsible for such effect of fenugreek seed.

There is no strong evidence that shows fenugreek leaves extract producing such effects. It strengthened the idea that these effects may be

1 ***Homeostatic model assessment (HOMA)*** *has been described by Matthews et al in 1985 and widely employed in clinical research to assess insulin sensitivity. HOMA is calculated as the product of the fasting values of glucose (expressed as mg/dL) times insulin (expressed as μU/mL) is divided by a constant (405): HOMA= Glucose x Insulin /405. The constant 405 should be replaced by 22.5 if glucose is expressed in S.I. units. The HOMA calculation compensates for fasting hyperglycaemia. HOMA and insulin values increase in the insulin-resistant patient while the Glucose/Insulin ratio decreases. The HOMA value correlates well with clamp techniques and has been frequently used to assess changes in insulin sensitivity after treatment.(Matthews et al. 1985)*

related to 4-hydroxyisoleucine as it is not that much inside the leaves.

In 1998, Sauvaire Y et al characterised 4-hydroxyisoleucine from fenugreek seeds and showed its anti-diabetic effect. Their study of administration of 4-hydroxyisoleucine directly to rat pancreas revealed insuliotropic effect of 4-hydroxyisoleucine in the concentration range 100 micromol/l to 1mmol/l. They also determined that 4-hydroxyisoleucine effect on stimulating insulin secretion from islets of Langerhans in pancreas strictly dependent on glucose concentration. 4-hydroxyisoleucine insulin stimulatory is ineffective in 3 and 5 mmol/l concentrations of glucose. Insulin secretion is induced by 4-hydroxyisoleucine at 6.6 to 16.7 mmol/l of glucose concentrations (Sauvaire *et al*. 1998).

Broca and his colleagues in 1999 demonstrated that administration of 50mg/kg/day of 4-hydroxyisoleucine to type 2 diabetes rat model significantly reduces basal hyperglycaemia and basal insulinemia. It also improved glucose tolerance test. They showed that 4-hydroxyisoleucine with concentration of 200 micromol/l induces insulin release from type 2 diabetic rat-isolated islets in exposure to high glucose concentration of 16.7 mmol/l (Broca *et al*. 1999). In 2000, Broca et al observed that the insuliotropic property of 4-hydroxyisoleucine only seen in the micromolar range of major isomer (2S,3R,4S)-4-hydroxyisoleucine (Broca *et al*. 2000). In 2004, Broca investigated extra-pancreatic effect of chronic 4-hydroxyisoleucine treatment in insulin resistant Zucker fa/fa rats and sucrose-lipid diet fed rats.

Study results demonstrated that 4-hydroxyisoleucine increases peripheral glucose utilization and decrease insulinemia in sucrose-lipid diet fed rats and reduces hepatic glucose production and progression of

hyperinsulinemia in insulin resistant Zucker fa/fa rats. He also showed that a single injection of 18 mg/kg of 4-hydroxyisoleucine increased PI3Kinase activity associated with IRS-1 in both muscle and liver same as insulin. Combining insulin and 4-hydroxyisoleucine did not increase PI3Kinase activity further. This study indicated that 4-hydroxyisoleucine has got insulin sensitizing property as well and increases insulin effect on utilizing peripheral glucose via interaction with early signalling of insulin (Broca *et al*. 2004).

Fenugreek seed extract containing minimum 40% 4-hydroxyisoleucine tested for genetic toxicity by Flammang et al from Abbott laboratories in America based on recommended US Food and Drug Administration (FDA) genetic toxicity evaluation protocols using a reverse mutation assay, mouse lymphoma forward mutation assay and mouse micronucleus assay. The negative assays results demonstrated that fenugreek seed extract and 4-hydroxyisoleucine are not genotoxic and safe to be consumed by human (Flammang *et al*. 2004).

There are some controversies in studies of 4-hydroxyisoleucine on skeletal muscle glycogen re-synthesis. Ruby et al evaluated oral fenugreek seed extract containing 4-hydroxyisoleucine with glucose drink and dextrose on trained male cyclist post-exercise skeletal muscle glycogen resynthesis after 90 minutes intense ride. They found that 4-hydroxyisoleucine plus glucose increases the rate of glycogen resynthesis by 63% compared to glucose alone. Results of dextrose showed the glycogen re-synthesis significantly enhanced by 4-hydroxyisoleucine compared to dextrose alone (Ruby *et al*. 2005). They repeated the experiments few years later by changing the protocol from 90 minutes intense exercise to 5 hours at 50% of peak cycling powder

and they reported that 4-hydroxyisoleucine does not improve glycogen resynthesis. They suggested that the difference between the results of the current study with previous work is due to differences in experimental protocol. Interestingly, comparison both study findings shows that 4-hydroxyisoleucine is effective in enhancing glycogen re-synthesis in high intensity muscle activity rather than long term low intensity muscle exercise (Slivka *et al*. 2008).

It is suggested that 4-hydroxyisoleucine supplement has preventive efficacy on obesity induced by high fat diet as shown in animal study that 4-hydroxyisoleucine reduces body weight gain and plasma triglyceride gain in obese fat induced by high fat diet and oil administration treated with fenugreek seed extract containing 4-hydroxyisoleucine (Handa *et al*. 2005). It has also been shown that 4-hydroxyisoleucine significantly decreases plasma triglyceride level by 33%, serum total cholesterol by 22% and plasma free fatty acid by 14% in dyslipidemic hamster (Narender *et al*. 2006).

Haeri et al in 2009 showed that long term treatment of fructose-fed rats (insulin resistance model) and streptozotocin-induced diabetes type 2 rats with 50 mg/kg/day 4-hydroxyisoleucine for 8 weeks restored significantly elevated liver damage markers including aspartate transaminase (AST) and alanine transaminase (ALT) to near control group values in fructose-fed rats but not in streptozotocin-induced diabetes type 2 rats. They also showed long term treatment with 4-hydroxyisoleucine is well tolerated in both models and 4-hydroxyisoleucine increased HDL level in streptozotocin-induced diabetes type 2 rats by 31% compared to control group (Haeri *et al*.2009).

Singh and his colleagues in 2010 published a study using 50 mg/kg/day 4-hydroxyisoleucine isolated from fenugreek seeds on C57BL/KsJ-db/db mice. They observed that 4-hydroxyisoleucine significantly decreases elevated blood glucose level, total cholesterol, LDL and triglyceride in well-characterised type 2 diabetes mice model. They also noticed a significant increase in HDL level. It was suggested in this study that 4-hydroxyisoleucine suppresses progression of type 2 diabetes by increasing insulin sensitivity and peripheral glucose uptake (Singh *et al*. 2010).

A recent published study by Jaiswal N et al on rat L6-GLUT4myc skeletal muscle cells treated with 5 and 25 µ/ml 4-hydroxyisoleucine for 16 hours showed that 4-hydroxyisoleucine significantly and dose dependently increases glucose uptake and time dependently GLUT4 translocation from plasma to the membrane of the cells after adding 100 nM insulin for 20 minutes. It was observed that incubation cells with 4-hydroxyisoleucine and insulin together for 16 hours has not significant effect on insulin-induced GLUT4 translocation compared to insulin alone as a control. Treatment with 4-hydroxyisoleucine did not change the cellular contents of GLUT1 and GLUT4 which prove that its effect on glucose uptake is due to modification of signalling leads to GLUT4 translocation. 4-hydroxyisoleucine-induced GLUT4 translocation completely blocked by adding 1 µ/ml of cycloheximide (CHX) which is a potent protein synthesis inhibitor. Cycloheximide (CHX) can not inhibit GLUT4 translocation alone and it was suggested that the increase in GLUT4 translocation by 4-hydroxyisoleucine requires synthesis of new proteins. It is also shown that adding wortmannin, a potent inhibitor of PI 3Kinase inhibits the 4-hydroxyisoleucine-induced GLUT4 translocation, which identifies the key role of PI 3Kinase in 4-

hydroxyisoleucine effect. 4-hydroxyisoleucine also significantly increased Akt (PKB) basal phosphorylation same as insulin without changing the mRNA expression of Akt (PKB), IRS-1 and GLUT4 in genetic level. This work confirms the effect of 4-hydroxyisoleucine on utilizing peripheral glucose and increasing insulin sensitivity via its signalling pathway as demonstrated in previous studies (Jaiswal *et al*. 2012).

1.12 Background (Isoleucine)

Ikehara and his colleagues published an animal study in 2008 about the effect of L-isoleucine in diabetes. They treated glucose intolerant high-fat diet (HFD) mice, severe type 2 diabetes db/db mice and normal mice with oral isoleucine at a dose of 30-300 mg/kg for 6 weeks. The results showed that isoleucine significantly and dose-dependently decreases blood glucose level in all groups including normal mice. It also significantly increases blood insulin levels only in high-fat diet (HFD) mice but not in other groups which suggests different mechanisms involved in hypoglycemic property of isoleucine apart from its insulinotropic activity on HFD mice. Insulin release dramatically reduced in high-fat diet (HFD) mice treated chronically with isoleucine after an oral glucose challenge without any changes in glucose tolerance curve which indicates insulin-sensitizing property of isoleucine. This study determined that both insulin secretion dependent and independent mechanisms are involved, but based on results, insulin secretion independent mechanism is playing a major role in its hypoglycaemic effect specially in normal and sever diabetic conditions (Ikehara *et al*. 2008).

There is evidence the L-isoleucine increases insulin release from pancreatic beta cells when added together with L-glutamine (Sener *et al*. 1981). *In-vitro* study on muscle C2C12 myotubes in the absence of insulin showed that isoleucine with concentration 1 mM significantly increased glucose consumption by 16.8% compared to control and l mM leucine. Isoleucine did not increase glycogen synthesis compared to leucine which significantly increased intra cellular glycogen synthesis. The study also suggested that isoleucine stimulates insulin-independent

glucose uptake mediated by activating PI3Kinase and independent of activation of mammalian target of rapamycin (mTOR) like leucine which is a potent activator of mTOR (Doi *et al*. 2003, Zhang *et al*. 2007). Another study by Doi et al showed that oral administration of 1.35 g/kg L-isoleucine to fasted rats for 18 hours significantly reduced blood glucose level compared to 1.35 g/kg L-leucine and control. L-leucine did not change the blood glucose level. Isoleucine increased skeletal muscle glucose uptake by 73% without changing glycogen synthesis and AMKP alpha-1(AMP-activated protein kinase). In contrary leucine increased glucose incorporation into glycogen without changing AMKP alpha-1. Isoleucine but not leucine significantly reduced AMKP alpha-2 activity. The study suggested that reduced AMPK alpha-2 activity by isoleucine administration is due to decrease in AMP content and the AMP/ATP ratio probably because of improvement of ATP availability (Doi *et al*. 2005).

Isoleucine has been shown to increase the expressions of PPAR-α and uncoupling protein 2 and 3 (UCP) in high fat diet rats treated for 6 weeks. Hepatic and skeletal muscle triglyceride concentration and hyperinsulinemia were significantly lower in isoleucine treated rats which show isoleucine is effective in modifying metabolic syndrome and preventing obesity (Nishimura *et al*. 2010).

1.13 Summary

It is well documented that both 4-hydroxyisoleucine and isoleucine have anti-diabetic and anti-dyslipidemic effects. Previous studies have shown both of these molecules have insulinotropic and insulin-sensitizing properties which are effective in diabetic and obesity conditions but still their mechanisms of action remain unclear. Two main differences, based on well established studies, exist between isoleucine and 4-hydroxyisoleucine in their mode of action as following:

- Isoleucine elicits its significant hypoglycaemic and anti-dyslipidemic actions at a higher dose above 100mg/kg (Ikehara *et al*. 2008) compared to 4-hydroxyisoleucine which is effective at a much lower dosage.

- The most important difference between these two molecules is that isoleucine is hypoglycaemic and anti-dyslipidemic activity is not dependent on glucose concentration and it can produce its effects in normal euglycemic conditions whereas the anti-diabetic and anti-dyslipidemic activity of 4-hydroxyisoleucine is completely dependent on glucose concentration and it is inactive at normal glucose concentration less than 6.6 mmol/l. 4-Hydroxyisoleucine's effects have a direct correlation with glucose concentration in which higher glucose concentration creates more profound effects of 4-hydroxyisoleucine (Sauvaire *et al*. 1998). This feature makes the 4-hydroxyisoleucine a unique molecule with higher safety and without any hypoglycaemic side effect in normoglycemic conditions, unlike most available anti-diabetic medications like sulfonylureas.

All available information about the action of 4-hydroxyisoleucine and isoleucine are summarised in the following table **(Table 1.5)**. After two decades from identification of 4-hydroxyisoleucine with unique properties from fenugreek seed extract still many questions remained unanswered and mechanisms involve in its glucose dependent anti-diabetic and hyperlipidemia modifying properties has not been clarified yet. Understanding mechanisms and modes of 4-hydroxyisoleucine's actions not only important of its use in management of diabetes but it is a great help to understand the fundamental metabolic and molecular pathogenesis and pathophysiology of diabetes which help in designing new remedies and management approach. All the previous works well demonstrated the significant hypoglycaemic and lipid lowering activity of 4-hydroxyisoleucine in both *in-vivo* and *in-vitro* conditions in the presence of insulin which mean 4-hydroxyisoleucine augments peripheral activity of insulin on glucose uptake. It is claimed that 4-hydroxyisoleucine anti-diabetic and anti-dyslipidemic activities are due to its insulinotropic and insulin-sensitizing properties in type 2 diabetes.

There is not sufficient published data about 4-hydroxyisoleucine efficacy on type 1 diabetes or any high glucose condition without the presence of insulin. There is also no study on modifying effect of 4-hydroxyisoleucine on uric acid level which rises in diabetic conditions specially type 1 diabetes and it is directly linked to diabetic nephropathy (Rosolowsky *et al*. 2008).

Effect	Fenugreek Seed Extract	4-Hydroxyisoleucine	Isoleucine
Blood Glucose level	↓ (Effective in diabetic, not normal blood glucose level)[1]	↓ (Effective in diabetic, not normal blood glucose level) [4]	↓(Effective in both diabetic and normal blood glucose level)[8]
Total Cholesterol	↓[1]	↓[4]	No Data
LDL (Low Density Lipoprotein)	↓[2]	↓[4]	No Data
HDL (High Density Lipoprotein)	↑/No change[3]	↑/No change[5]	No Data
Triglyceride	↓	↓[4]	↓[9]
Uric Acid	No Data	No Data	No Data
Glucose Uptake	↑[1]	↑[4]	↑[8]
Glut4 Translocation	No Data	↑[6]	No Data
PI3 Kinase activity	No Data	↑[7]	↑(Not Confirmed)[10]
Glucose Concentration Dependent	No Data	Yes[4]	No
Insulinotropic	No Data	Yes[4]	Yes[8]
Insuin-sensitizing Action	Yes[1]	Yes[4]	Yes[8]
Insulin-independent hypoglycaemic activity	No Data	No Data	Yes[8]

Table 1.5. Summary of findings about fenugreek seed extract, 4-hydroxyisoleucine and isoleucine based on previous studies covered in the background section.

1- Sharma *et al*. 1990, Abu saleh *et al*. 2006, Analava *et al*. 2006

2- Narendar *et al*. 2006 3- Gupta *et al*. 2001

4- Sauvaire *et al*. 1998, Broca *et al*. 1999 and 2000

5- Singh *et al*. 2010 6- Jaiswal *et al*. 2012 7- Broca *et al*. 2004

8-Ikehara *et al*. 2008 9- Nisimura *et al*. 2010

10- Doi *et al*. 2003, Zhang *et al*. 2007

1.14 Questions and study design notions

The study in this work was designed to investigate the anti-diabetic properties of 4-hydroxyisoleucine in-vivo and in-vitro with minimised insulin effect.

Question 1: Is 4-hydroxyisoleucine capable of decreasing blood glucose level and modify dyslipidemia, as studied in previous works in type 2 diabetes or insulin-resistant animal models in type 1 diabetes model with minimal insulin presence? If yes, is it effective in modifying the uric acid level as well as plasma glucose and lipid levels?

Finding the answer for question 1 is very important which shows that 4-hydroxyisoleucine is dependent on insulin for its action or not for the first time in animal models.

Broca showed that infusion of 4-hydroxyisoleucine directly to rat isolated pancreas islet cells with high concentration glucose stimulates insulin release (Broca *et al*. 1999) but there is no other study about the direct effects of 4-hydroxyisoleucine on pancreatic beta cells. It has been shown that isoleucine stimulates insulin secretion as well but there is no direct study on beta cells.

Question 2: Does 4-hydroxyisoleucine increase insulin secretion from beta cells in the presence of different concentrations of glucose? Does it increase the consumption of glucose (Uptake) in beta cells? Does isoleucine increase the insulin secretion and glucose consumption in beta cells? If yes is is it dependent on glucose concentration? Is isoleucin action dependent on glucose concentration same as 4-hydroxyisoleucine?

The reason behind the question 2 is based on the fact that insulin secretion has got direct correlation with glucose concentration and glucose uptake by beta cells as explained in the early part under insulin secretion section.

There are evidences that 4-hydroxyisoleucine activates insulin signalling via increasing phosphorylation of PI3Kinase and Akt. It also has been demonstrated that 4-hydroxyisoleucine modulates glucose consumption by increasing GLUT4 translocation mediated by PI3Kinase in glucose disposal cells including adipose tissue and muscle cells (Jaiswal *et al*. 2012). The previous study about isoleucine suggested isoleucine activates PI3Kinase and reduces the activity of AMPK alph-2 due to decrease of AMP and ATP improvement (Doi *et al*. 2005).

Question 3: In beta cells GLUT4 is not the major glucose transporter, and GLUT1 and GLUT2 are mainly responsible for glucose transportation. If the 4-hydroxyisoleucine and isoleucine increase glucose consumption in beta cells, it is possibly related to GLUT1 and GLUT2. Does inhibition of PI3 Kinase in beta cells affect glucose consumption in beta cells treated with 4-hydroxyisoleucine and isoleucine?

Based on available knowledge of biochemistry how can we find a unified explanation for all the effects of 4-hydroxyisoleucine mentioned in table 5 considering its glucose dependency mode of action? Many studies found that most of these activities can be explained by the energy content of the cell. All the signalling pathways within the cells are handled by a group of enzymes, which are activated and deactivated by phosphorylation and ATP is the major source of this phosphorylation. Logically, if the ATP/ADP ratio within the cell is increased by converting more ADPs to ATPs, it is probable that many of these sensitizing activities happens. Our aim was to identify any connection between 4-hydroxyisoleucine and isoleucine and ATP within the cells.

Question 4: Do 4-hydroxyisoleucine and isoleucine increase ATP production and ATP content of beta cells? If yes, can they create the same effect in other cell types like HepG2 as a cancerous cell with minimal aerobic respiration? Does PI3Kinase inhibitor affect ATP production in beta cells treated with 4-hydroxyisoleucine and isoleucine?

As explained before insulin release is completely and directly regulated by the ATP/ADP ratio. As previous studies claimed on insulinotropic effect of both 4-hydroxyisoleucine and isoleucine, this effect may be connected to the ATP level within the pancreatic beta cells. This was the concept for selecting beta cell as a model to address question 4.

Chapter II

Material and Methods

Part I

Animal Trial

2.1 Animals

Male Wistar rats were purchased from the Pharmacological Research Centre of Tehran University of Medical Sciences. Eight week-old rats weighing between 220 and 250 g were used at the start of each treatment protocol. Rats were housed in separate cages in an animal room kept at constant temperature (25 ◦C) with a 12 h light–dark cycle. Standard rat chow pellet and water were provided ad libitum throughout the experimental period. The animals were maintained in accordance with the Animal Ethics Committee of the University of Medical Science, Qom, Iran.

2.2 Preliminary Study

A preliminary pre-experimental screening, animal test was set up to examine different streptozotocin (STZ) induction protocols for reaching the optimized condition before proceeding to main experiment. The aim of the project was to study the effect of 4-hydroxyisoleucine in an animal model in which there was minimal insulin secretion. STZ was used to reduce insulin secretion to make it possible to study the effects of 4-hydroxyisoleucine on glucose metabolism that are independent of insulin. STZ can be administered intravenously (iv) or intraperitoneally (ip) to destroy pancreatic beta cells to create a diabetes type 1 rat model. Intra-peritoneal administration is less effective than intravenous as inject directly into the blood stream can produce a more profound effect with lower dose but it was more suitable as it combines less risk of infection and other side effects in rats during long term experiments because direct injecting of STZ to vein via rat tail may increase the risk of transferring germs directly to the blood stream and also there is no option

to control bleeding after the injection. STZ was purchased from Sigma-Aldrich (London, UK) and prepared according to standard protocols (Motyl *et al.* 2009) by mixing STZ with freshly prepared Na-citrate buffer (1.47 g of Na-Citrate into 50 ml deionized water, adjusted PH to 4.5) immediately before injection. Eight rats with weight range of 190-230 g were divided into four equal groups with two rats in each group. STZ was injected with doses of single 60mg/kg, single 150 mg/kg and 60 mg/kg for five consecutive days. One group was allocated as control without STZ injection as vehicle control (injecting Na-citrate buffer without STZ). Glucose levels were measured using a micro-drop strip glucometer (Accu-check,Germany) before the injection of STZ and five days after injection to monitor glucose level. Glucometer was calibrated by glucose solution prior to test to make sure accurate reading of blood glucose. The blood glucose levels of all rats were in the range of 68 to 84 mg/dl as an indication of normoglycemic status as explained in chapter 3. Two weeks after receiving the injection rats were killed by volatile inhalational anaesthetics (Ether) using gas scavenging apparatus for rodent and blood collected directly from the left ventricle of heart by blood sampling vacutainer for glucose and insulin measurement. The blood glucose level increased in all groups apart from the control group. The blood insulin level was measured using an ELISA insulin kit (DRG International, NJ, USA) to measure the amount of insulin five days after the injection as described below.

2.3 Main Experiment

Rats were divided into three groups each of six, comprising normal controls (NC),diabetic controls (D) and diabetic rats treated with 4HO-Ile (D4H). Rats were rendered type 1 diabetic by intraperitoneal injection

of streptozotocin (60 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) for five days consecutively (Motyl *et al*. 2009). After one week blood glucose concentration was measured with a Glucometer on a drop of blood from the tail. Rats were considered to be diabetic if blood glucose levels were greater than 300 mg/dl. The treatment group of diabetic rats were intubated daily with a solution of 4-hydroxyisoleucine in saline (Extracted from fenugreek seeds with 98% HPLC purity). 4-Hydroxyisoleucine was a preparation extracted from fenugreek seeds with 98% HPLC purity (Haeri *et al*. 2009), and was given at a dose equivalent to 50 mg/kg/day for four weeks. Normal control rats and diabetic control rats were intubated with an equivalent volume of saline alone.

2.4 Insulin Assay

The Blood samples immediately centrifuged at 1000 × g for 15 min. Plasma was removed and stored at −20 ◦C and insulin level was measured in each sample by DRG Insulin ultra sensitve ELISA Kit (DRG International, NJ, USA) which is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle with measurement range of 0.02 to 5.5 µg/l . The microtiter wells of kit are coated with a monoclonal antibody directed towards a unique antigenic site on the Insulin molecule.

25 µL of each standard, control and samples were dispensed with new disposable tips into appropriate wells. 25 µL enzyme conjugate was added into each well and thoroughly mix for 10 second following 30 minutes incubation at room temperature. 50 µL of enzyme complex were added to each well after 3 times wash with a diluted wash solution (400 µL per well) and incubated at room temperature for 30 minutes. 50 µL of

substrate solution were added to each well after 3 times wash and incubated at room temperature for 15 minutes. Enzymatic reaction was stopped by adding 50 µL of stop solution to each well and absorbance (OD) of each well was measured at 450 ± 10 nm with a microtiter plate reader. A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. The concentration of the samples was read directly from this standard curve.

2.5 Serum lipid profile, glucose and uric acid

At the end of the experiment rats were killed by volatile inhalational anaesthetics (Ether) using gas scavenging apparatus for rodent and blood samples were collected by cardiac puncture from the left ventricle into heparinised tubes and immediately centrifuged at 1000 g for 15 min. Plasma was removed and stored at −20 °C. Fasting plasma glucose, triglyceride, cholesterol and LDL concentration were measured on a fully automated autoanalyser (Biosystem, Spain) by the university of Qom's medical school reference laboratory.

Plasma uric acid was measured using a timed endpoint method on a fully automated Beckman Coulter Synchron LX20 clinical system which is an automated computer-driven, general chemistry analyser designed for the in vitro determination of a variety of general chemistries, therapeutic drugs, and other chemistries (Rosolowsky *et al*. 2008). The tests were done and verified by university of Qom's medical school reference laboratory.

2.6 Statistical analysis

Data were analysed using SPAW version 18.0 and are expressed as mean ± SD. ANOVA with Tukey and Bonferroni post hoc tests were used to determine significance of differences between groups. Preventing the type I error (considering significant a difference that actually isn't significant) was the main reason we chose to use both Tukey and Bonferroni post hoc approaches. We tested all the results with Bonferroni and repeated the analysis with Tukey test to make sure that we make a correct translation of the results by eliminating type 1 error. $P < 0.05$ was considered to be statistically significant.

All the in-vitro results were analysed using only Bonferroni post hoc test.

Part II

Laboratory In-vitro Studies

2.7 Cell line and cell culture

BRIN-BD11 is a hybrid pancreatic islet cell line formed by the electrofusion of a primary culture of NEDH rat pancreatic islets with the rat insulinoma cells (RINm5F cell line). BRIN-BD11 is a stable, glucose-responsive insulin secreting beta cell line expressing insulin, glucokinase, Islet Amyloid Polypeptide (IAPP or Amylin) as detected by immunocytochemistry, and also expresses the GLUT2 glucose transporter (McClenaghan *et al*. 1996). BRIN-BD11 was purchased from the HPA (Health Protection Agency, United Kingdom). 4 x 10^4 Cells were cultured in RPMI1640 with 2 mM glutamine and 10% FCS containing 11 mM glucose and incubated in 5% CO_2 at 37°C. The cell population doubled approximately within 24 hours and reached more than 80% confluency within 48 hours in a 75 cm^2 flask. Cells were seeded for experiments in 24 well plates at 300,000 cells in each well in 2 ml normal RPMI-1640 with 11 mM glucose for 24 hours (more than 80% confluent). The medium was replaced by RPMI-1640 with 2 mM glutamine and 10% FCS containing 22 mM glucose and loaded with treatment compounds at different concentrations, incubated for 24 hours with 5% CO_2 at 37°C. All the inhibitors and activators used in the current study were loaded at the same time as isoleucine and 4-hydroxyisoleucine added to culture media. The glucose concentration of medium with 22 mM glucose was measured each time before loading using an optimized glucometer as described below in order to create a baseline for the glucose concentration at zero time.

2.8 Glucose measurement

Glucose levels in the cell culture medium were measured using code-free glucooxygenase based strip technology. The glucometer measurement accuracy was evaluated by 50 independent measurements of glucose in RPMI-1640 containing 11 and 22 mM glucose separately. The uncertainty intervals and relative deviation from true value were calculated by following formulation:

Uncertainty Intervals = (highest reading value – Mean)+(Mean- lowest reading value)
Relative Deviation = [(Mean – True Value)/True Value] x 100

The test showed that the machine reading achieved uncertainty intervals of 0.5 and 0.3 mM and relative deviation of 8.34% and 4.29% in 11 and 22 mM RPMI-1640 respectively. The consistency and accuracy of reading were important as we used the technique to compare glucose changes in the medium. The glucose level of each sample of cell culture medium was measured three times using the same batch of strips and corrected using relative deviation.

2.9 ATP assay

Cell ATP content was measured by a colorimetric assay from Abcam (Cambridge, UK) using the manufacturer's standard protocol as follows:
300,000 cells were lysed using 100 µl ATP assay buffer provided in the kit and centrifuged at 15,000 x g and at 4$^{\circ C}$ for 2 minutes to pellet insoluble materials. 50 µl of supernatant was added to each well of a 96-well plate, and the final volume adjusted to 100 µl by adding 50 µl

ATP assay buffer. 50 µl of ATP reaction mix containing 44 µl ATP assay buffer, 2 µl ATP probe, 2 µl ATP converter and 2 µl developer mix which prepared based on manufacturer protocol provided in the kit, was added to each well and incubated for 30 minutes at room temperature. A standard curve was prepared using the standards provided standards in the kit with the range of 0 to 10 nmol. Absorption was measured at 570 nm using BMG Labtech FLUOstar reader.

2.10 Insulin measurement

Insulin in the medium was measured using an HTRF (Homogeneous Time-Resolved Fluorescence) insulin assay kit from Cisbio Bioassays, (France) (Youl *et al*. 2010). HTRF is newly developed generic assay technology to measure analytes in a homogenous format by combining fluorescence resonance energy transfer technology (FRET) with time-resolved measurement (TR). In TR-FRET (HTRF) assays, a signal is generated through fluorescent resonance energy transfer between a donor and an acceptor molecule when in close proximity to each other which significantly reduces the buffer and media interference using dual-wavelength detection. The final signal is proportional to the extent of product formation which makes the HTRF assay sensitive and robust for many antibody-based assays including the insulin.

The HTRF® insulin assay is a sandwich immunoassay involving two antibodies, one labeled with Europium Cryptate (Eu-K) and the other with cross-linked allophycocyanin (XL665). As the two antibodies bind to insulin molecules, fluorescence resonance energy transfer (FRET) occurs between the Eu-K and XL665 tracers. This HTRF®

signal is thus proportional to the insulin concentration. The fluorescence emitted from the well is recorded at both 665nm (XL665 emission wavelength) and 620nm (Eu-K emission wavelength) in a time-resolved manner to differentiate signal from the medium background and the free acceptor XL665 from the HTRF signal, using a BMG Labtech FLUOstar plate reader using the following settings: excitation filter: 337 nm, emission filters: 620 nm and 665 nm; integration delay (lag time): 60 μs; Integration time:400 μs; number of flashes: 200; gain: 2300 for 620 and 665 nm. The fluorescence ratio, expressed as F_{665} / F_{620} x10,000, was calculated for each well in order to minimize medium interferences. A standard curve with a range from 0 to 12.5 ng/ml was prepared using standard insulin supplied by the manufacturer of the kit.

50 μl of each sample (Culture medium), 25 μl Ab-XL665 (supplied in the kit) and 25 μL anti-insulin Ab-XL665 respectively, were added to each well of 96 well-plate and covered with a plate sealer and incubated for 2 hours at room temperature then the result read by BMG Labtech FLUOstar plate reader.

2.11 Compounds

4-Hydroxyisoleucine: Synthetic 4-hydroxyisoleucine was purchased from TRC, (Noth York, Canada) and had 98% purity as confirmed by NMR spectroscopic and mass spectrometric analysis, according to the manufacturer's data sheet. It contained (2S,3R,4S) 4-hydroxyisoleucine as the major isomer (> 94%) and (2R,3R,4S) 4-hydroxyisoleucine as the minor isomer (< 4%). 100 mM stock solution was prepared in RPMI-1640 plain medium, filter sterilized and stored at 4 °C.

Isoleucine: Isoleucine was purchased from Sigma-Aldrich (Poole, UK) and had a purity of 99% (HPLC grade). 100 mM stock solution in RPMI-1640 plain medium filter sterilized and stored at 4 °C.

Other compounds: the following table contains the list of specialist compounds used in experiments.

Name	Molecular Weight	Effect	Stock solution
Cycloheximide (Abcam, UK)	281.35	Protein synthesis inhibitor	16mM (10X)
Kaempferol (Abcam, UK)	286.24	Inhibition of topoisomerase-1, MAO, COX, estrogenic effects and activation of the mitochondrial Ca2+ uniporter.	20mM
KU 0063794 (Sigma-Aldrich, UK)	465.5	Selective inhibitor of mammalian target of rapamycin (mTOR) (IC50 ~10 nM for mTORC1 and mTORC2). Displays no activity at PI 3-kinase or 76 other kinases tested. Inhibits activation and hydrophobic motif phosphorylation of Akt, S6K and SGK, but not RSK. Suppresses cell growth and induces G1 cell cycle arrest in vitro. (10nM)	10mM
Ruthenium Red (Abcam, UK)	786.35	Inhibitor of calcium signalling with multiple actions. Inhibits the mitochondrial Ca^{2+} uniporter, Ca^{2+} dependent ATPase, troponin C and calmodulin. Attenuates capsaicin-induced cation channel opening and inhibits Ca^{2+} release from ryanodinesensitive intracellular Ca^{2+} stores. Blocks large-conductance Ca^{2+} activated potassium channels.	10 mM(10X)

STF-31 (Merck)	423.5	A cell-permeable sulfonamide that selectively inhibits the growth of VHL-deficient renal cell carcinomas (RCCs) dose-dependently (0-5 μM) by directly targeting glucose transporter 1 (GLUT1), which is up-regulated by HIF (hypoxia-inducible factor) transcription factor in VHL-deficient cells. It elicits decreased oxidative phosphorylation associated with aerobic glycolysis and leads to necrosis, which is consistent with the effect induced by GLUT1 RNA interference. It does not bind to other glucose transporters, and does not inhibit a broad range of 50 tested kinases.	10mM
Wortmannin (Sigma-Aldrich, UK)	428.43	PI3 Kinase inhibitor	10mM
UK-5099 (Sigma-Aldrich, UK)	288.3	Inhibitor of plasma membrane monocarboxylate transporters (MCTs) and the mitochondrial pyruvate carrier (MPC).	20mM

2.12 Cell metabolism and mitochondrial respiration analysis

The Seahorse Bioscience XF24 instrument was developed and designed to measure the rate of change of dissolved oxygen and pH in the media immediately surrounding living attached cells cultured in a microplate in real time mode. Changes in the extracellular media are caused by the consumption or production of oxygen and protons by the cells. Therefore, a sensitive measurement of the media flux can be used to determine rates of cellular metabolism with great precision and in a totally non-invasive, label-free manner. Accurate and repeatable measurements of consumption and production of oxygen and proton in as little as five minutes are the unique feature of the XF technology. This is accomplished by isolating an extremely small volume (less than 10 µl) of media above the cell monolayer. Cellular metabolism causes rapid, easily measured changes to the "microenvironment" in this small volume. Typically, a measurement cycle is performed for 2-5 minutes. During this time, the media are gently mixed and is followed by a short temperature recovery period. The analyte levels are then measured until the oxygen concentration drops approximately 20-30% and media pH declines approximately 0.1-0.2 pH units. The measurement is performed using 24 optical fluorescent biosensors embedded in a sterile disposable cartridge that is placed into the Seahorse 24 well tissue culture microplate. The XF analyser measures oxygen consumption rate (OCR) as an indicator of mitochondrial respiration and extracellular acidification rate (ECAR) as the result of glycolysis at intervals of approximately 2-5 minutes. Baseline metabolic rates typically measure 3-4 times, and are reported in pmol/min for OCR PPR (Protons Production Rate) and in mpH/min for ECAR. The compound is then added to the media and mixed for 5 minutes, and then the post-treatment

OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR changes. Cellular oxygen consumption (respiration) and proton excretion (glycolysis) causes rapid, easily measurable changes to the concentrations of dissolved oxygen and free protons in this "transient microchamber" which are measured every few seconds by solid state sensor probes residing 200 microns above the cell monolayer. Seahorse analyser continues to measure the concentrations until the rate of change is linear and then calculates the slope to determine OCR and ECAR, respectively (Seahorse Bioacience; http://www.seahorsebio.com).

2.13 Optimisation of cell number for Seahorse XF-24

In this experiment, BRIN-BD 11 cells were seeded at four different densities, 20000, 30000, 40000 and 50000 per well with five replicates to indicate the best density of cells for seeding to achieve optimum responses in Seahorse cell culture plates. The basal oxygen consumption (OCR) rates were measured to the establish baseline rate in different densities of cells in order to identify the optimum cell density for experiment repetition.

Cells were dispensed into each well in 100µL RPMI-1640 with 11 mM glucose and left to attach; the plate was left out in the vertical laminar flow hood (VLFH) for about 30 minutes before returning them to the incubator as this helped to evenly disperse cells across the well. All the wells were topped up with medium to 300µL and placed into CO_2 incubator as normal. The growth medium was removed from each well and the cells were washed with 1ml of pre-warmed RPMI-1640 without bicarbonate and with 1% FBS because bicarbonate and

FBS affect the buffer capacity of the medium and interfere with protons measurement of the Seahorse XF-24 sensors. Each well was loaded with 625 µl of RPMI-1640 without bicarbonate and with 1% FBS medium and incubated in a 37˚C incubator without CO_2 for 60 minutes to allow cells to pre-equilibrate with the assay medium.

BRIN-BD11 cells were seeded onto 20 wells of a 24-well Seahorse special microplate and 4 wells were left and filled with RPMI-1640 only for calibration purposes, as instructed by the manufacturer's standard protocol, and shown in the following layout **(Fig 2.1)**. Sensor Cartridge (Proper orientation: notch at lower left) was loaded to the machine and pre-warmed RPMI-1640 running medium (without bicarbonate and with1% FBS) plus 0.3% DMSO was loaded to control wells (A1, B4, C3 and D6). The Seahorse XF-24 command was adjusted to 3x loop start and 3 minute measurement at each loop.

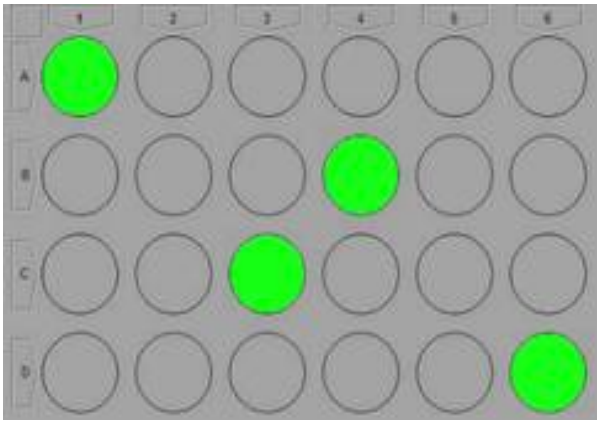
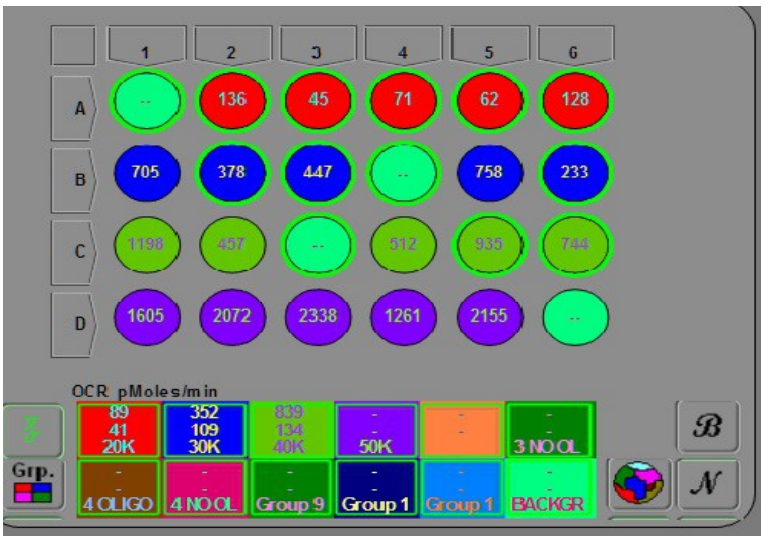


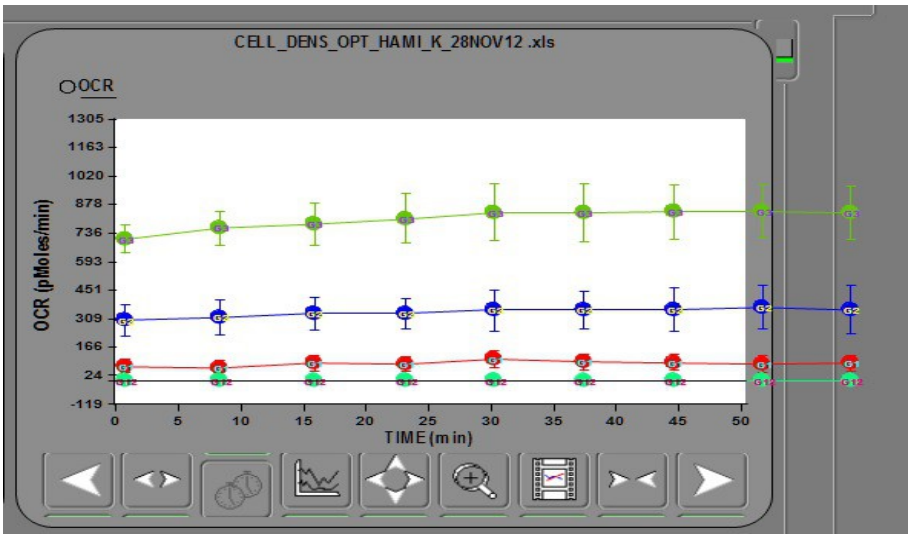
Figure 2.1. The layout of negative controls in the Seahorse microplateplate. The green wells were filled with RPMI-1640 medium without cells for the purpose of machine calibration during test runs.

2.14 Results of cell number optimization

The results of two preliminary experiments to verify the best density of BRIN-BD 11 cells for use in Seahorse XF-24 microplates showed that the density of 40000 cells per well achieved the best oxygen consumption rate (OCR) measurements compared with other cell densities within the machine reading range **(Fig 2.2)**. The density of 50000 cells produced out of range results. As a conclusion, the density of 40000 BRIN-BD11 cells is the maximum cells which can be loaded to create enough cells for a robust mitochondrial respiration measurement with Seahorse XF-24. To eliminate probable bias, five replicates for each density were measured simultaneously.



a)



b) **Red: 20K Blue: 30K Green: 40K (Green in the bottom is baseline without cells)**

Figure 2.2. Five replicates of 20000, 30000, 40000 and 50000 BRIB-BD 11 cells were seeded respectively from column A to D of the microplate. A1, B4, C3 and D6 were filled by RPMI-1640 without cell for calibration during the test (a). The measurements of oxygen consumption rate (OCR) in 50 minutes shows the density of 40000 BRIN-BD11 cell creates more robust reading compared to others and density of 50000 cells creates out of range result (b).

Chapter III

Animal Trial

All the facilities for carrying this animal study were provided by Qom medical university, Iran and Dr Mohammadreza Haeri. He also helped me in all the steps of handling animals, collecting samples and analysing data. Dr Kenneth White also provided me with immense help in statistically analysing data and writing the paper which has been published in Phytomedicine journal (Appendix I)

3.1 Type 1 diabetes animal model

The main characteristic of type 1 diabetes is a lack of insulin production due to the destruction of the pancreatic beta cells, which can be achieved in animal via chemical destruction of beta cells or breeding a genetically modified rodent who develop pancreatic beta cell destruction due to spontaneous autoimmune response. Steptozotocine (STZ) and alloxan are two main compounds which use for chemically induced type 1 diabetes. They compete with glucose to enter pancreatic beta cells due to their structural similarity with glucose. Chemically induced diabetes is an easy and cheap technique to create a type 1 diabetes model for testing drugs or therapies where the main mechanism of action is lowering blood glucose in a non beta cell dependent or insulin independent manner (Sheshala *et al*., 2009). Both STZ and alloxan can be toxic at other organs of the body and this should be considered when drugs are being tested in these models. STZ tolerates better than alloxan and has a wider diabetogenic ratherthan alloxan with a narrow diabetogenic dose. Alloxan light overdosing can cause general toxicity, especially to the kidney (Szkudelski,2001).

Streptozotocin (STZ) is particularly toxic to pancreatic beta cells and has been used for several decades to induce the diabetic state in rodents (Davidson *et al*. 1977, Rees *et al*. 2005). STZ is a glucosamine nitrosourea compound similar to glucose, that is recognised and absorbed by GLUT2, but not other glucose transporters. STZ induces DNA damage by activation of poly ADP-ribosylation within the beta cells (Szkudelski. 2001), and is particularly toxic to pancreatic beta cells because they express high level of GLUT2 (Wang *et al*.1998).

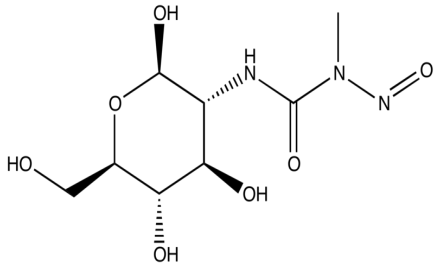


Figure 3.1. Streptozotocin chemical structure. (Adapted from Sigma Aldrich, http://www.sigmaaldrich.com)

The type 1 diabetes rat model can be produced using single high dose STZ and multiple injection of STZ over 5 days. Single high dose STZ administration could create a simple hyperglycaemia model, but it is not stable for long term model due to spontaneous regeneration of endogenous beta cells (King. 2012). Multiple injection of STZ could create more stable damage to pancreatic beta cell which could not be regenerated after a few weeks (King. 2012). Chemically induced animal is not a appropriate model for studying the pathogenesis of type 1 diabetes. The spontaneous models of autoimmunity is more close to human disease for such study (Reddy *et al*. 1995). We adapted the STZ technique to create a suitable model for our study as our aim is to investigate the effect of 4-hydroxyisoleucine on blood glucose level in an animal model close to type 1 diabetes not studying the pathogenesis of the disease.

We were looking to create a type 1 diabetes rat model with minimum insulin secretion in which the animal can survive throughout the experimental period without the need to administer insulin. This would allow us to assess the effect of 4-hydroxyisoleucine on condition with a significant reduction in insulin production ability and the amount of insulin in circulation.

We compared three protocols of STZ administration, using intra-peritoneal (IP) injection to achieve a stable model with high blood

glucose level and significantly reduced insulin production capability. A single IP injection of STZ at doses of 60 mg/kg or 150 mg/kg were compared with the procedure of daily single IP injections of STZ at 60 mg/kg for five consecutive days (Motyl *et al*. 2009). We ran a preliminary test to compare these different protocols to achieve the best possible selection of a suitable rat model for the study. Eight rats with the weight of 190-230 g were selected and divided into 4 equal groups with two rats in each group **(Table 3.1)**. The glucose level was measured using a micro-drop strip glucometer before the injection of STZ, at day 5 after injection. The rats were killed two weeks after injection and blood collected directly from the heart for glucose and insulin measurement.

Group A	Single dose STZ 60 mg/kg IP injection
Group B	Single dose STZ 150 mg/kg IP injection
Group C	Multiple dose STZ 60mg/dl consecutive 5 days IP Injection
Group D	Normal Control without STZ

Table 3.1. Different protocols of intraperitoneal injection of STZ.

Single doses of STZ induced increases in glucose levels, but not to levels greater than 300 mg/dl, the threshold for a diabetic phenotype **(Table 3.2)**. Repeated doses of STZ induced glucose levels of 520 ± 13 mg/dl compared with 79 ± 3 mg/dl for controls (n = 2), and insulin levels were below the limit of detection (0.2 µg/l) using automated clinical reader compared with an average of 1.79 µg/l in controls. We did not use ultra sensitive ELISA kit for preliminary study for its high cost, but the method of detection was accurate enough for our purpose. We used different insulin measurement using ultra sensitive rat insulin kit with detection range of 0.02 µg/l to 5.5 µg/l. On this basis the repeated doses of

STZ could be used to induce a state in rats as close as possible to type 1 diabetic. Multiple STZ injection created a desired model with hyperglycaemia and minimized insulin production, which suggest a massive destruction of pancreatic beta cells. Using such model could make it possible the activity of 4-hydroxyisoleucine in different angle.

Group	**Blood Glucose mg/dl**	**Blood Glucose mg/dl**	**Blood Glucose mg/dl**	**Insulin µg/l**
A1	82	118	127	1.19
A2	77	109	123	0.94
	SD ± 3.54	SD ± 6.36	SD ± 2.83	SD ± 0.77
B1	68	201	247	0.59
B2	79	177	262	0.51
	SD ± 7.78	SD ± 16.97	SD ± 10.61	SD ± 0.06
C1	71	497	530	lower than
C2	84	441	511	measurement level
	SD ± 9.19	SD ± 39.6	SD ± 13.4	
D1	71	80	77	1.81
D2	76	73	81	1.76
	SD ± 3.54	SD ± 4.95	SD ± 2.83	SD ± 0.04

Table 3.2. Comparison of blood glucose and insulin in rats treated with different STZ injection protocols. Two rats were allocated in each group as explained in Table 1. Blood glucose levels before injection, in day 5 and two weeks after injection was shown in columns 2, 3 and 4 respectively. Group C shows highest sustained blood glucose level with no trace of insulin within 2 weeks of 60mg/kg STZ injection for five consecutive days. Rats in group C survived with high blood glucose level for two weeks and it demonstrates a suitable diabetic rat model close to diabetes type 1 for our experiment .

3.2 Animal study results and discussion

Rats were divided into three treatment groups, each with six animals. Two groups were treated with a single dose of 60 mg/kg STZ for five consecutive days and the third group received a vehicle control. Animals treated with STZ were left for seven days to develop diabetes, as confirmed by measurement of blood glucose (glucose level of tail blood over 300 mg/dl) before starting treatment with 4-hydroxyisoleucine. The diabetic treatment group was intubated with 50 mg/kg/day of 4-hydroxyisoleucine, and the diabetic control group with saline vehicle, continuously for four weeks. One week after STZ administration rats had a markedly elevated plasma glucose compared with controls, and this was sustained for a further four weeks **(Fig 3.1, groups D and D4H)**. Fasting blood glucose, triglyceride, cholesterol, LDL, HDL and uric acid were measured using methods described in chapter II and data analysed statistically with the ANOVA Bonferroni and Tukey tests to avoid type 1 error. The correlation with *P* value less than 0.05 in both tests was accepted as significant. Treatment of diabetic rats with 4-hydroxyisoleucine for four weeks induced a reduction in blood glucose from 500[±SD] mg/dl to 330[±SD] mg/dl which is statistically significant ($P<0.05$). The blood glucose level in both control and diabetic control groups remained steady during the experimental period without significant changes, comparing blood glucose levels at the beginning and the end of the experiment in each group **(Fig 3.1)**. It shows that damaged pancreatic beta cells were not restored naturally in the STZ induced diabetic group during the treatment period of four weeks and the diabetic status was constant. The fact that blood glucose levels remained steady over the four week period of the experiment in the non-diabetic group, after being housed

in the same conditions as the other groups, suggests that the prevailing environmental and dietary conditions did not influence blood glucose levels. Generally the diabetic animals treated with 4-hydroxyisoleucine had an improved appearance and it was noticeable that the heavy ocular vascularization induced by STZ was being reversed as the treatment with 4-hydroxyisoleucine progressed (Haeri et al. unpublished data).

Insulin was measured by ultrasensitive ELISA kit with the detection limit of 0.02 µg/l to 5.5 µg/l. All the samples measurement were within the detection range. Insulin levels in the diabetic groups were significantly lower than normal controls with levels decreased by 60 % from 0.7 µg/l to less than 0.3 µg/l. There is not a significant difference in insulin levels between the diabetic control and diabetic group treated with 4-hydroxyisoleucine, after four weeks **(Fig 3.2)**.

These data suggest that restoration of pancreatic beta cells does not play a role in improving diabetic conditions in the 4-hydroxyisoleucine treatment group.

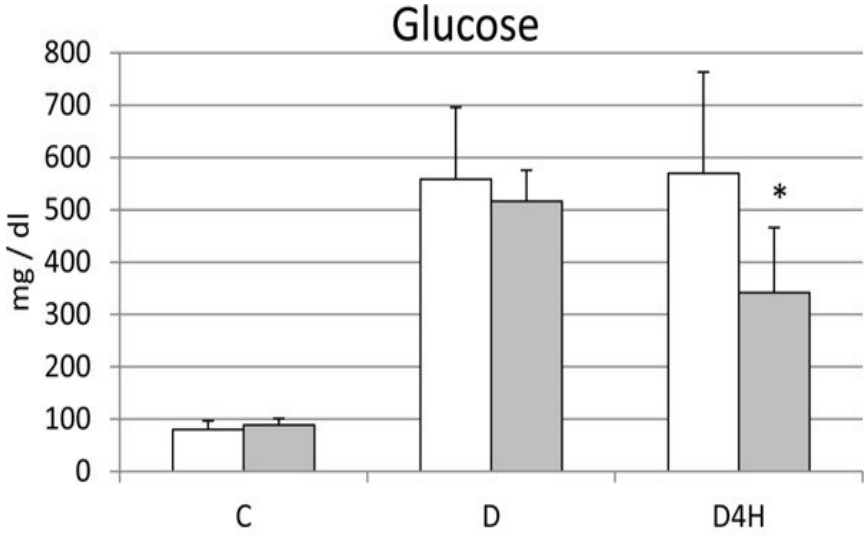


Figure 3.1. Fasting plasma glucose at the beginning and end of the 4-hydroxyisoleucine treatment. Mean and SD plasma glucose of five or six rats per group. Control rats (C). Diabetic (D) and treatment (D4H) groups were made diabetic by repeated doses of STZ. Treatment with 4-hydroxyisoleucine started one week after the STZ treatment and continued for four weeks. Glucose was measured at the start of the 4-hydroxyisoleucine treatment (White Columns) and at the end of four weeks treatment (Grey Columns). 4HO-Ile induced a significant (*$p < 0.05$) decrease in plasma glucose within the treatment group after four weeks.

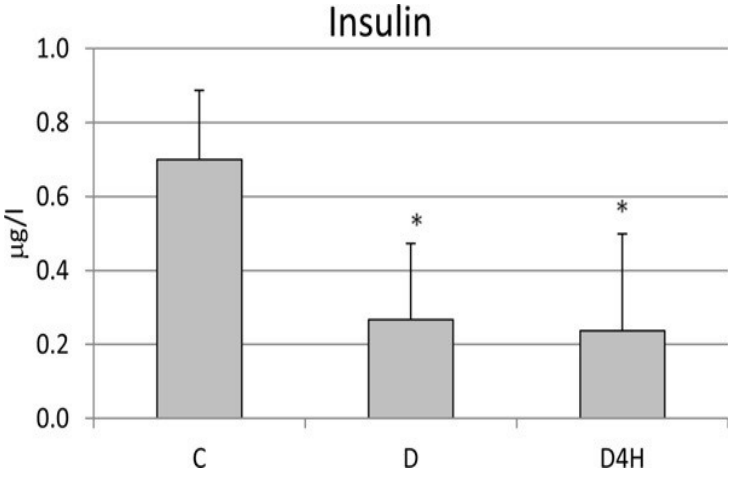


Figure 3.2. Plasma insulin after treatment with 4-hydroxyisoleucine. Plasma insulin was measured by ultrasensitive insulin ELISA kit with the detection limit of 0.02 µg/l to 5.5 µg/l. Data are mean + SD of five or six rats per group at the end of the treatment period. Insulin levels were significantly lower in diabetic groups [D, D4H] compared with control [C] (*$p < 0.05$).

The diabetic rats had a markedly higher intake of food and water compared with controls **(Fig 3.3)**, but the hyperphagia did not cause any increase in body weight in the diabetic rats compared with controls. Body weights (mean ± SD) at the end of the study were 289 ± 31 g (C), 269 ± 15 g (D) and 267 ± 5 (D4H). There was no significant difference between groups.

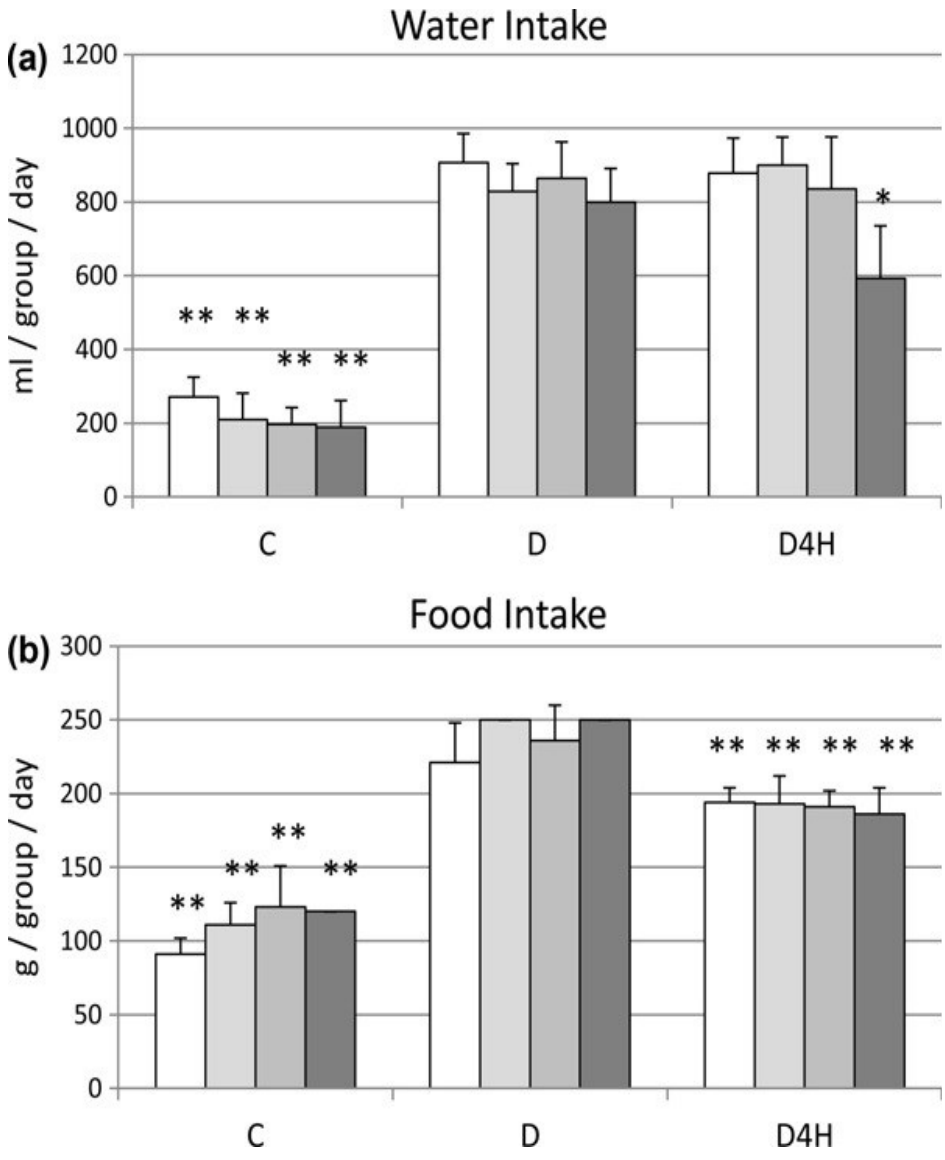


Figure 3.3. Water and food intake over treatment period. Intake of water (a) and food (b) over the four week period of 4-hydroxyisoleucine treatment. Data are mean + SD daily intake per animal over weekly periods for control (C), diabetic (D) and diabetic rats treated with 4-hydroxyisoleucine (D4H). Data are for the four consecutive weeks of the period of treatment with 4HO-Ile: week 1 (□), 2 (■), 3 (■), 4 (■). Significant differences between means of group D compared with groups C or D4H, for a given week, are indicated where $p < 0.05$ (*) or $p < 0.01$ (**).

The lipid profile of the diabetic rats was also consistent with a diabetic phenotype, with significantly elevated triglyceride, cholesterol, HDL (High Density Lipoprotein) and LDL (Low Density Lipoprotein) compared with the control group **(Fig 3.4)**. The changes in glucose and lipids coupled with the marked decrease in insulin indicate that a type 1 diabetic phenotype is induced by the repeated intraperitoneal doses of STZ. The levels of triglyceride, LDL and HDL in the treated diabetic animals were not significantly different to those of control, non-diabetic rats and total cholesterol was reduced to near control levels **(Fig 3.4b)** More strikingly the 4-hydroxyisoleucine treatment also resulted in significant decreases in all lipid markers compared with untreated diabetic rats.

The levels of triglyceride, LDL and HDL in the treated diabetic animals were not significantly different to those of control, non-diabetic rats and total cholesterol was reduced to near control levels indicating that treatment with 4-hydroxyisoleucine had restored the diabetic lipid profile to an almost normal one. In type 1 diabetes levels of HDL typically decrease but in our model the STZ-treated animals had elevated HDL.
The reason for this is unclear, but similar behaviour in STZ-rats have been found by others (Islam. 2011). In our study the treatment with 4-hydroxyisoleucine could reverse the changes in HDL induced by STZ and restore levels close to that of control non-diabetic animals **(Fig 3.4)**.

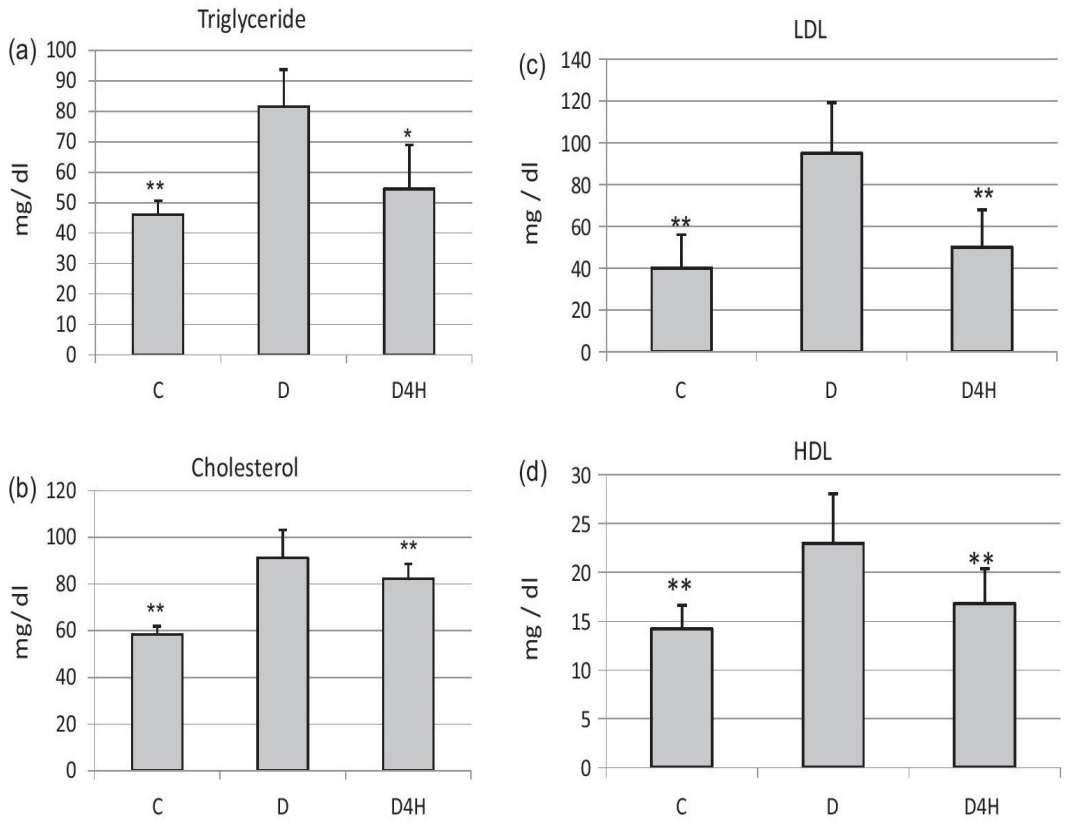


Figure 3.4. Serum lipid profile of rats after four weeks treatment with 4-hydroxyisoleucine. Triglyceride (a), cholesterol (b), LDL (c) and HDL (d) measured at the end of the treatment with 4-hydroxyisoleucine are shown as the mean + SD of five or six rats per group. Levels were significantly lower in control (C) and treated diabetic (D4H) groups compared with untreated diabetic animals (D) (*$p < 0.05$, **$p < 0.01$).

Uric acid levels in the STZ-induced diabetic rat group were elevated significantly, about 40% compared to the non-diabetic group, and this increased hyperuricemia of the diabetic rats was restored to levels found in normal rat controls after treatment with 4-hydroxyisoleucine **(Fig 3.5)**. There is a relationship and biochemical interaction between high serum glucose and high serum uric acid levels as uric acid is

increased during hyperglycaemia (Cook *et al*. 1986). The relationship between serum uric acid and metabolic diseases has been explored in a number of studies. There is a positive relationship between levels of blood insulin and blood uric acid level and uric acid concentration is closely related to pancreatic beta cell activity (Robles-Cervantes *et al*. 2011, Sinagra *et al*. 1996). Hyperuricemia is directly linked to damage to the kidney, which is one of the major causes of morbidity and mortality in diabetes (Rosolowsky *et al*. 2008). There is increasing evidence from clinical data that high levels of uric acid are an independent risk factor for kidney disease (Miao *et al*. 2011). It also has been demonstrated that high blood uric acid level is directly associated with macro and microangiopathies and also independently associated with coronary heart disease and renal dysfunction in diabetic patients (Ito *et al*. 2011). It is notable in this study that the reduction in hyperuricaemia caused by treatment with 4-hydroxyisoleucine is more pronounced than the reduction in hyperglycaemia, in that uric acid levels return to those of normal controls **(Fig 3.5 compares groups C and D4H)** whereas levels of blood glucose remain three times higher than normal controls **(Fig 3.1 compare groups C and D4H)** and insulin levels remained unchanged **(Fig 3.2 compare groups D and D4H)**. It remains to be seen whether or not the effects of 4-hydroxyisoleucine on serum uric acid are mediated through improved, but not fully restored glucose levels, or by another mechanism without increasing insulin level.

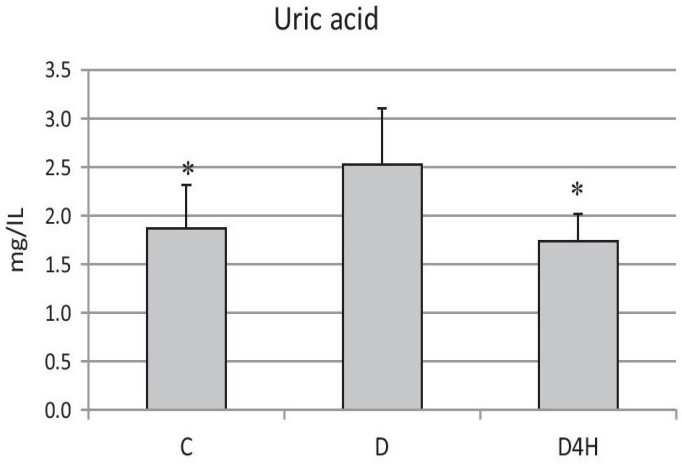


Figure 3.5. Serum uric acid after four weeks treatment with 4-hydroxyisoleucine. Data are mean + SD serum uric acid in rats at the end of the period of treatment with 4-hydroxyisoleucine. Levels were significantly lower in control (C) and treated diabetic (D4H) groups compared with untreated diabetic animals (D) (*$p < 0.05$).

The effective correction of the diabetes induced dyslipidemia by 4-hydroxyisoleucine is similar to results of the 4-hydroxyisoleucine treatment of rats or mice with a type 2 diabetes phenotype, in which 4-hydroxyisoleucine induced improvements in both lipid profile and hyperglycaemia (Haeri *et al*. 2009; Singh *et al*. 2010). anti-dyslipidemic activity of 4-hydroxyisoleucine has also been demonstrated in a hamster model of dyslipidemia (Narender *et al*. 2006). The findings presented in this thesis show that 4-hydroxyisoleucine is capable of modifying diabetes type 1 condition as a new mode of action in addition to the known beneficial effects of 4-hydroxyisoleucine in type 2 diabetes.

Previous studies of the anti-diabetic properties of 4-hydroxyisoleucine have focused on diabetes type 2 and animal models of insulin resistance and have suggested that 4-hydroxyisoleucine produces its anti-diabetic

effects via stimulating insulin secretion from pancreatic beta cells (Insulinotropic) and by an insulin sensitizing mechanism (Sauvaire Y *et al*. 1998, Broca *et al*. 1999, Broca *et al*. 2000, Jette *et al*. 2009). In the work presented in this thesis it is notable that 4-hydroxyisoleucine did not induce an increase in insulin levels in diabetic rats compared with untreated diabetic controls **(Fig 3.2)**. Both groups had insulin levels of about 0.3 g/l, which were approximately 65% lower than the non-diabetic control group **(Fig 3.2)**.

These data show that 4-hydroxyisoleucine has no insulinotropic activity in this model of type 1 diabetes, despite the high levels of glucose in the diabetic rats. Thus, even though insulin levels were low, indicative of a small amount of pancreatic activity, they were unchanged by treatment with 4-hydroxyisoleucine.

The improvement by treatment with 4-hydroxyisoleucine on the metabolic parameters of glucose, lipid profile and uric acid in a model of type 1 diabetes model with low level of insulin availability and pancreatic beta cell activity as well as diabetes type 2 and insulin resistant conditions suggest that 4-hydroxyisoleucine has a systemic effect on metabolically active tissues, including liver, muscle and adipose tissue, that is independent of insulin. The data from our study and from previous literatures cited above suggest that 4-hydroxyisoleucine could be used as a treatment for both type1 and type 2 diabetes independently or in combination with other treatments. Analysing the data from the current study show that 4-hydroxyisoleucine's main mechanism of action is not insulin stimulating or insulin sensitizing as was determined in previous studies.

We assume that 4-hydroxyisoleucine produces its anti-diabetic effects by facilitating glucose utilization in tissues independent of insulin considering the fact that 4-hydroxyisoleucine exhibits its activity in a high glucose concentration without glucose lowering effect in normal blood glucose level (Sauvaire *et al*. 1998). 4-hydroxyisoleucine could be a promising candidate for of diabetes treatment and understanding its mechanism of action to control diabetes metabolic disturbances also could shed a light to new treatment approaches and designing new medications.

3.3 Animal study findings at a glance

- 4-hydroxyisoleucine is effective in modifying diabetes type 1 metabolic conditions as well as those of diabetes type 2 and insulin resistance.

- 4-hydroxyisoleucine produces its anti-diabetic effects independent of insulin as no increase in insulin was observed through the duration of the study. It challenges the previously suggested mechanism of action such as an insulinotropic activity for 4-hydroxyisoleucine.

- 4-hydroxyisoleucine restores LDL (Low Density Lipoprotein) and triglyceride close to normal levels in diabetic rats, which demonstrate its effectiveness in reducing the risk of cardiac and vascular complications of diabetes.

- The effect of 4-hydroxyisoleucine on uric acid has been studied for the first time and it was demonstrated that 4-hydroxyisoleucine can restore diabetic hyperuricemia without increasing insulin secretion or pancreatic beta cell activity.

Chapter IV
In-Vitro Study
BRIN-BD11 Cell

In-vitro studies discussed in this chapter have been carried out with the help of my colleague, Dr Ravi Velga. He helped me during all the steps for preparation, cell culture and running tests. I would like to thank him for all his help. I would like to thank Dr Kenneth White for his constructive advices throughout this phase of the study.

4.1 Introduction

The animal studies described in Chapter III showed that 4-hydroxyisoleucine decreases plasma glucose, concentration and modifies other diabetes-related adverse effects such as hyperlipidemia and hyperuremia, independently of insulin. This mode of action contrasts with previous suggestions that 4-hydroxyisoleucine's mode of action is as an insulinotropic and insulin sensitizing agents (Broca *et al*. 2004, Sauvaire *et al*. 1998, Al-Habori *et al*. 1998), and raises the question as to how 4-hydroxyisoleucine decreases blood glucose levels. Interestingly, isoleucine, which is structurally close to 4-hydroxyisoleucine, also had an insulin-independent hypoglycaemic effect on diabetic mice (Ikehara *et al*. 2008), and it increased glucose uptake in muscle of fasted rats without changing insulin levels (Doi *et al*. 2007). Accordingly isoleucine was included in cell based studies, described below, which attempted to elucidate the mechanism of action of 4-hydroxyisoleucine.

In view of the published literature about isoleucine and of the data from our animal study, we hypothesized that 4-hydroxyisoleucine increases glucose uptake and utilisation in peripheral tissues, which leads to its anti-diabetic effects. Evidence to support the hypothesis that 4-hydroxyisoleucine increases glucose utilization in peripheral tissues which is not insulin mediated includes the observation that chronic, but not acute, treatment with 4-hydroxyisoleucine produces hypoglycaemia (Broca *et al*. 2004), that the activity of 4-hydroxyisoleucine is insulin independent (Haeri et al. 2012), that the anti-diabetic effects of 4-hydroxyisoleucine directly correlate with glucose concentration (Sauvaire *et al*. 1998) and that the chemical structure is similar to isoleucine, which has been shown to increase

glucose uptake independent of insulin (Doi *et al*. 2007). To investigate the insulin-independent effects of 4-hydroxyisoleucine and isoleucine on glucose uptake and utilization a cell model was used which is not insulin-responsive and dependent on the insulin signalling pathway for glucose uptake. Use of the model provides the opportunity to study the mechanisms of action of both isoleucine and 4-hydroxyisoleucine independently.

The model will also shed light on the question whether 4-hydroxyisoleucine is an insulin sensitizing agent, or whether its independent effect on increasing basal glucose utilization facilitates insulin action as well - if any molecule can increase glucose consumption independently in peripheral tissues, it can increase the insulin sensitivity.

4.1.1 BRIN-BD 11 cell as a model for In-vitro study

Neural cells, kidney tubular cells and pancreatic beta cells all possess an insulin-independent glucose uptake mechanism. Neural cells possess both insulin independent and insulin mediated glucose uptake mechanisms (Schulingkamp *et al*. 2000). Glucose uptake in pancreatic beta cells is not mediated by insulin due to their glucose sensory nature and any insulin mediated glucose uptake mechanism would interrupt the sensory function. Glucose uptake is independent of insulin in pancreatic beta cells. Insulin has autocrine activity on beta cells as a modulator, and has different roles such as enhancing beta cell survival and inducing proliferation, through mechanisms that are unclear (Emilyn *et al*. 2010).

In contrast to adipose tissue and muscle cells, which express high levels of GLUT 4, an insulin regulated glucose transporter, the glucose transporters mainly expressed in beta cells are GLUT 1 and GLUT 2. In

mouse beta cells GLUT 2 is expressed at higher levels than GLUT 1 and is responsible for glucose-stimulated insulin secretion, while in human beta cells both GLUT 1 and GLUT 2 are responsible for glucose-stimulated insulin secretion (Ohtsubo *et al*. 2011).

Considering the physiological and biological properties of a variety of cell lines, beta cells make an appropriate model for investigating the effects of 4-hydroxyisoleucine and isoleucine on glucose uptake by minimising the interference of insulin. The search for a cell model that has the normal functionality of beta cells, with a well established record and the ability to be grown in normal laboratory conditions, guided us towards the BRIN-BD11 cell line. BRIN-BD11 is a hybrid cell line formed by the electrofusion of a primary culture of NEDH rat pancreatic islets with RINm5F (a cell line derived from a NEDH rat insulinoma) and unlike other tumoral cell lines, it spreads and grows evenly in culture as monolayers with an epithelioid morphology (McClenaghan *et al*. 1996).

BRIN-BD11 cells also have the unique advantages that they retain key attributes of normal pancreatic beta cells for glucose responsiveness, insulin synthesis and secretion (McClenaghan *et al*. 1996). They are also immortalised and provide stable growth and function up to 50 passages. Other attributes include a high capacity for glucose sensing, specificity, transport and metabolism, and appropriate responses to a range of non-glucose nutrients and receptor-mediated modulators of beta cell function, including amino acids, neurotransmitters and sulphonylurea drugs. Preliminary studies showed that amino acids may act on BRIN-BD11 cells by diverse mechanisms, including metabolism to ATP and uptake of cationic amino acids (McClenaghan *et*

al. 1996). BRIN-BD11 cells are also suitable for nutrient interaction studies of the regulation of insulin secretion by sulphonylureas and other compounds.

4.2 The effect of 4-hydroxyisoleucine and isoleucine on glucose consumption in BRIN-BD11 cells

In the first set of experiments using BRIN-BD11 cells, different concentrations of glucose were used to assess the effects of 4-hydroxyisoleucine and isoleucine on glucose uptake.

The glucose concentration of the culture medium is one of the criteria considered to model the diabetic status in order to evaluate the effect of isoleucine and 4-hydroxyisoleucine on glucose consumption. Exposure of BRIN-BD11 to 500 μM of isoleucine and 4-hydroxyisoleucine added to RPMI-1640 medium contains 5, 11 and 22 mM of glucose for 24 hours, showed a very significant increase in glucose consumption in cells incubated with 4-hydroxyisoleucine with 22 mM glucose RPMI1640 compared with control and other groups **(Fig 4.1)**. Glucose consumption increased constantly and significantly in all groups compared to 4-hydroxyisoleucine which showed activity in 11 and 22 mM glucose containing medium.

The results of the first experiments demonstrated the correlation between 4-hydroxyisoleucine activity and glucose concentration, in which 4-hydroxyisoleucine is only active at higher concentrations of glucose and the activity increases with increase in glucose, and are consistent with previous observations (Sauvaire *et al*. 1998). The effect of isoleucine, to stimulate a relatively constant increase in glucose uptake in all concentrations of glucose, indicates its activity is independent of glucose concentration.

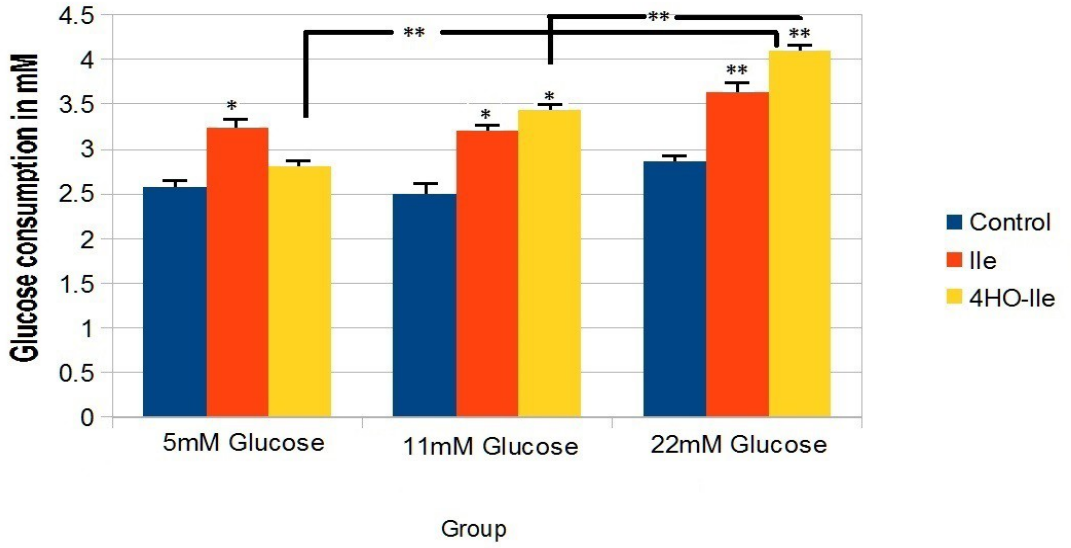


Figure 4.1. **The Effect of Ile and 4HO-Ile on glucose consumption by BRIN-BD11 cells.** Comparing glucose consumption in BRIN-BD 11 cells incubated with 500 µM isoleucine and 4-hydroxyisoleucine for 24 hours in variable concentrations of 5, 11 and 22 mM of glucose in RPMI-1640 medium. Glucose consumption is increased very significantly by both 4-hydroxyisoleucine and isoleucine in 22 mM group ($p<0.01$) and significantly in 11 mM group ($p<0.05$). The experiment was repeated three times with five sets of replicate in each experiment and results analysing by ANOVA Bonferroni test. Isoleucine shows a consistent increase in glucose consumption independent of glucose concentration compared to 4-hydroxyisoleucine which its activity increased correlatively with glucose concentration. Both 4-hydroxyisoleucine and isoleucine exhibit optimal activity in 22 mM concentration of glucose.

4.3 The effect of 4-hydroxyisoleucine and isoleucine on insulin secretion from BRIN-BD11 cells

The experiment was set up to assess the effect of 4-hydroxyisoleucine and isoleucine on insulin secretion in BRIN-BD11 cells. The amount of insulin within the medium has been measured as a reflection of insulin secretory activity of BRIN-BD 11 cells. There was no significant increase observed in insulin secretion between 4-hydroxyisoleucine and isoleucine compared to control in all groups **(Fig 4.2)**. The increase in insulin secretion was consistent with the increase in glucose concentration, as expected, which confirms the normal glucose responsiveness of the insulin secretion activity of BRIN-BD 11 cells and hence the reliability of using BRIN-BD 11 cells as a model for normal pancreatic beta cells.

T

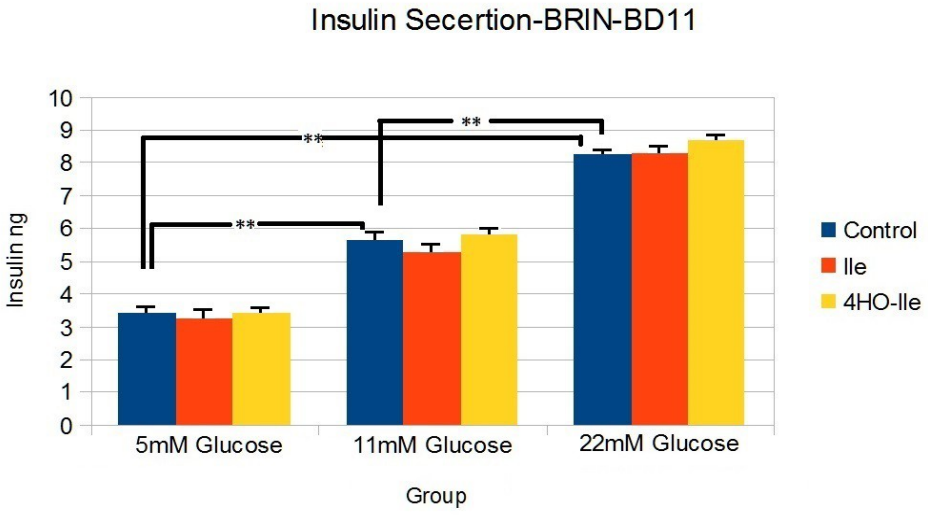


Figure 4.2. The Effect of isoleucine and 4-hydroxyisoleucien on insulin secretion by BRIN-BD11 cells. The measurement of insulin in cell culture medium showed a very significant increase in insulin secretion by increasing glucose concentration ($p<0.01$). There is no significant differences in insulin secretion in cells incubated with 500 µM 4-hydroxyisoleucine and isoleucine for 24 hours. The experiment was repeated three times with five sets of replicate in each experiment and data were analysed by ANOVA Bonferroni test.

Insulin measurements revealed that both 4-hydroxyisoleucine and isoleucine did not stimulate insulin secretion, whilst they both increased glucose uptake **(Fig 4.1)**. This finding confirms the results from animal studies in which 4-hydroxyisoleucine lowered blood glucose without increasing plasma insulin. The current results from BRIN-BD 11 cells and animal studies do not support the findings of Sauvaire in 1998 and Broca 1999 (Sauvaire *et al*. 1998, Broca *et al*. 1999) which suggested that 4-hydroxyisoleucine produces its anti-diabetic effect by stimulating insulin release from pancreatic beta cells. Current observations reconfirm an insulin independent mechanism of 4-hydroxyisoleucine in utilizing glucose consumption. A similar conclusion was arrived at when it was shown that 4-hydroxyisoleucine stimulated glucose uptake in skeletal muscle L6 cells in the absence of insulin (Jaiswal *et al*. 2012).

Significant increase in glucose consumption in beta cell exposed to high concentration of glucose (22 mM) and long incubation with 4-hydroxyisoleucine without changing insulin secretion explains an insulin-independent glucose utilization mechanism involves in 4-hydroxyisoleucine anti-diabetic effects. Isoleucine follows the pattern of 4-hydroxyisoleucine with a substantial difference in dependency to glucose concentration. The glucose concentration dependent activity of 4-hydroxyisoleucine makes it an interesting molecule with a unique mode of action, only active in hyperglycaemic condition and does not show hypoglycaemic effect in lower glucose concentrations. It is active when it needs to create an effect.

4.4 The effect of 4-hydroxyisoleucine and isoleucine on ATP content of BRIN-BD11 cells

Incubation of BRIN-BD 11 cells with 4-hydroxyisoleucine and isoleucine did not increase ATP levels in BRIN-BD 11 cells after 24 hours whilst the glucose uptake was increased. The increase of ATP level was in direct correlation with the glucose concentration of the culture medium as expected **(Fig 4.3)**. Doi's study shows that isoleucine did not change the ATP level in liver cells whilst it increases glucose uptake (Doi *et al*. 2007). Current study iterates that isoleucine produces the similar effect in beta cell as well as observed in liver cell. Exposure of the beta cell to higher glucose concentration stimulates insulin secretion by increasing the ATP production within the beta cell and the amount of insulin secreted from cell has a direct correlation with glucose concentration. ATP measurement results in BRIN-BD 11 also showed that insulin secretion increases in higher concentrations of glucose **(Fig 4.2)** which suggest that BRIN-BD11 resembles the normal beta cell attributes.

The glucose concentration of 22 mM was adapted as a standard glucose concentration for all the experiments during the current study. There were no significant differences observed in glucose consumption in BRIN-BD 11 in the presence of 22 mM glucose between the variable range of 4-hydroxyisoleucine and isoleucine concentration from 100 micro molar to 1 mM. It is in line with Sauvaire et al study in 1998 which suggested, 4-hydroxyisoleucine glucose lowering activity is present in concentration range from 100 micro molar to 1 mM. This finding also shows that 4-hydroxyisoleucine dose-dependent effect pattern in lower concentration (lower than 50 micro mole) as observed in

Jaiswal et al in 2012, is not seen in dose above 100 micro mole up to 1mM. The concentration of 500 micro molar of both 4-hydroxyisoleucine and isoleucine was used for all the future experiments in this study as an average effective dose. The pattern of glucose consumption and cellular ATP level were consistent with the findings as shown in figure 1 and 2 in all the run through the study **(Fig 4.4)**.

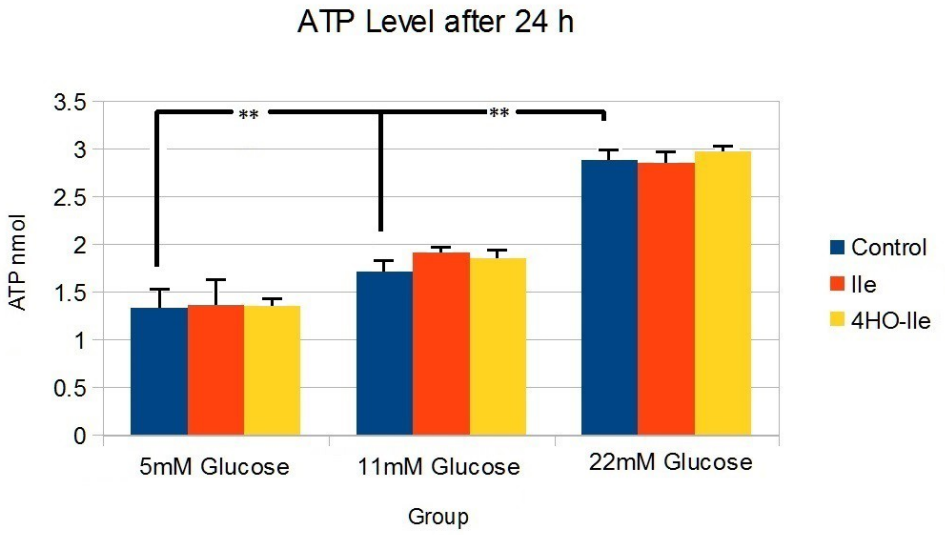
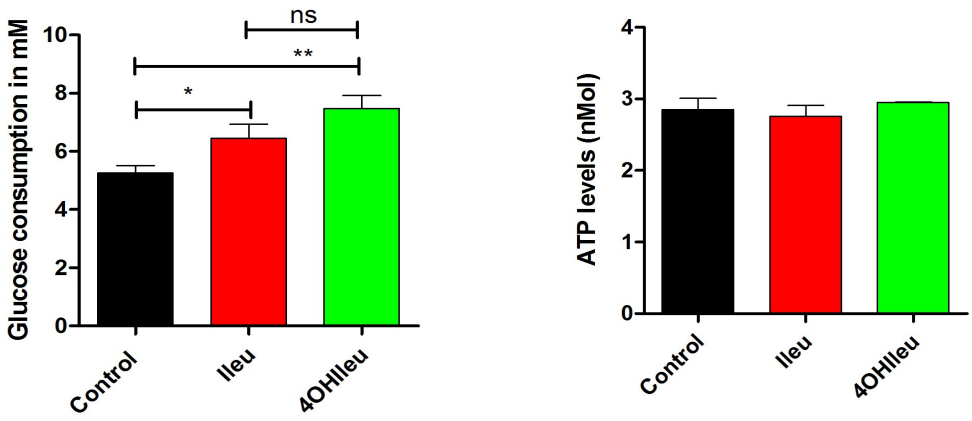


Figure 4.3. **The Effect of isoleucine and 4-hydroxyisoleucine on ATP content of BRIN-BD11 cells after 24 hours.** ATP level in BRIN-BD 11 cell measured after 24 hours with HTRF (homogeneous time-resolved fluorescence) technique. There is a very significant increase in the amount of ATP within the cells in medium with11 mM and 22 mM glucose concentration compared to each other and medium with 5mM glucose ($p<0.01$). There is no significant difference in ATP level of 4-hydroxyisoleucine and isoleucine groups. The experiment was repeated three times with five sets of replicate in each experiment and data were analysed by ANOVA test.



a) b)

Figure 4.4. **Glucose consumptions in the same batch of BRIN-BD 11 cells used for ATP content measurements.** Glucose consumption in BRIN-BD11 cells increases very significantly in treatment group with 500 µM 4hydroxyisoleucine ($p<0.01$) and significantly with 500 µM isoleucine ($p<0.05$) compared to control group after 24 hours in the presence of 22 mM glucose (a). There is no significant changes in ATP level between groups (b).

4.5 Cycloheximide (CHX) and protein synthesis role in 4-hydroxyisoleucine and isoleucine mechanism of actions

A recent published study by Jaiswal *et al* in 2012 showed that increase in glucose uptake and GLUT 4 translocation in L6-GLUT4myc skeletal muscle cells in the presence of 4-hydroxyisoleucine are blocked by adding 1 microg/ml of cycloheximide (CHX) which is a potent protein synthesis inhibitor. CHX is a selective inhibitor of eukaryotic protein synthesis which blocks tRNA binding and release from ribosome (Obrig *et al*. 1971). Incubation of BRIN-BD11 cells with 4hydroxyisoleucine and isoleucine in the presence of 0.8 μM (1 μg/ml) of CHX showed that CHX significantly inhibits the effect of 4-hydroxyisoleucine on increasing glucose uptake but not isoleucine **(Fig 4.5)**. ATP measurement also did not show any changes in the content of ATP within the cells in front of CHX after 24hours **(Fig 4.5)**.

Isoleucine significantly increases the glucose consumption in the presence of CHX, suggests a synergistic effect in isoleucine mechanism and also signifies that isoleucine glucose lowering effect does not dependent in synthesis of new proteins whilst 4-hydroxyisoleucine mechanism is noticeably dependent on protein synthesis in beta cells and skeletal muscles which previously observed by Jaiswal (Jaiswal *et al*. 2012). Observing the same result in different cells and different dose extends the conclusion that 4-hydroxyisoleucine hypoglycaemic effect and in some extent anti-diabetic properties directly or indirectly are mediated by a protein or proteins which need to be synthesised. It can be concluded that protein or proteins are involved in 4-hydroxyisoleucine mechanism do not have a long life, may be due to rapid degradation cycle or not abundantly available in normal condition within the cells

which need to be synthesised newly. This observation also suggests that there are different mediators are involved in isoleucine mechanism of action from 4hydroxyisoleucine.

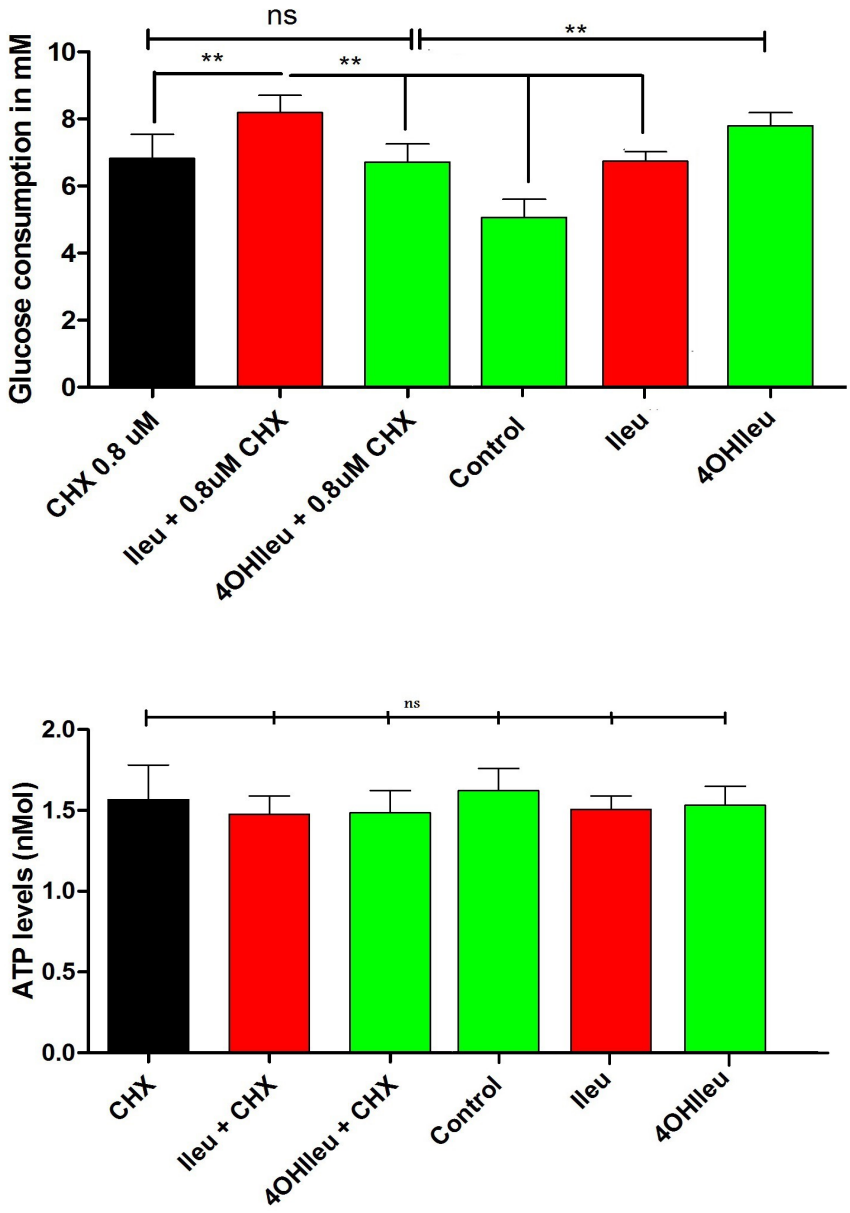


Figure 4.5. **The effect of cycloheximide (CHX) on glucose uptake and ATP content in BRIN-BD 11 cells.** BRIN-BD11cells incubated with 500 µM 4-hydroxyisoleucine and isoleucine in the presence of 0.8 µM cycloheximide (CHX). There is a significant changes in glucose consumption between 4-hydroxyisoleucine and 4-hydroxyisoleucine control group. There is significant increase in glucose consumption observed in isoleucine group ($p<0.01$). There is no changes in ATP level in all groups.

4.6 Wortmannin and PI3Kinase role in 4-hydroxyisoleucine and isoleucine activities in BRIN-BD11 cells

Broca *et al*. (2004) showed that 4-hydroxyisoleucine can increase PI3Kinase activity in both muscle and liver independently of insulin in the presence of high glucose concentration, and the study by Jaiswal *et al*. (2012) confirmed that adding wortmannin, a potent inhibitor of PI 3Kinase, inhibits the 4-hydroxyisoleucine-induced GLUT4 translocation and glucose uptake in muscle. These studies suggest a key role for PI 3Kinase in mediating the effects of 4-hydroxyisoleucine. Adding 5nM of wortmannin (IC50 = 2 - 4 nM) to the culture medium of BRIN-BD11 at the same time with a 4-hydroxyisoleucine and isoleucine and incubated for 24 hours did not inhibit 4-hydroxyisoleucine increasing effect on glucose consumption **(Fig 4.6)**. It was interestingly observed that wortmannin blocks isoleucine effect even glucose consumption significantly reduces in front of wortmannin compared to isoleucine control group **(Fig 4.6)**. It suggested that the PI3Kinase plays role in isoleucine effects on increasing glucose uptake but not in 4-hydroxyisoleucine in BRIN-BD 11 cells. It seems that inhibiting PI3Kinase activity in BRIN BD 11 cells does not inhibit 4-hydroxyisoleucine action contrary to previous findings in muscles reported by Jaiswal *et al*. It can be concluded that there is a strong relation between the mechanism of hypoglycaemic action of isoleucine and PI3 Kinase activity as reported by Doi *et al* in 2003.

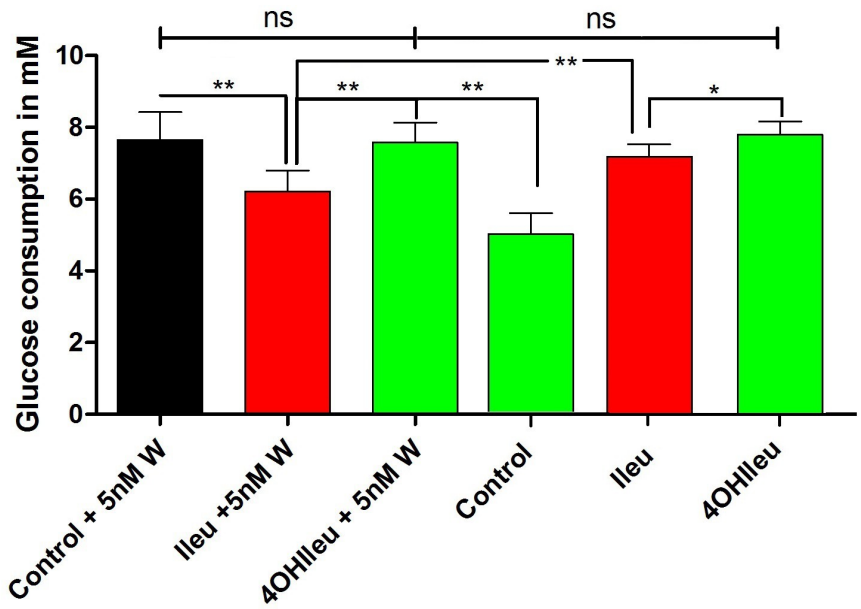


Figure 4.6. Glucose consumption in BRIN-BD11 cell was not affected in 4-hydroxyisoleucine group in front of 5 nM wortmannin after 24 hours. There is a significant reduction in glucose consumption in the isoleucine group in front of wortmannin ($p<0.01$).

4.7 Wortmannin and Cycloheximide (CHX) increase glucose consumption in BRIN-BD11 cells

It was noticeable in our several runs with wortmannin and CHX that glucose consumption in experiments with both compounds was significantly higher individually compared to the control without any compound **(Fig 4.6)**. To verify it we set up an independent trial to investigate the effect of 10 nM and 5 nM wortmannin and 0.8 µM CHX on BRIN-BD11 cell's glucose consumption. We used both cell count and protein assay together for normalisation to reduce the bias as much as possible. The results confirmed our previous findings, which both wortmannin and CHX significantly increased glucose consumption in BRIN-BD 11 cells incubated with RPMI-1640 medium contains 22mM glucose after 24 hours **(Fig 4.7)**.

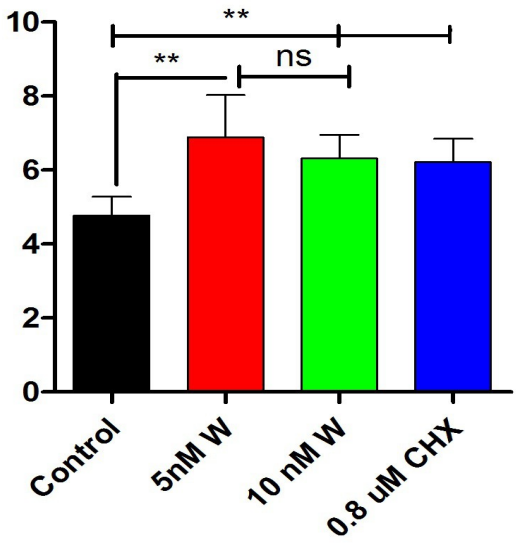


Figure 4.7. BRIN-BD11 cells were incubated with 5 nM and 10 nM wortmannin (W) and 0.8 µM CHX in RPMI-1640 with 22 mM glucose for 24 hours. Glucose measurements show a very significant increase in glucose consumption in both wortmannin and CHX groups ($p<0.01$). No significant difference was observed between 5 nM and 10 nM wortmannin group (ns).

The same finding has been observed in rat epididymal fat pad exposed to 0.8 mM (1 microg/ml) CHX and there is no clear explanation for it (García-Sáinz *et al*. 1977). Current study shows that CHX produces same effect on beta cell with unknown mechanism. It also clarifies that increase of glucose uptake baseline in all CHX groups respect to non-CHX groups are due to the CHX stimulatory effect on glucose uptake and adding 4-hydroxyisoleucine to the cells before CHX unlike isoleucine does not stimulate the glucose uptake further than CHX control group baseline.

Current study finding about the effect of wortmannin on increasing glucose uptake in beta cell is new as there is no such a report found in the literatures. It was shown in previous works that wortmannin as inhibitor of PI3Kinase, amplifies glucose-stimulated insulin secretion in beta cells (Zawalich *et al*. 2002) and it has also suggested that wortmannin induces insulin secretion in high glucose concentration (16.7 mM) by inhibiting phosphodiesterase to increase cAMP content which indicates that PI3-kinase inhibits insulin secretion by activating phosphodiesterase to reduce cAMP content (Nunoi *et al*. 2000). Considering wortmannin stimulatory effect on glucose uptake and glucose-dependent insulin secretion hypothesizes that inhibition of PI3Kinase may activates parallel compensatory mechanisms within the beta cell which results in a glucose influx into the cell and insulin secretion respectively. It creates unique research opportunity to address this finding.

4.8 The mTOR role in 4-hydroxyisoleucine and isoleucine mechanism of actions

PI3kinase plays an essential role in insulin-stimulated glucose uptake, GLUT 1 and GLUT 4 translocations in adipose and other peripheral tissues responsible for glucose disposal which is blocked completely by wortmannin (Okada *et al*. 1994, Clarke *et al*. 1994). mTOR (mammalian Target of Rapamycin) as downstream molecules in PI3Kinase-AKT (protein kinase B) pathway is a critical cellular regulator. The mTOR signalling pathway integrates a large number of environmental changes and cellular stresses, including reactive oxygen species, hyperosmotic and nutrient depletion stress as well as growth factor and cytokine signalling (Corradetti *et al*. 2006). Previous studies showed that mTOR enhances the intrinsic activity of GLUT1 transporters (Wieman *et al*. 2007), GLUT 1 expression and glucose uptake (Buller *et al*. 2008). It was observed that isoleucine stimulates insulin-independent glucose uptake mediated by activating PI3Kinase and independent of activation of mammalian target of rapamycin (mTOR) like leucine which is a potent activator of mTOR (Doi *et al*. 2003, Zhang *et al*. 2007). In previous experiments it was determined that PI3 Kinase activity is directly involved in both isoleucine and 4-hydroxyisoleucine ability in stimulating glucose uptake in the beta cell. Considering the role of mTOR in PI3 Kinase signalling pathway and glucose uptake, the effect of isoleucine and 4-hydroxyisoleucine were studied in the presence of 10 nM KU 0063794, a selective inhibitor of mammalian target of rapamycin (mTOR) (IC50 =10 nM for mTORC1 and mTORC2) without any activity at PI3-kinase or 76 other kinases within the cells (García-Martínez *et al*. 2009).

Results revealed that inhibition of mTOR solely with a specific inhibitor does not change the effect of isoleucine and 4-hydroxyisoleucine on increasing glucose consumption in beta cells **(Fig 4.8)** which reiterate the Doi's findings about isoleucine and extends it to 4-hydroxyisoleucine. Persistent results in each set of experiments by KU 0063794 determine that mTOR does not play essential role in the mechanisms of action of isoleucine and 4-hydroxyisoleucine.

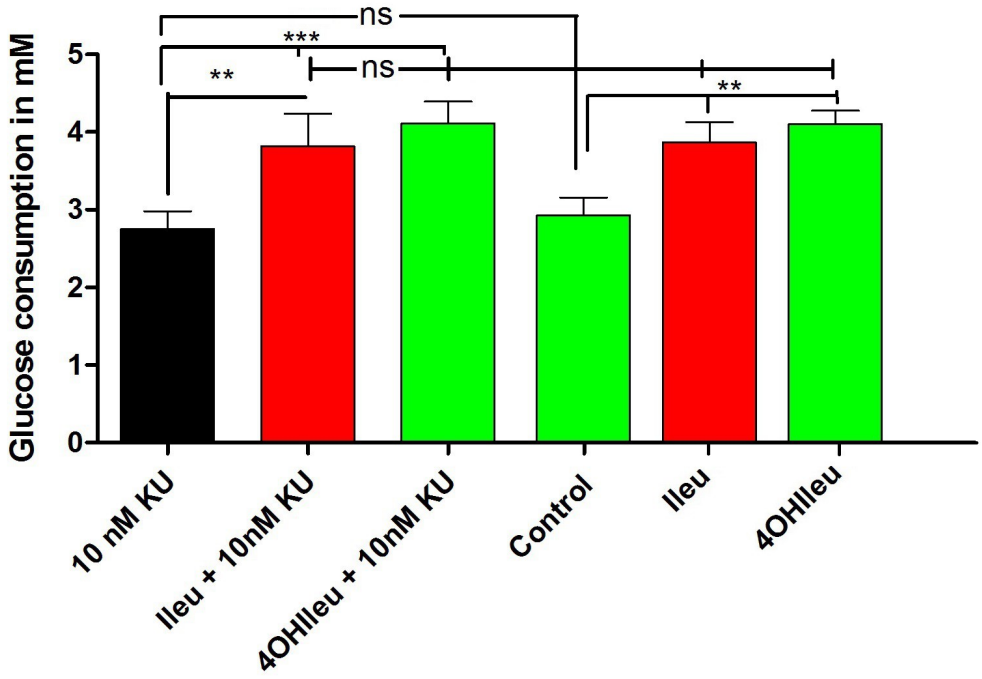


Figure 4.8. **The effect of mTOR inhibitor on glucose consumption rate of 4-hydroxyisoleucine and isoleucine in BRIN-BD11 cell.** mTOR inhibition does not change the glucose uptake in BRIN-BD11 cells indicates that mTOR does not play main role in uptaking glucose by pancreatic beta cells. There are also no changes in the activity of isoleucine and 4-hydroxyisoleucine in the presence of 10 nM KU 0063794, a selective mTOR inhibitor. Isoleucine and 4-hydroxyisoleucine significantly increased glucose consumption in BRIN-BD11 cells incubated with RPMI-1640 with 22 mM glucose ($p<0.01$).

PI3 Kinase-AKT-mTOR pathway plays important role in glucose uptake regulation as one of the major downstream intracellular signalling pathways in response to binding of many substances like growth factor and insulin to their receptors on the surface of the cell. Activation of PI3 Kinase-AKT-mTOR pathway increases glucose uptake via interacting with GLUT 1 and in less extent GLUT 3 expression and activity through mTOR **(Fig 4.9)** (Bryan *et al*. 2005). GLUT1 plays critical role in maintaining basal glucose uptake required to sustain cellular respiration in all cells. Expression levels of GLUT1 in cell membranes are increased by reducing glucose levels and decreased by increased glucose levels. GLUT1 is able to compensate for GLUT2 deficiency or under expression in beta cells and maintains an appropriate rate of glucose uptake to sustain glucose metabolism in pancreatic beta-cells (Liang *et al*. 1997).

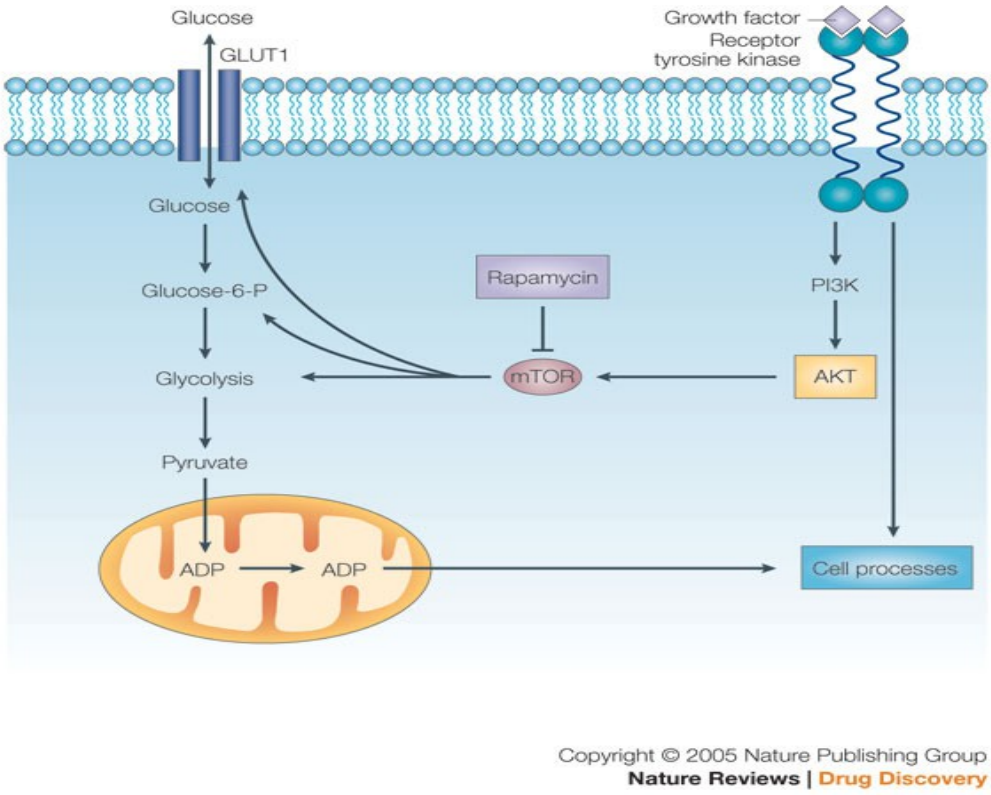


Figure 4.9. AKT and mTOR are activated by membrane receptors and stimulate glycolysis in part by AKT-induced localization of the glucose transporters, including GLUT1 and GLUT3, to the cell surface, and maintenance of hexokinase function in the absence of extrinsic factors with resultant glucose production. PI3K, phosphatidylinositol-3-kinase. (Adapted from Bryan *et al*. 2005)

It drew the attention to investigate the role of the GLUT 1 in the effect of isoleucine and 4-hydroxyisoleucine on glucose uptake considering that the GLUT1 is the most abundant glucose transporters in the beta cell which facilitates a main glucose influx into the cell.

4.9 Role of GLUT1 in 4-hydroxyisoleucine and isoleucine mechanism of actions in BRIN-BD11 cells

BRIN-BD11 cells were incubated 24 hours with isoelucine and 4-hydroxyisoleucine in RPMI 1640 medium containing 22 mM glucose in front of 5 μM STF 31, a cell-permeable sulfonamide that selectively inhibits GLUT1 and glucose uptake (Chan *et al*. 2011) to achieve a specific GLUT1 inhibiting status without compromising other transporters in order to evaluate the role of GLUT1 in the effect of isoleucine and 4-hydroxyisoleucine.

STF 31 was shown to inhibit the growth of VHL-deficient renal cell carcinomas (RCCs) dose-dependently (5 μM) by directly targeting GLUT1 without binding to other glucose transporters, and it does not inhibit a broad range of 50 tested kinases (Chan *et al*. 2011). 4-hydroxyisoleucine glucose consumption increasing effect in BRIN-BD11 cells reduced in front of STF 31 but GLUT1 inhibition did not show any significant changes in the activity of isoleucine on glucose consumption in BRIN-BD11 cells **(Fig 4.10a)**. ATP measurements confirmed a remarkable reduction in ATP content of the cell in STF 31 groups after 24 hours **(Fig 4.10b)**. The findings determine that 4-hydroxyisoleucine mechanism of action is profoundly dependent on GLUT1 activity and it is in line with its mode of actions, insulin-independent increase of basal glucose uptake in peripheral tissues as GLUT1 is responsible for basal glucose uptake in cells.

The beta pancreatic cell membrane has a very high capacity of transporting glucose into the cytoplasm compared to other cells. High capacity of glucose transport in beta pancreatic cell is due to GLUT2 which has a

very high Vmax and high Km of 15-20 mM (Guillam *et al*. 1997). GLUT1 with low Km is also expressed in pancreatic beta cells. A previous study showed thatGLUT1 expression is higher when glucose is low, and GLUT2 expression is more profound when glucose is high in the culture media (Tal *et al*. 1992). Incubation of BRIN-BD11 cell with 22 mM glucose and selective GLUT1 inhibitor, STF 31 for 24 hours increases glucose uptake significantly, which implicates that GLUT1 inhibition may lead to over-expression of GLUT2 or shifting towards other uptake mechanisms as a compensatory phenomena. Rabuazzo *et al* in 1993 showed that inhibition of only GLUT1 when GLUT2 is functioning in hamster-derived pancreatic beta cell line (HIT) enhances insulin release. In our study, STF 31 increased glucose consumption, which can be explained by referring to Rabuazz's findings as an insulin release enhancement depends on higher rate of glucose transport which means more glucose consumption. If GLUT1 inhibition enhances insulin secretion in beta cell as shown in Rabuazzo study and insulin secretion process consumes energy (ATP) and logically the ATP content should drop after an increase prior to release. The end point measurement of the cell ATP content after 24 hours incubated with STF 31 showed significant drop.

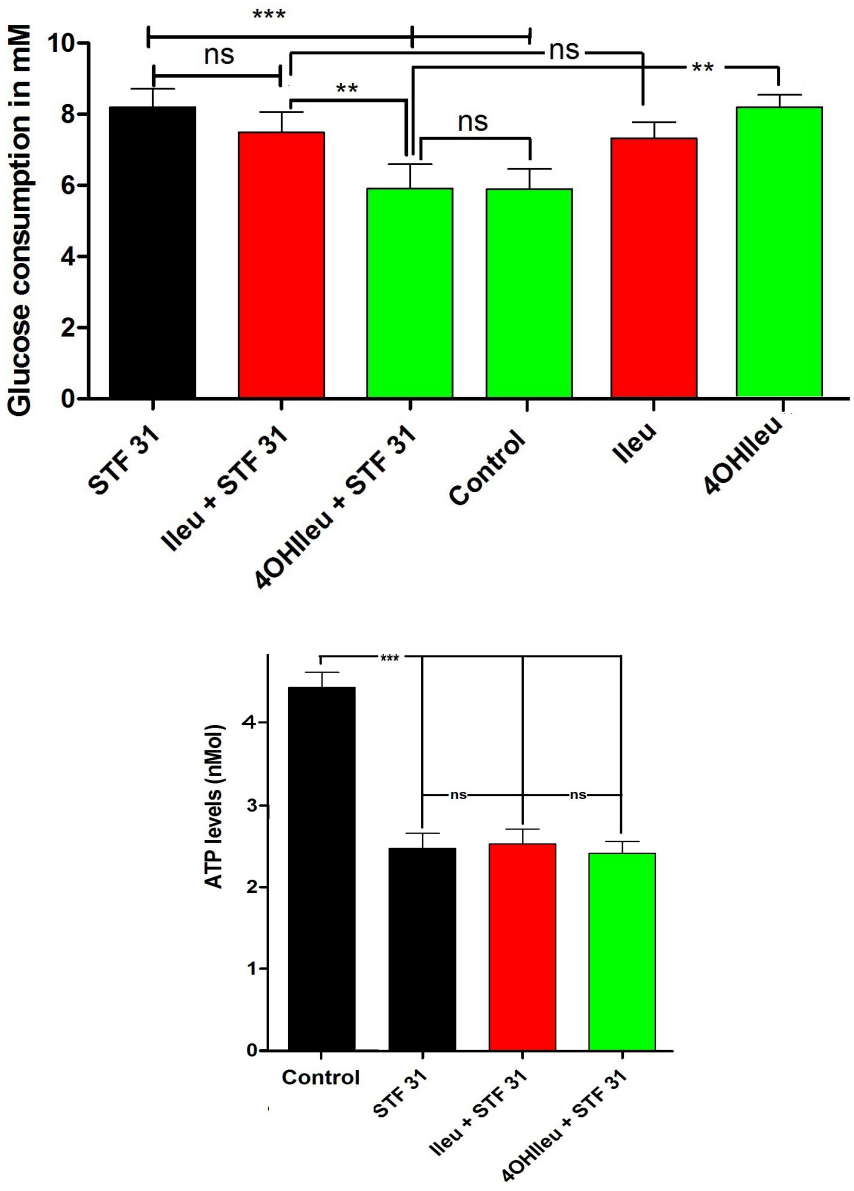


Figure 4.10. **Glucose consumption and ATP content in BRIN-BD11 cell in the presence of STF 31.** Glucose consumption was increased significantly ($p<0.01$) in BRIN-BD11 cell in the presence of 5 μM of STF 31 after 24 hours incubation. The glucose consumption increase was inhibited significantly in 4-hydroxyisoleucine group in front of STF 31($p<0.01$) but not in isoleucine group (a). The ATP content of the cells decreased dramatically by STF 31 ($p<0.001$) and remained unchanged in all STF 31 groups including isoleucine and 4-hydroxyisoleucine (b).

GLUT1 inhibition dramatically interferes with 4-hydroxyisoleucine activity as in all our previous findings the 4-hydroxyisoleucine had the higher increasing effect on glucose consumption in BRIN-BD11 cell compared to isoleucine but its effect reduced significantly in front of STF 31. It can be hypothesized by reviewing current findings that GLUT1 plays a major role in 4-hydroxyisoleucine mechanism of action but not in isoleucine.

4.10 The role of calcium on the effects of 4-hydroxyisoleucine and isoleucine on glucose consumption rate and ATP content in BRIN-BD11 cells

The glucose transporter 1 (GLUT1) is expressed in a wide variety of cell types and is largely responsible for maintaining the basal level of glucose uptake. A previous study suggested that cytosolic calcium plays a role in modulating basal glucose uptake and GLUT1 activity. It also showed that lowering extracellular calcium decreases basal glucose uptake in rat epithelial cells (Quintanilla *et al*. 2000). It was observed that intracellular calcium stimulates glucose uptake in skeletal muscle cell and inhibition of calcium release from the sarcoplasmic reticulum (SR) reduces sugar uptake, whereas the increase of calcium release from SR stimulates glucose transporter activity (Youn *et al*. 1991). A recent study using shRNA-mediated silencing showed that the recently-identified mitochondrial calcium uniporter (MCU) is essential for a sustained glucose-induced increase in the cytosolic ATP/ADP ratio in pancreatic beta cells (Tarasov *et al*. 2012). The mitochondrial calcium uniporter (MCU) is located in the mitochondrial inner membrane that regulates calcium uptake into mitochondria and plays a key role in mitochondrial calcium homeostasis and cellular physiology. It regulates cytoplasmic

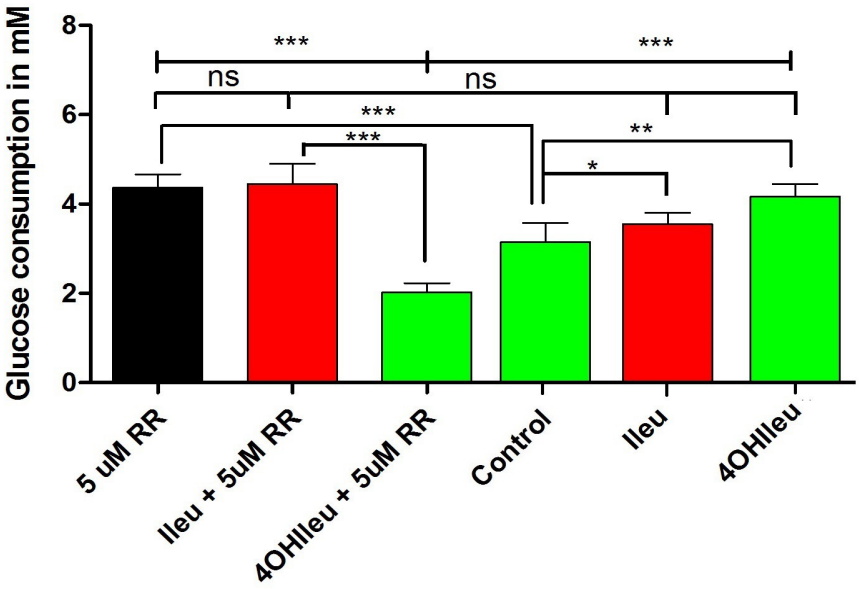
calcium signals, cell bioenergetics and also glucose-dependent insulin secretion in pancreatic beta-cells by regulating mitochondrial calcium uptake (Alam *et al*. 2012, Mallilankaraman *et al*. 2012).

Considering the related available data for the role of calcium and MCU as a mitochondrial calcium homeostasis regulator drew the attention to evaluate the role of mitochondrial calcium uniport channels in isoleucine and 4-hydroxyisoleucine effects in BRIN-BD 11 cell glucose uptake. To study the calcium role, Ruthenium Red has been used. Ruthenium Red is an inhibitor of mitochondrial calcium uniporter (MCU) which blocks calcium uptake and release from mitochondria (Bernardi *et al*. 1984). It also inhibits calcium release from ryanodine-sensitive intracellular stores and blocks cell membrane-located capsaicin-activated cation channels (Bernardi *et al*. 1984, Szallasi *et al*. 1999, Israelson *et al*. 2008). It was shown that ruthenium red inhibits neurotransmitter release by blocking voltage-sensitive Ca^{2+} channels (Xu L *et al*. 1999). Ruthenium red efficiently blocks the L-type calcium channel in a dose-dependent manner which reaches an inhibition of 85% at 5 μM (Malécot *et al*. 1998).

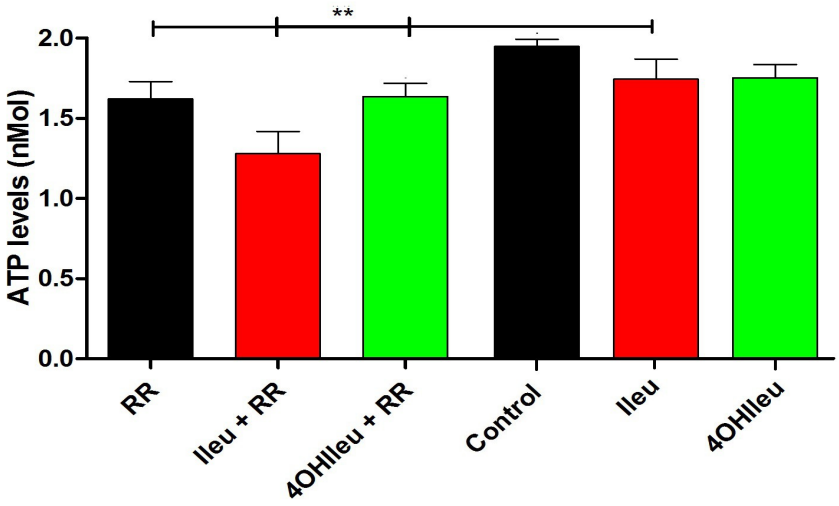
To investigate the role of calcium in the modulation of glucose uptake in BRIN-BD11 cells by isoleucine and 4-hydroxyisoleucine, cells were treated with 5 μM of ruthenium red simultaneously with 500 μM isoleucine and 4-hydroxyisoleucine leaded to very significant decrease in glucose consumption in 4-hydroxyisoleucine group and inhibition of the glucose consumption increase in isoleucine groups **(Fig 4.11a)**.

Several experiment repeats with five replicates in each set, showed a persistent result which implicates the roles of calcium in both isoleucine and 4-hydroxyisoleucine mechanisms of action. Inhibition of mitochondrial calcium uniporter (MCU), calcium channels and calcium

signalling by ruthenium red not only inhibits 4-hydroxyisoleucine activity but creates a reverse activity in 4-hydroxyisoleucine. The glucose consumption decreased significantly in 4-hydroxyisoleucine group treated with ruthenium red compared to controls **(Fig 4.11a)**. Contrary to 4-hydroxyisoleucine group, glucose uptake remarkably increased in isoleucine group treated by ruthenium red compared to controls**(Fig 4.11a)**. It seems that intracellular calcium signalling does not play an equal role in 4-hydroxyisoleucine and isoleucine mechanism of actions. It may be explained by referring to the previous findings about the relation of calcium and glucose uptake that inhibition of cytoplasmic calcium channels by ruthenim red alongside with MCUs result in decrease of cytoplasmic calcium due to reducing the diffusion of extracellular calcium (Bernardi *et al*. 1984, Szallasi *et al*. 1999, Israelson *et al*. 2008). Reducing cytoplasmic calcium triggers the calcium release from sarcoplasmic reticulum (SR) and stimulates glucose transporter activity (Youn *et al*. 1991). We also observed that ruthenium red is solely increasing glucose consumption in BRIN-BD11 cells **(Fig 4.11a)**. The reduction in cellular ATP content after 24 hours in all ruthenium red groups and more significantly in isoleucine plus ruthenium red **(Fig 4.11b)** shows that inhibition of calcium channels affects bioenergetic status of the cell and indicates important role of calcium in both in isoleucine and 4-hydroxyisoleucine effects. BRIN-BD11 cell's glucose uptake paradoxical behaviour in isoleucine and 4-hydroxyisoleucine groups in the presence of ruthenium red determine a distinct difference in 4-hydroxyisoleucine and isoleucine mechanisms of action which is in line with our previous findings. The ruthenium red neutralizes the glucose uptake stimulatory effect of 4-hydroxyisoleucine but on the other hand it has synergy with isoleucine glucose uptake activity.



a)



b)

Figure 4.11. **The effect of ruthenium red on Isoelucine and 4-hydroxyisoleucin activities in BRIN BD11 cells.** The glucose consumption in the presence of 5 μM ruthenium red in BRIN-BD11 cell in RPMI-1640 with 22 mM glucose after 24 hours (a). Glucose consumption decreased very significantly in 4-hydroxyisoleucine group treated simultaneously with ruthenium red ($p<0.01$) and there is no sign of inhibition in isoleucine group. Isoleucine showed higher glucose consumption in front of

ruthenium red compared to isoleucine control without ruthenium red (a) which shows paradoxical behaviour of 4-hydroxyisoleucine in combination with ruthenium red on glucose uptake. Ruthenium red is solely increasesing glucose consumption significantly ($p<0.01$) (a). The ATP concentration of cells at the end of the experiment shows a significant reduction in ATP content in all ruthenium red treated groups ($p<0.01$) and with greater decreases in isoleucine group (b) Non treatment groups did not show any significant difference in ATP content (c).

The intracellular calcium level is regulated by the sarcoplasmic reticulum (SR) as a reservoir of calcium, mitochondria and cytoplasmic membrane calcium channels. Intracellular calcium uptake into the SR regulates by the sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA 2a) calcium pump and remaining of cytoplasmic calcium is removed via the Na^{+}/Ca^{2+} exchanger (NCX) or taken up into mitochondria by the mitochondrial calcium uniporter (MCU) (Bodi *et al*. 2005, Cingolani *et al*. 2007). Mitochondrial oxidative phosphorylation as a source of ATP production is dependent on mitochondrial calcium uptake via a ruthenium red-sensitive mitochondrial calcium uniporter (MCU). The MCU calcium transport rate is slow and its affinity for calcium is low (Km 10-20 mM). The Na^{+}/Ca^{2+} exchanger is responsible for extruding calcium from mitochondria (Denton *et al*. 1985,Gunter *et al*. 1990). Calcium release from SR is controlled by ryanodine receptors (RyR) and inositol triphosphate receptors (IP_3R). Depletion of the SR calcium reservoir is sensed by stromal interaction molecules (STIMs) located on the SR membrane and monitor its calcium content. Sensing of the low calcium content of SR by STIMs activates store-operated calcium channels (SOCE) on the membrane leading to calcium influx stimulation **(Fig 4.12)** (Mascia *et al*. 2012). G protein coupled receptor activation will increase the release of calcium from SR through the

production of IP3, (Inositol 3 Phosphate) which binds to IP3 receptor on the surface of SR (Mascia *et al*. 2012).

Considering cellular calcium homeostasis shows that ruthenium red reduces calcium release from SR in pancreatic beta cells by inhibiting ryanodine receptors (RyR), calcium influx by blocking calcium channels on cytoplasmic membrane and mitochondrial calcium uptake by inhibiting MCUs which lead to intracellular calcium depletion. The cellular protective mechanism will try to compensate intracellular calcium depletion via increasing calcium release from the SR by activating IP3 receptors (parallel gate which is not blocked by ruthenium red) through triggering production of IP3 by intrinsic enzymatic activity, reducing calcium uptake by sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2) and stimulation of different types of membrane calcium channel to increase calcium influx. Activation of parallel unblocked calcium gates on SR leads to depletion of SR calcium and activation of stromal interaction molecules (STIMs) as SR calcium sensor. Stromal interaction molecules (STIMs) stimulate calcium influx as well. Other important pumps could help the cell to compensate the lack of cytosolic calcium in such a condition is a sodium-calcium exchanger (NCX).

The sodium-calcium exchanger (NCX) is a high capacity antiporter membrane protein uses the electrochemical gradient energy, allowing sodium to flow down its gradient across the plasma membrane in exchange for the counter-transport of calcium ions. The NCX is important in cellular calcium homeostasis expresses in many different cell types and is capable of exchanging a single calcium ion with three sodium ions in both ways depend on a gradient (Yu SP *et al*. 1997, Dipolo *et al*. 2006).

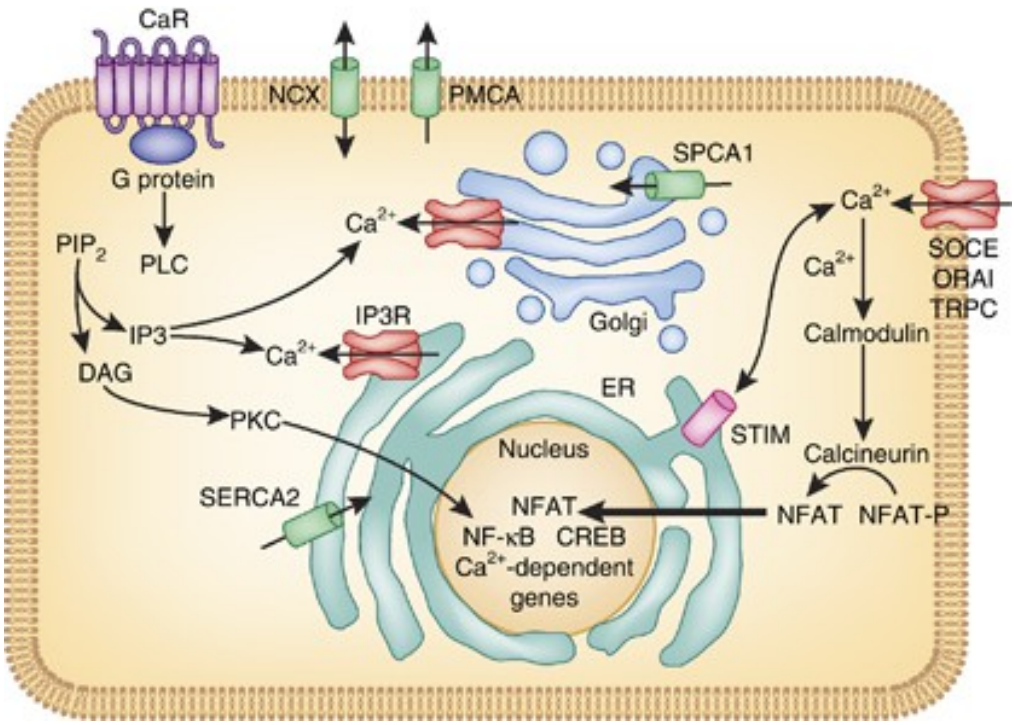


Figure 4.12. Integration of the calcium signaling circuitry. The major regulators of calcium homeostasis in keratinocytes are depicted. Plasma membrane pumps and channels (PMCA, NCX, and SOCE) regulate flux in and out of the cytosol. G-protein–coupled receptors (calcium-sensing receptor (CaR) and others not shown) initiate signals that modify compartmentalized calcium stores (e.g., IP3). Calcium ATPases on organelles (SERCA, SPCA1) monitor and replenish intracellular storage sites. CCa^{2+}, calcium; DAG, diacylglycerol; ER, endoplasmic reticulum; IP3R, inositol 1,4,5-trisphosphate receptor; NCX, Na^{+}/Ca^{2+} exchanger; NFAT, nuclear factor of activated T cells; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, sarco (endo)plasmic recticulum Ca^{2+} ATPase; SOCE, store-operated calcium channels; STIM, stromal interaction molecule; TRPC, transient receptor potential C. The figure is modified from Savignac *et al*. (2011) with permission from Elsevier. (Completely adapted from Mascia *et al* . 2012)

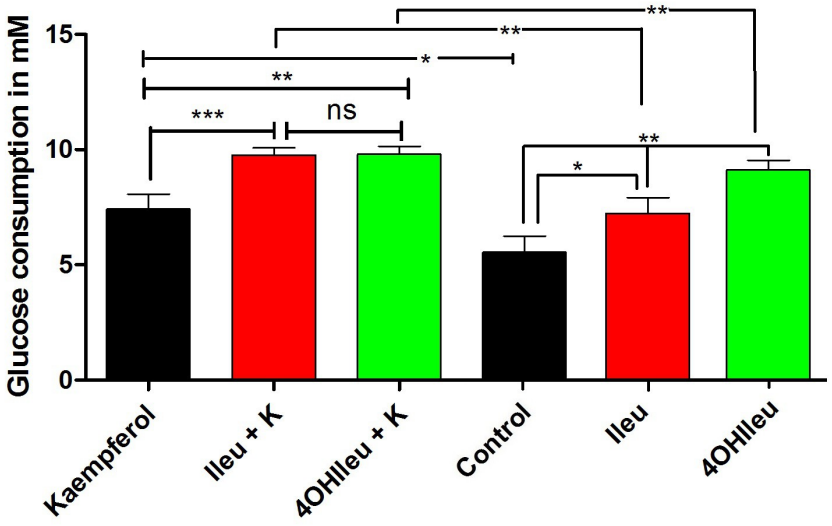
All these pathways for balancing disturbed calcium homeostasis need energy and ATP production may slow down due to MCU blockage by

ruthenium red **(Fig 4.11b)**. To compensate for slow energy production, cells attempt to take up more fuel by increasing glucose uptake to boost up energy production in mitochondria. On the other hand increasing glucose uptake **(Fig 4.11a)** itself leads to beta cell membrane depolarization and subsequent calcium influx (Srinivasan *et al*. 2002). Ruthenium red indirectly increases glucose uptake via disturbing intracellular calcium homeostasis. Previous animal trials showed that verapamil, a calcium channel blocker which blocks L-type calcium channels mainly and prevents cellular calcium influx, reduces blood glucose level and increased insulin levels in STZ-induced diabetic mice by increasing glucose uptake in beta cells and enhancing pancreatic beta cell function (Xu G *et al*. 2012).

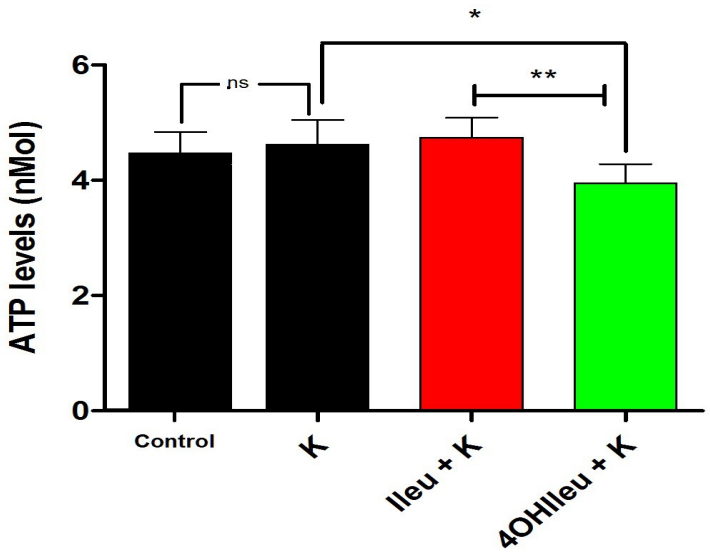
Mitochondrial calcium uniporters (MCUs) and calcium roles in 4-hydroxyisoleucine and isoleucine effects attracted the attention by observing the effect of ruthenium red as an MCU inhibitor on isoleucine and 4-hydroxyisoleucine activity. Does MCUs activator alter isoleucine and 4-hydroxyisoleucine effects on glucose consumption, If it is assumed based on current findings that MCUs inhibition can eliminate the effect of the 4-hydroxyisoleucine. To answer the question, BRIN-BD 11 cells were incubated with 500 µM isoleucine and 4-hydroxyisoleucine for 24 hours in the presence of 20 µM of kaempferol, a naturally occurring flavonoid found in Gingko biloba and red wines that activates the mitochondrial calcium uniporters (MCUs) (EC50 = 7 µM) (Montero *et al*. 2004, Vay *et al*. 2007).

Kaempferol is able to increase glucose uptake in 3T3-L1 adipocytes and act as an anti-diabetic agent via multiple targets such as acting as a partial agonist of PPARgamma (Fang *et al*. 2008). It was also observed

that Kaempferol metabolite, kaempferol 3-neohesperidoside, is capable to increase glucose uptake in myocytes mediated by PI3Kinase and protein kinase C (PKC) pathways (Zanatta *et al*. 2008). Glucose consumption in BRIN BD11 cells increased significantly after 24 hours incubation with 20 μM of kaempferol without noticeable changes in cellular ATP contents at the end of the experiment **(Fig 4.13a,b)**. This finding is in line with previous studies mentioned above and confirms its glucose uptake stimulatory property. Kaemferol produced the similar amount of increase in glucose consumption as isoleucine alone, but significantly lower than the 4-hydroxyisoleucine which shows that 4-hydroxyisoleucine stimulates the glucose uptake in much greater extent to the kaempferol and isoleucine.



a)



b)

Figure 4.13. **The effect of kaempferol on glucose consumption in BRIN-BD11cells.** Glucose consumption was significantly increased by 20 µM of kaempferol ($p<0.01$) and dominantly in both isoleucine and 4-hydroxyisoleucine groups incubated simultaneously with kaempferol ($p<0.001$) (a). A synergistic effect of kaempferol with 4-hydroxyisoleucine and in greater extent with isoleucine has been observed (a). ATP content of cells does not change in the presence of kaempferol and kaempferol plus isoleucine but a noticeable decrease was observed in 4- hydroxyisoleucine plus Kaempferol group ($p<0.05$) (b).

Incubation of BRIN BD11 cells with 4-hydroxyisoleucine and isoleucine alongside with 20 μM kaempferol in the same time for 24 hours showed a remarkable increase in glucose consumption in both 4-hydroxyisoleucine and isoleucine groups with kaempferol compared to controls **(Fig 4.13a)**. Interestingly, the glucose consumption in isoleucine with Kaempferol group increased very significantly by more than 3 mM compared to isoleucine alone ($p<0.001$). In all our previous findings, 4-hydroxyisoleucine solely created greater effect than isoleucine but the combination of isoleucine and kaempferol could create the equal effect as 4-hydroxyisoleucine with or without kaempferol. These findings indicate a strong synergistic effect between kaempferol and both 4hydroxyisoleucine and isoleucine on stimulating glucose uptake in BRIN BD 11 cells and also demonstrate the role of calcium and MCU in mechanisms of action of 4-hydroxyisoleucine and isoleucine. The results also show the important role of calcium and mitochondrial calcium uniporters in glucose uptake mechanism. ATP measurements did not show changes in ATP content between kaempferol and isoleucine plus kaempferol group but a significant decrease were observed in ATP content of BRIN-BD 11 cells in 4-hydroxyisoleucine plus kaempferol group **(Fig 4.13b)**.

A previous gene expression study using microarray and real-time PCR identified a set of metabolically relevant genes were induced by 20 μM kaempferol after 24 hours including peroxisome proliferator–activated receptor coactivator-1, carnitine palmitoyl transferase-1, mitochondrial transcription factor 1, citrate synthase, and uncoupling protein-3 (da-Silva *et al*. 2007). It is also shown that kaempferol increases the oxygen consumption and cellular energy expenditure via increasing cyclic AMP in skeletal muscle (da-Silva *et al*. 2007). Synergistic effect between isoleucine, 4-hydroxyisoleucine and kaempferol suggests that the

previously identified metabolically relevant genes induced by kaempferol in da-Silva *et al* study could have direct or indirect roles in the mechanisms of action of 4-hydroxyisoleucine and isoleucine which create a new interesting area of further investigation.

The results from ruthenium red and kaempferol experiments support that intracellular calcium signalling and mitochondrial calcium uniporters (MCUs) play an important role in 4-hydroxyisoleucine and isoleucine glucose uptake stimulatory effect but their roles are not similar in isoleucine and 4-hydroxyisoleucine mechanisms of action.

Analysing data from the effects of ruthenium red **(Fig 4.11)** as an MCU and calcium signalling inhibitor and kaempferol as an MCU activator **(Fig 4.13)** on isoleucine and 4-hydroxyisoleucine activity in BRIN-BD 11 cells indicate:

- Interference with calcium signalling and mitochondrial calcium uniporter (MCU) using inhibitor, inhibits 4-hydroxyisoleucine effect on increasing glucose consumption, but not the effect of isoleucine whereas using MCU activator enhances both 4-hydroxyisoleucine and in greater degree isoleucine effects which means the important roles of calcium signalling and MCU in 4-hydroxyisoleucine and isoleucine mechanisms of action.

- Isoleucine activity exhibits a significant increase in the presence of both MCU activator and inhibitors , which is different from 4-hydroxyisoleucine behaviour. Unlike isoleucine, inhibition of 4-hydroxyisoleucine effect on stimulating glucose uptake in the presence of ruthenium red indicates that calcium signalling is an

essential for its mechanism of action. It also reiterates that calcium's role in the mechanism of action of 4-hydroxyisoleucine is different from isoleucine.

- These findings support that different mechanisms and pathways are involved in actions of isoleucine and 4-hydroxyisoleucine apart from some similarities in their effects and molecular structures.

4.11 The effect of the UK-5099 as a mitochondrial pyruvate carrier (MPC) inhibitor on 4-hydroxyisoleucine and isoleucine glucose utilisation in BRIN-BD11 cells

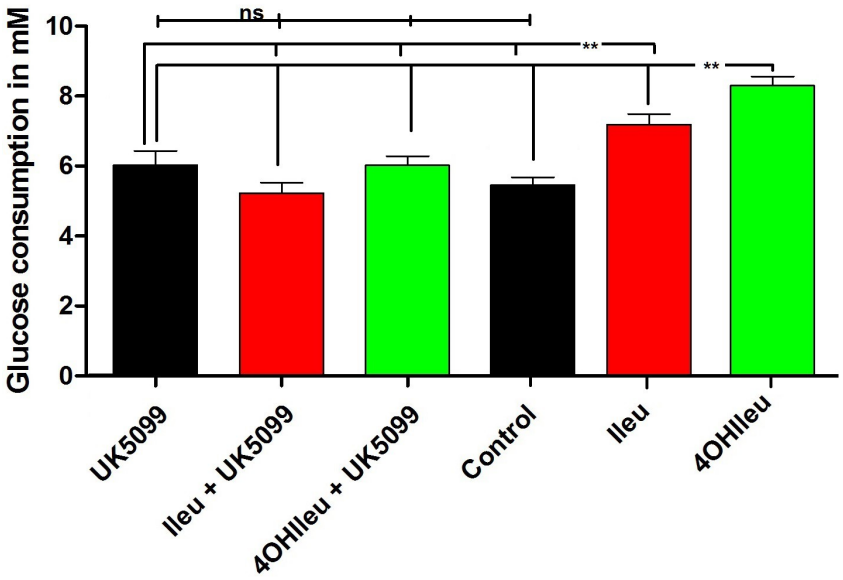
The results of previous experiments with ruthenium red and kaempferol demonstrated the role of calcium and in a broader spectrum, the mitochondria in 4-hydroxyisoleucine and isoleucine effects on glucose uptake in BRIN-BD 11 cells. We also observed in all the previous experiments that the ATP content of the cells using end point cell ATP content measurement at the end of each experiment did not change considering the increase in glucose consumption induced by 4-hydroxyisoleucine and isoleucine. The BRIN-BD 11 cell is a beta pancreatic cell which consumes glucose to make energy in the form of ATP for its biological activities unlike adipose cell which can store excess glucose by transforming it to triglyceride. As the glucose uptake is an energy consuming activity and considering the previous observations in the current study showing the glucose consumption increases in front of 4-hydroxyisoleucine and isoleucine while the ATP content unchanged , suggest that energy production process in

mitochondria is essential for 4-hydroxyisoleucine and isoleucine effects. To evaluate the role of mitochondria and energy production cycle in the effect of isoleucine and 4-hydroxyisoleucine, we designed an experiment to assess 4-hydroxyisoleucine and isoleucine effects on glucose consumption in BRIN-BD 11 cells in front of a mitochondrial pyruvate carrier (MPC) blocker. Pyruvate is the end product of glycolysis and a major substrate for the tricarboxylic acid (TCA) cycle in mitochondria for producing ATP. Pyruvate is produced in cytosol and across the inner mitochondrial membrane via MPCs which are located in inner mitochondrial membrane (Herzig *et al*. 2012). Mitochondrial pyruvate carrier (MPC) is essential for producing ATP.

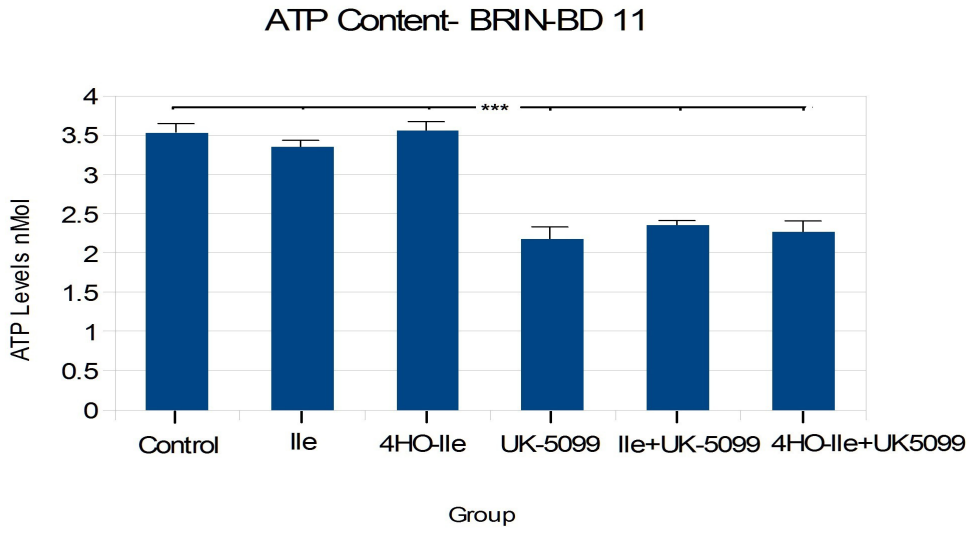
The BRIN-BD 11 cells were incubated with 500 µM 4-hydroxyisoleucine and isoleucine simultaneously with 35 µM 0f UK-5099, an a-cyanocinnamate analogues which acts as specific and potent inhibitors of mitochondrial pyruvate carrier (MPC) activity (Halestrap. 1978). A recent study by Divakaruni et al showed that UK 5099 increases glucose uptake in myocytes which directly proportional to the extent of respiratory inhibition by UK 5099 but our study results indicated that UK 5099 did not change the glucose consumption in BRIN-BD 11 while the ATP content dramatically decreased after 24 hours incubation **(Fig 4.14)**. This finding is not in line with Divakaruni et al study. The difference between the biology of beta pancreatic cell and myocytes which used in Divakaruni study could explain different behaviours. On the other hand we expected to see a significant reduction in glucose consumption in the UK 5099 control group as intervening with ATP production cycle should reduce glucose uptake due to cellular energy depletion but the glucose consumption in the UK 5099 groups remained unchanged compared to no treatment control. However we did not detect an increase in glucose uptake in the UK5099 group same as

Divakaruni observed in his study in myocytes but unchanged glucose consumption in BRIN-BD 11 in front of the UK 5099 shows that mitochondrial respiratory inhibition in beta cell may activate a feedback to push the cell to take up more glucose for compensating the disrupted energy production cycle.

Interestingly isoleucine and 4-hydroxyisoleucine effects were diminished significantly in front of the UK 5099 **(Fig 4.14)** compared to the controls. The results indicate that mitochondria and ATP production cycle within the mitochondria are essential for both 4-hydroxyisoleucine and isoleucine mechanisms of action. It raises a question for further study about the effect of isoleucine and 4-hydroxyisleucine on mitochondrial activity and glycolysis pathway in the cell. Theoretically based on these findings, it is expected that the mitochondrial activity may increase in front of 4-hydroxyisoleucine and isoleucine to drive the cells towards more glucose uptake then it could justify why mitochondrial pyruvate carrier inhibition blocks 4-hydroxyisoleucine and isoleucine effects. It is very important to investigate the effect of 4-hydroxyisoleucine and isoleucine on mitochondrial respiration because of the essential role of mitochondria in diabetes and insulin resistance.



a)



b)

Figure 4.14. **The effect of UK 5099 on glucose consumption in BRIN-BD 11 cells.** There is no significant change in glucose consumption in BRIN-BD 11 cell after 24 hours incubation with 35 µM UK 5099 (a). Glucose consumption shows a dramatic decrease in both isoleucine and 4-hydroxyisoleucine in front of UK 5099 ($p<0.01$) compared to UK 5099 and no treatment control groups (a). ATP level significantly reduced in all UK 5099 groups ($p<0.01$) without difference between isoleucine and 4-hydroxyisoleucine groups (b).

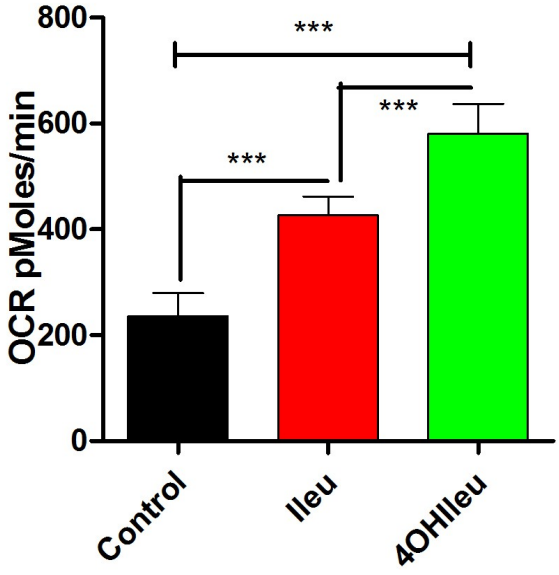
4.12 Analysis of the effects of 4-hydroxyisoleucine and isoleucine on mitochondrial respiration in BRIN-BD 11 cells with Seahorse XF-24 autoanalyser

We used the Seahorse cell metabolism analyser to evaluate the mitochondrial activity and glycolysis within the BRIN-BD 11 cells after 24 hours incubation with 500 µM isoleucine and 4-hydroxyisoleucine. Seahorse XF (Extracellular Flux) analyser is the platform for metabolic assays which simultaneously and in real time measures the two major energy producing pathways of the cell, mitochondrial respiration and glycolysis, in a microplate. The seahorse XF autoanalyser measures oxygen consumption rate (OCR) as the indicator of mitochondrial respiration and extracellular acidification rate (ECAR) which is predominately the result of glycolysis at intervals of approximately 2-5 minutes. We measured the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in BRIN-BD11 cells after 24 incubation with isoleucine and 4-hydroxyisoleucine for 30 minutes in three replicates for each group to study changes in the activity of mitochondria and glycolysis. Results after normalisation using cell counts showed that oxygen consumption rate (OCR) in BRIN-BD 11 cells was significantly higher in cells incubated with isoleucine and 4-hydroxyisoleucine compared to control **(Fig 4.15a)**. The OCR was significantly higher in 4-hydroxyisoleucine group compared to isoleucine group **(Fig 4.15a,b)**. Increasing in OCR indicates that both 4-hydroxyisoleucine and isoleucine increase the mitochondrial activity, but 4-hydroxyisoleucine effect on mitochondrial metabolism is much greater than isoleucine in BRIN-BD11 cells. Extracellular acidification rate (ECAR) measurement showed a significant rise in 4-hydroxyisoleucine group, but not in isoleucine group **(Fig 4.15c)** which indicates the higher

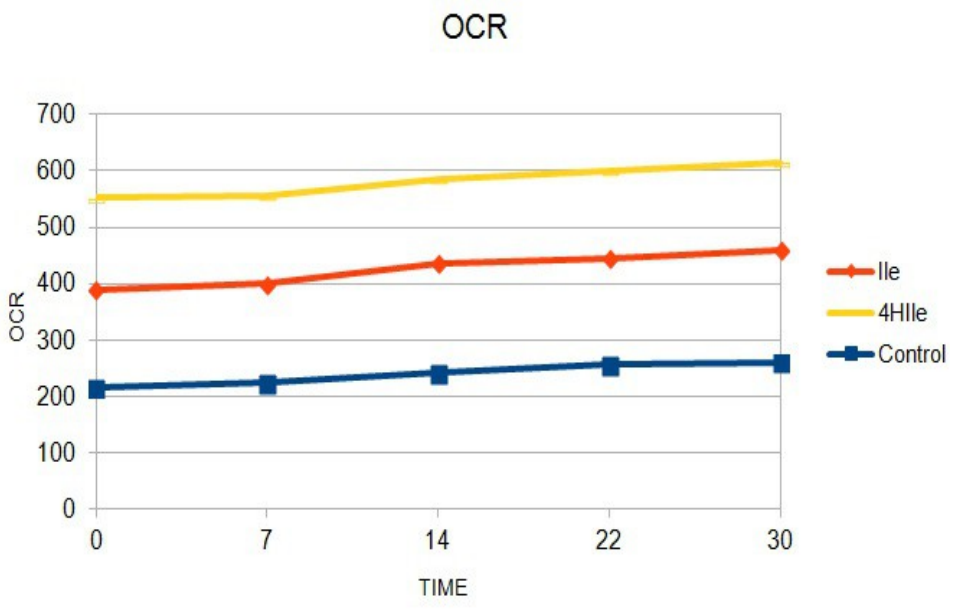
glycolysis rate in the cells incubated with 4-hydroxyisoleucine compared to isoleucine. The OCR/ECAR ratio is significantly higher in both isoleucine and 4-hydroxyisoleucine groups compared to control **(Fig 4.15d)** with no significant difference between the 4-hydroxyisoleucine and isoleucine groups. Seahorse study revealed that 4-hydroxyisoleucine increases both mitochondrial metabolism rates shown as OCR and glycolysis rate shown as ECAR but isoleucine only increases mitochondrial metabolism rate without changing the glycolysis rate significantly. These findings explain the inhibitory effect of the UK5099 as an MPC blocker on both 4-hydroxyisoleucine and isoleucine activities in BRIN-BD 11 cells. It also indicates that their activities firstly dependent on mitochondrial activity and secondly are not limited to glucose uptake increase but they can increase cell metabolism. There is possibility that increasing glucose uptake by 4-hydroxyisoleucine and isoleucine is not a direct action due to increased glucose transporters or glucose uptake pathways within the cell, but it could be the indirect result of increasing the cell basal metabolism in the deepest layer. One of the important common underlying pathophysiology in both diabetes type 1 and 2 is cellular metabolic defect due to lack of insulin and insulin resistance, resulted in diabetic metabolic complications. Considering the facts that our previous animal study showed a comprehensive modifying effect of 4-hydroxyisoleucine on different metabolic parameter such as blood sugar, triglyceride, cholesterol in diabetes type 1 models without changing the level of insulin, and the role of calcium signalling specially mitochondria calcium transporters, support the idea that 4-hydroxyisoleucine and in lesser extent with probably different mechanism isoleucine exert their anti-diabetic properties through increasing cellular metabolism. Theoretically, if the metabolism of cell increases, it needs to produce more energy and accordingly it requires to

uptake more glucose from the peripheral. It means that facilitating the glucose uptake or increasing insulin sensitivity as suggested in some previous literatures may not be a direct mechanism of 4-hydroxyisoleucine's anti-diabetes effect like any available anti-diabetic medications but they could be the results of its effect.

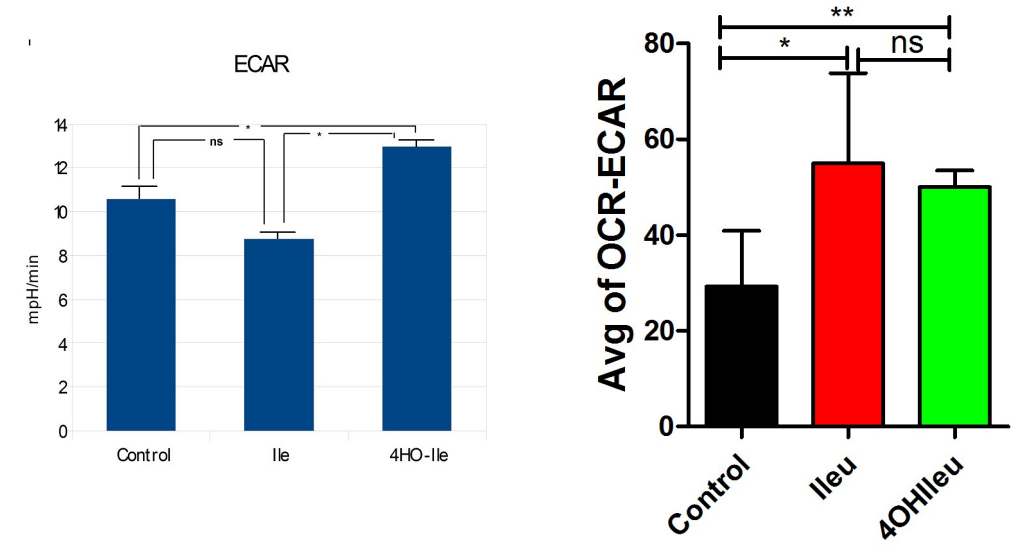
Seahorse measurement findings showed a lower glycolysis rate in isoleucine compared to 4-hydroxyisoleucine which could support the previous findings of the current study in the sense that 4-hydroxyisoleucine and isoleucine mechanisms of action are not similar in the cellular level and interacts with wider targets and pathways within the cell leading to facilitate glucose utilisation probably via enhancing glycolysis beside other mechanisms.


800
600
400
200
0
OCR pMoles/min
***
***
***
Control
Ileu
4OHIleu

a)


OCR
700
600
500
400
300
200
100
0
OCR
0
7
14
22
30
TIME
Ile
4HIle
Control

b)



c) d)

Figure 4.15. **Seahorse XF-24 autoanalyser results.** Oxygen consumption rate (OCR) in BRIN-BD11 cells increased very significantly after 24 hours incubation with 500 µM isoleucine and 4-hydroxyisoleucine ($p<0.001$) (a). OCR measurement indicated much greater increase in mitochondrial metabolism in 4-hydroxyisoleucine groups in comparison with isoleucine groups ($p<0.01$) (a,b). Extra cellular acidification rate as an indicator of glycolysis increased significantly in 4-hydroxyisoleucine groups ($p<0.05$) but not in isoleucine groups (c). A non statistically significant reduction in ECAR has been observed in isoleucine groups (c). Average OCR/ECAR ratio shows significant increase in metabolic rate in cells incubated with isoleucine and 4-hydroxyisoleucine (d). Average OCR/ECAR ratio is lower in 4-hydroxyisoleucine because of increase in both ECAR and OCR which is not observed in isoleucine (d).

Chapter V

Conclusions

We tried to create a diabetic rat model as close as possible to diabetes type 1 condition with high blood glucose level and significant reduced plasma insulin and insulin secretion capability of pancreatic beta cell using a multiple intraperitoneal streptozotocine injection. Multiple dose of streptozotocine injection for creating a stable type 1 diabetes animal model is a well established and affordable technique which leads to a significant reduction in pancreatic beat cells number and concomitant reduction in insulin secretion capacity (King AJ. 2012). There is always a limitation to create a right animal model, but our diabetic animal model exhibits all expected metabolic complications of diabetes, including high blood glucose, triglyceride, cholesterol and uric acid with noticeable reduction in insulin compared to non diabetic animals. Treatment of diabetic animals with 4-hydroxyisoleucine for four weeks modified the metabolic complications of diabetes without changing insulin level compared to the diabetic control group. Our study findings follow the same results from previous studies with different animal models close to type 2 diabetes (Broca *et al*. 2004, Sauvaire *et al*. 1998, Singh *et al*. 2010 and Haeri *et al.* 2009). It shows that 4-hydroxyisoleucine is not necessarily dependent on insulin for exhibiting its anti-diabetic properties as concluded in previous works. 4-hydroxyisoleucine could increase glucose uptake without increasing insulin, otherwise in diabetes type 2 animal models with inulinemia in Broca's study we should see more sever insulinemia instead of significant reduction in insulinemia. It also sheds a light on the fact that 4-hydroxyisoleucine effect is not limited to insulin and it could exert more profound effect in utilizing extra glucose and also modifies metabolic pathways.

We chose a pancreatic beta cell as a model to investigate the 4-hydroxyisoleucine for further clarification of our animal study findings. Studying the 4-hydroxyisolecine effect on pancreatic beta cell which

does not exhibit insulin induced glucose uptake pathway allowed us assess its effect without insulin interference. Isoleucine was chosen as a positive control because of its close molecular and anti-diabetic properties to 4-hydroxyisoleucine. Incubation of BRIN-BD 11 cells as a pancreatic beta cell model in a medium with 22mM glucose concentration and 4-hydroxyisoleucine and isoleucine simultaneously for 24 hourse did not show any significant increase of insulin secretion compared to control despite the significant increase in glucose consumption. This finding was in line with our previous animal study with 4-hydroxyisoleucine showing its insulin independent glucose lowering effect and confirms that 4-hydroxyisoleucine does not act as a insulinotropic agent. Increase of glucose uptake in beta pancreatic cell without changes in insulin secretion and decrease of blood glucose level in our diabetic rat model without significant change of plasma insulin level, indicate that the mechanism of action involves in 4-hydroxyisoleucine is different from previously understood. All the suggested mechanisms such as insulin sensitizing and stimulation of insulin secretion by previous researchers are not a direct effect of 4-hydroxyisoleucine but they could be the indirect effects which resulted from the activity of 4-hydroxyisoleucine on other mechanisms within the cells. It seems that both 4-hydroxyisoleucine and isoleucine utilize the glucose consumption via modulating the cellular basal glucose consumption because they are capable of stimulation of glucose uptake in pancreatic beta cell which does not express GLUT 4. It means that the increase in glucose uptake is handled by GLUT 1 or GLUT 2. Considering the fact that GLUT 2 is mainly expressed in beta cells not other peripheral cells and both 4-hydroxyisoleucine and isoleucine are able to show a hypoglycaemic effect in animal models and peripheral cells, such as adipose and muscle cells, GLUT 1 could be a potential

candidate for handling increased glucose uptake. It is also in line with the role of GLUT 1 in maintaining basal glucose uptake required for a sustain cellular respiration in most of the peripheral cells.

Endpoint measurements of ATP content of the BRIN-BD11 cells did not show any significant changes between 4-hydroxyisoleucine, isoleucine and control groups as well as an insulin level measurement in culture medium after 24 hours. Study with different concentrations of glucose (5, 11 and 22 mM) indicated a direct correlation between the increase of glucose uptake, ATP content and insulin secretion **(Fig 4.2,4.3)** which confirm the physiologically responsiveness of BRIN-BD 11 cell as expected. It means that the cell's behaviour follows the physiological pattern, increasing glucose concentration in medium as expected leads to more glucose uptake and more ATP production, resulted in more insulin secretion.

Endpoint measurement of cell's ATP contents in a specific point of time is not a accurate and proper method to investigate the ATP production within the cell. As ATP continuously recycles within the cell, we need more accurate quantitative method to measure ATP in a real-time format. It raises the question that how the cell utilizes the extra glucose, which entered the cell. It would be a key point in mechanisms of action of both 4-hydroxyisoleucine and isoleucine, which requires more detailed and sophisticated study.

Rendering these data suggest that unlike insulin, which increase immediate glucose uptake via translocation of GLUT4 in peripheral tissues, 4-hydroxyisoleucine and isoleucine may increase the cellular basal glucose consumption by interacting directly with the cellular metabolism and energy production cycle. It also could explain why their hypoglycaemic effects appear in the long term not short treatment as it requires more time that basal glucose consumption picks up.

Inhibiting a variety of pathways related to glucose uptake and cell metabolism using selected inhibitors revealed that there are substantial differences between isoleucine and 4-hydroxyisoleucine mechanisms of action unlike their similar glucose uptake stimulation effect and anti-diabetic properties which is greater in 4-hydroxyisoleucine **(Fig 5.1)**.

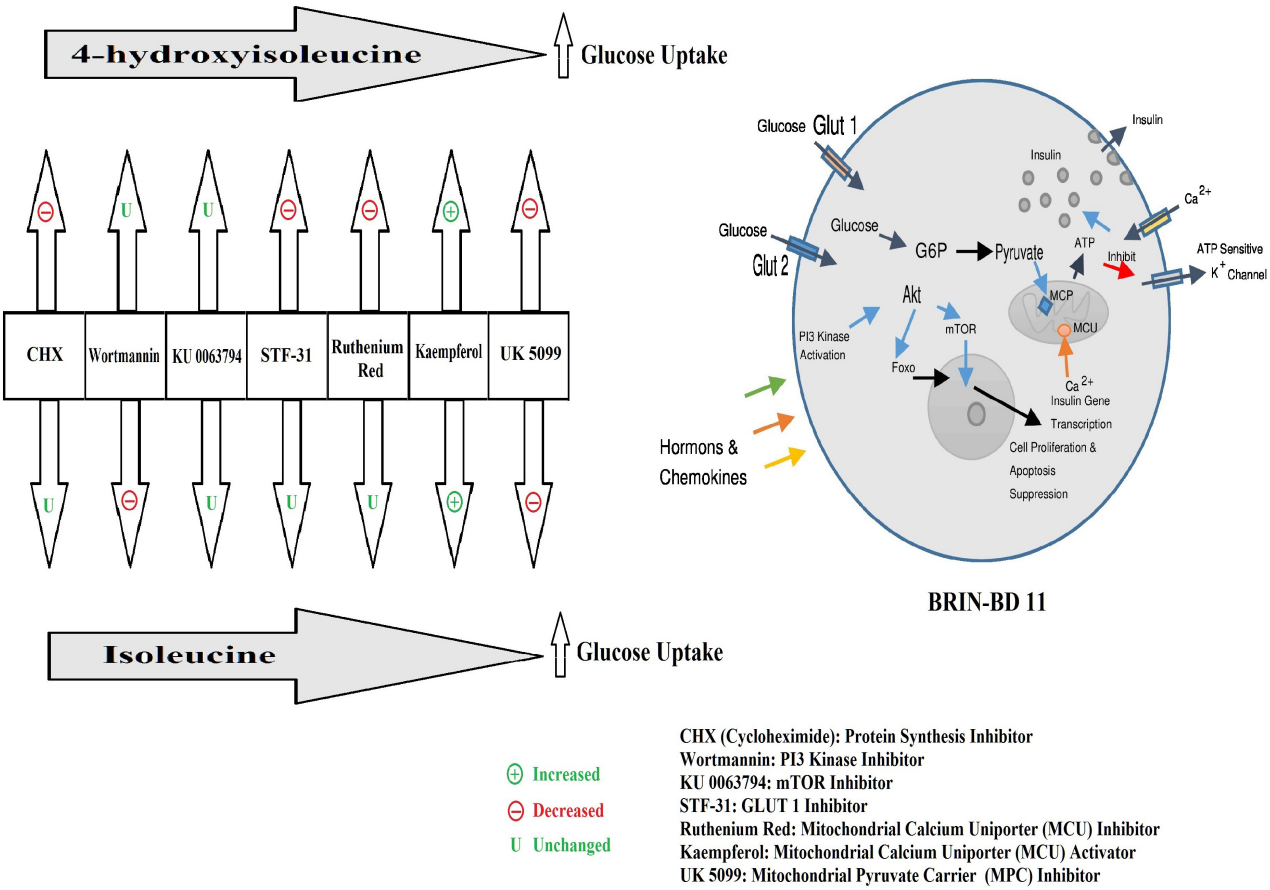


Figure 5.1. Summary of the in-vitro studies, testing the effect of different inhibitors and activator on 4-hydroxyisoleucine and isoleucine glucose uptake stimulation activity.

As previous study showed that 4-hydroxyisoleucine effect was blocked by protein synthesis inhibition using CHX (Jaiswal *et al*. 2012), we tested the effect of CHX on isoleucine and 4-hydroxyisoleucine activity in BRIN-BD 11. Adding CHX to BRIN-BD11 cells loaded simultaneously with 4-hydroxyisoleucine and isoleucine revealed that unlike isoleucine, 4-hydroxyisoleucine glucose uptake stimulatory effect reduced significantly in front of protein synthesis inhibitor. It suggests that intermediary protein or proteins are involved in 4-hydroxyisoleucine mechanism of action but not isoleucine. It could add explanation to why 4-hydroxyisoleucine exhibits its hypoglycaemic effect in long term treatment. 4-hydroxyisoleucine is dependent on the synthesis of specific proteins and up-regulation of protein expression requires time. It requires more investigation to study the effect of 4-hydroxyisoleucine on proteins and genes expression in BRIN-BD 11 cell and other cell models such as adipose and skeletal muscle. It could be an important subject for the future research, providing more detailed insight about the mechanisms are involved in 4-hydroxyisoleucin activity.

As explained in the beginning, GLUT 1 plays a major role in maintaining the basal glucose uptake required to sustain cellular respiration in all types of cell. GLUT 1 is the most abundant glucose transporters in the beta cells and it is able to compensate for the GLUT 2 deficiency or under expression in beta pancreatic cells and maintain an appropriate rate of glucose uptake to carry on glucose metabolism and insulin secretion activity in pancreatic beta cells (Liang *et al*. 1997). Partially blocking the GLUT 1 activity in BRIN-BD 11 cells with STF-31 as a highly selective GLUT 1 inhibitor, showed a direct dependency of 4-hydroxyisoleucine on GLUT 1 in comparison with isoleucine. The direct correlation between GLUT 1 activity and 4-hydroxyisoleucine glucose uptake stimulation, suggests that 4-hydroxyisoleucine directly or

indirectly increases cellular basal glucose uptake in long term and it may increase GLUT 1 expression as well. It supports the notion that 4-hydroxyisoleucine exhibits its activity in the long term treatment in animal models and it does not show an immediate glucose lowering effect like some anti-diabetic medications which increase insulin mediated glucose uptake. Unfortunately, it was out of our available resources to examine GLUT 1 expression in protein and gene level in BRIN-BD 11 but it is recommended for the future research to study GLUT 1 protein and gene expression in BRIN-BD 11 cell incubated with 4-hydroxyisoleucine in high glucose concentration. Based on our findings, GLUT 1 plays a critical role, but only increasing GLUT 1 expression or activity do not completely explain all 4-hydroxyisoleucine's anti-diabetic properties and other mechanisms are involved.

GLUT 1 inhibition by STF-31 reduces the glucose uptake in 4-hydroxyisoleucine group but not isoleucine group. On the other hand, isoleucine effect was diminished by inhibiting PI3Kinase activity in BRIN-BD11 cells with Wortmannin without significant change in 4-hydroxyisoleucine activity. PI3Kinase plays a variety of roles in beta pancreatic cells including increase of insulin gene transcription, cell proliferation and suppress appotosis (Cerf. 2013). It was shown that PI3Kinase inhibition in pancreatic beta cell, increased glucose uptake and glucose-stimulated insulin secretion (Zawalich *et al*. 2002). Broca's study in 2004 showed that 4-hydroxyisoleucine administration increased PI3Kinase activity associated with IRS-1 in both muscle and liver same as insulin but combining insulin and 4-hydroxyisoleucine did not increase PI3Kinase activity further. Our finding does not support the direct connection between 4-hydroxyisoleucine and PI3Kinase. Increasing of PI3Kinase activity as detected in previous study could be

result of other pathways activation within the cell. In contrary with 4-hydroxyisoleucine, our finding supports the role of PI3Kinase in isoleucine activity. It is important to emphasis that our experiment only provide a snap shot of interaction between 4-hydroxyisoleucine and isoleucine with PI3Kinase and it requires more investigation to assess the role of PI3Kinase in both 4-hydroxyisoleucine and isoleucine mechanism of actions as PI3Kinase is involved in many pathways within the cell.

It is an interesting finding of the opposite behaviour of 4-hydroxyisoleucine and isoleucine in front of different inhibitors, despite their similar effect on increasing the glucose uptake in BRIN-BD11 cells. The different behaviours of isoleucine and 4-hydroxyisoleucine in front of selected inhibitors clearly demonstrate a difference in mode of actions between 4-hydroxyisoleucine and isoleucine.

Insulin independent increase of glucose uptake in BRIN-BD 11 cells incubated by 4-hydroxyisoleucine and isoleucine raised a question about the effect of these molecules on the cellular metabolism. As previously explained, 4-hydroxyisoleucine seems to increase basal glucose uptake in longer treatment in both in-vitro and in-vivo studies. Theoretically, It requires that 4-hydroxyisoleucine facilitate the glucose utilization within the cell via increasing cytoplasmic metabolism and mitochondrial respiration.

The first step towards understanding the role of mitochondria was assessed using mitochondrial calcium uniporter (MCU) inhibitor and activator. It allows to study the role of calcium in 4-hydroxyisoleucine and isoleucine mechanisms of action as well as mitochondria. The increase of intracellular calcium and release of calcium from the sarcoplasmic reticulum (SR), stimulate glucose uptake (Youn *et al*. 1991)

which shows the role of calcium in glucose uptake mechanisms. MCU plays an essential role for a sustained glucose-induced increase in the cytosolic ATP/ADP ratio in pancreatic beta cells (Trasov *et al*. 2012). Partial inhibition of MCU in BRIN-BD 11 using ruthenium red created an opposite effect in 4-hydroxyisoleucine and isolecuine groups. 4-hydroxyisoleucine effect was diminished significantly, but isoleucine showed some degree of synergistic effect with ruthenium red in increasing glucose uptake. Both 4-ydroxyisoleucine and isoleucine showed a significant increase in glucose uptake caused by activation of MCU using kaempferol as an MCU activator. It does not only support the crucial role of mitochondria and calcium in their mechanisms, but shows different mechanisms are involved. It seems both 4-hydroxyisoleucine and isoleucine interact with mitochondria but in via seperate mechanisms. This experiment raises a strong idea to develop a new research in the future to examine the role of calcium in 4-hydroxyisoleucine and isoleucine activities which could be one of the important aspects of 4-hydroxyyisoleucine and isoleucine mechanisms of action.

Mitochondrial metabolism interruption in BRIN-BD11 cell using the UK5099, a specific mitochondrial pyruvate carrier (MPC) inhibitor when loaded simultaneously with 4-hydroxyisoleucine and isoleucine led to significant inhibition of both 4-hydroxyisoleucine and isoleucine glucose uptake stimulation effect. It proves the notion about the role of mitochondrial respiration in their mode of actions. Real time metabolic activity analysis of the BRIN-BD11cells after 24 hours incubation with 4-hydroxyisoleucine and isoleucine in the presence of 22mM glucose with seahorse autoanalyser confirmed such a role of mitochondria. A significant increase in metabolic activity was observed in cells after 24

hours incubation with 4 hydroxyisleucine and isoleucine but significantly greater in 4-hydroxyisoleucine group. It explains the greater glucose uptake in front of the same concentration of 4-hydroxyisoleucine compared to isoleucine which was persistent throughout our experiments. Reviewing the seahorse data shows a meaningful difference between isoleucine and 4-hydroxyisleucine apart from their effect on mitochondrial activity. Both oxygen consumption rate and extracellular acidification rate were significantly increased by 4-hydroxyisoleucine but no significant increase was detected in extracellular acidification rate as the indication of glycolysis in isoleucine group. 4-hydroxyisoleucine interacts with glycolysis cycle in the cytoplasm as well as mitochondrial respiration but isoleucine increases the mitochondria respiration without affecting the glycolysis rate. It could explain the different behaviours of isoleucine and 4-hydroxyisoleucine in front of some inhibitors in this study.

Seahorse autoanalyser results ignited the idea that there may be a link between 4-hydroxyisoleucine activity and glucokinase. Glucokinase requires a much higher glucose concentration for maximal activity. Glucokinase is found in the liver and pancreatic beta cells and it plays an important role in liver due to its high Vmax, allowing the liver to effectively remove excess glucose, and minimize hyperglycaemia after eating. Glucokinase unlike hexokinase which is inhibited by the product of its reaction, is not inhibited by glucose 6 phosphate (G6P). Previous studies showed that expression of glucokinase in cultured human muscle cells treated with AdCMV-GKL results in proportional increases in insulin-independent glucose disposal, and that muscle glucose storage and utilization becomes controlled in a glucose concentration-dependent manner in AdCMV-GKL-treated cells (Baqué *et al*. 1998). It can raise the hypothesis that 4-hydroxyisoleucine may involve in facilitating

glucokinase activity as the main regulator of glycolysis alongside with other possible mechanisms which may be in common with isoleucine considering the facts that the hypoglycaemic activity of 4-hydroxyisoleucine is seen in higher concentration of glucose. The Seahorse autoanalyser results could support the idea of involvement of 4-hydroxyisoleucine in the glucokinase pathway as it increases glycolysis as well as mitochondrial respiration unlike isoleucine which does not affect glycolysis. It will require more detailed studies to be done in the future to investigate the interaction between glucokinase and 4-hydroxyisoleucine. Unfortunately, we could not carry such study due to limited resources and facilities, but it would be a great idea for the further research project.

We can summarise the conclusions from animal and cellular studies about 4-hydroxyisoleucine as follows:

- 4-hydroxyisoleucine has a unique long term anti-diabetic activity as observed and it possibly could increase the cellular basal glucose consumption in high glucose concentration over a longer period of time without a significant immediate effect on glucose uptake similar to insulin.

- 4-hydroxyisoleucine anti-diabetic effects are independent of insulin but its effect on increasing basal glucose uptake in long term treatment can contribute to the stronger insulin effect on peripheral tissues. Insulin binding to its receptor, creates a surge in basal glucose uptake by promoting GLUT 4 translocation to the plasma membrane. If the basal glucose uptake increases, the same amount of insulin can create a stronger effect on lowering glucose

level. Increased basal glucose uptake in peripheral cells via 4-hydroxyisoleucine could explain the insulin-sensitizing property of 4-hydroxyisoleucine in insulin resistant diabetic models studied before.

- 4-hydroxyisoleucine modifies metabolic complications of diabetes, most importantly dyslipidemia by decreasing triglyceride, cholesterol, low density lipoprotein (LDL) levels in blood. It also reduces plasma uric acid in diabetes as well as hyperglycaemia and dyslipidemia. It is concluded that 4-hydroxyisoleucine is not only a hypoglycaemic agent, but it is capable of limiting the disabling complications of diabetes resulted from metabolic defects over a long period.

- 4-hydroxyisoleucine unlike its similarity to isoleucine in the perspective of molecular structure and hypoglycaemic property, has wider activities via interacting with pathways which are not affected by isoleucine. 4-hydroxyisoleucine molecular structure core is similar to isoleucine, but its extra hydroxyl group at position four creates unique distinctive properties from isoleucine.Current in-vitro study results indicate that isoleucine and 4-hydroxyisoleucine have different modes of action. Its glucose lowering effect is dependent on glucose concentration and protein synthesis that suggests a different pathway for 4-hydroxyisoleucine and could explain the other extra effects of 4-hydroxyisoleucine in modifying diabetes adverse effects compare to isoleucine.

- 4-hydroxyisoleucine activity is strongly dependent on new protein synthesis and GLUT1 activity. GLUT1 is widely available in most of the cells and it controls the basal glucose uptake independent of insulin. The connection between GLUT1 and 4-hydroxyisoleucine effect in cellular level, supports the idea that 4-hydroxyisoleucine utilises the glucose basal consumption and uptake of cells.

- PI3 Kinase plays essential role in the isoleucine mode of action but not 4-hydroxyisoleucine as proposed in previous literatures. Increasing of the PI3Kinase activity in tissues treated by 4-hydroxyisoleucine as observed in previous studies could be an indirect effect of 4-hydroxyisoleucin.

- Both 4-hydroxyisoleucine and isoleucine activities are strongly dependent on mitochondrial respiration, but 4-hydroxyisoleucine up-regulates glycolysis significantly which is not affected by isoleucine. The connection between mitochondria calcium signalling and contradictory behaviour of 4-hydroxyisoleucine and isoleucine in front of reuthenium red support the very important role of calcium in their mechanisms of action.

- The improving effects of 4hydroxyisoleucine on metabolic parameters in diabetes including plasma glucose, lipid profile and uric acid in animal study, and increasing glucose consumption, glycolysis and mitochondrial activity in BRIN-BD11 cells, suggest that 4hydroxyisoleucine has a systemic effect on metabolically active tissues, including the liver, adipose, muscle and pancreatic beta cells that are independent of insulin. It does not exclude the role of insulin in combination with 4-hydroxyisoleucine which

may amplify its effect as shown previously, but current study findings exclude the insulin secretion stimulation and interaction directly with insulin as the key mechanism of action of 4-hydroxyisoleucine.

- Current study findings indicate that both 4-hydroxyisoleucine and isoleucine have mechanisms beyond the insulin regulatory pathway and affect metabolism and energy modulation in both cytoplasmic and mitochondrial levels which requires more profound and meticulous study.

We tried to create an overview and basis about potential mechanisms involved in 4-hydroxyisoleucine in our study to open a window for more researches in the future. The evaluation of 4-hydroxyisoleucine and isoleucine on expression of a variety of genes in different types of cell using microarray technique could provide an immense data about their mechanisms of action.

4-hydroxyisoleucine is an anti-diabetic molecule with multiple actions, which is not only reduces plasma glucose in type1 and 2 diabetes, but modifies other metabolic complications of diabetes and it is only active in high glucose concentration unlike other antidiabetic medications. There have been no reports of any adverse effects or toxicity of 4hydroxyisoleucine in previously published studies which indicate that it is well tolerated and not toxic (Flammang *et al.* 2004). It makes the 4-hydroxyisoleucine an intelligent molecule which only active in diabetic conditions and its consumption do not produce the hypoglycaemic effect in normoglycaemic or non-diabetic condition. It is a safe choice, unlike other medications if it is consumed by non-diabetic patients or misused.

Analysing the data from our animal and in-vitro studies determine that 4-

hydroxyisoleucine could be a promising candidate for type 1 and 2 diabetes solely or in combination with other therapeutic regimen. Unfortunately, there is no precise information about the dosage of 4-hydroxyisoleucine for such application in human. There are some suggestions based on traditional medicine for using fenugreek seed extract but there is no verifiable scientific data about the dosage of fenugreek. The lack of such useful information makes pharamcokinetic studies an important field to understand 4-hydroxyisoleucine metabolism in the body, interaction with other medications and dose response.

5.1 Future recommended studies

There are some ideas for the future studies recommended in this chapter based on the current study finding to provide a better and clearer understanding of 4-hydroxyisoleucine mechanisms of action. Carrying more fundamental and accurate studies which obviously require more resources and supports to discover 4-hydroxyisoleucine mechanisms of action which not only allow us to develop more efficient and safer therapeutics, but also guide us towards a better understanding of the pathophysiology of diabetes and insulin resistance.

There are a summary of suggested future studies for 4-hydroxyisoleucine which may be used as ideas for designing better research projects in the future.

- Investigation of the GLUT 1 expression and its gene transcription in pancreatic beta cell, liver, adipose and skeletal muscle cell models incubated with 4-hydroxyisoleucine in the presence of high glucose concentration. It is suggested to use a dosage titration to identify the relation of any changes with 4-hydroxyisoleucine.

- As we showed the role of calcium in 4-hydroxyisoleucine activity, it is important to study the role of calcium signalling pathways within the cell in more details not only in pancreatic beta cell but in other types of cells incubated with 4-hydroxyisoleucine and isoleucine for comparison.
- Based on the Seahorse autoanalyzer results which showed increased activity in both glycolysis and mitochondrial respiration, It is recommended to look into the interaction of 4-hydroxyisoleucine with glucokinase and other enzymes within the Krebs cycle. There is a potential possibility of direct or indirect interaction between 4-hydroxyisoleucine and Krebs cycle's enzyme. Investigation of the expression and activity of the Krebs cycle enzymes in pancreatic beta cell and other cell models such as liver and adipose could provide a valuable data about the role of 4-hydroxyisoleucine in energy modulation via interacting with mitochondrial respiration.
- Screening the whole genome expression level in the cell or tissue models treated with 4-hydroxyisoleucine and isoleucine in front of high concentration of glucose compared to non treatment control using a microarray technique could reveal a valuable data about their mechanisms of action. It also creates a database for different kind of analysis to provide a wider perceptive about 4-hydroxyisoleucine and isoleucine activities in gene level. Unfortunately, carrying such experiment is expensive and requires substantial resources and facilities.
- A limited human clinical trial could be very informative to evaluate the efficacy of 4-hydroxyisoleucine in diabetic patients solely or in combination with other available medication. It requires pharmacokinetics study prior to clinical study to set out a

dose response and more information about 4-hydroxyisoleucine metabolism. It also requires more cost effective chemistry method to produce an affordable synthetic 4-hydroxyisoleucine because it is an expensive and a rare molecule from being used in milligram dosage for clinical study.

References

Abu Saleh M. Moosa,Mamun Ur Rashid, A.Z.S. Asadi, Nazma Ara,M. Mojib Uddin and A. Ferdaus. Hypolipidemic effects of fenugreek seed powder.Bangladesh J Pharmacol 2006; 1: 64-67.

Accili, D., and Arden, K.C. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell. 2004. 117, 421–426.

ADA, Standards of Medical Care in Diabetes - 2010. Diabetes Care, 2010

Alam M.R., Groschner L.N., Parichatikanond W., Kuo L., Bondarenko A.I., Rost R., Waldeck-Weiermair M., Malli R., Graier W.F. Mitochondrial Ca^{2+} uptake 1 (MICU1) and mitochondrial Ca^{2+} uniporter (MCU) contribute to metabolism-secretion coupling in clonal pancreatic beta-cells. J. Biol. Chem. 2012, 287:34445-34454.

Alcock NW, Crout DHG, Gregorio MVM, Lee E, Pike G, Samuel CJ. Stereochemistry of the 4-hydroxyisoleucine from Trigonella foenum graecum. Phytochem. 1989; 28 (7): 1835 – 41.

Al-Habori M, Al-Aghbari AM, Al-Mamary M. Effects of fenugreek seeds and its extracts on plasma lipid profile: a study on rabbits. Phytother. Res. 1998; 12: 272 – 5.

Amin Gh. Popular medicinal plants of Iran. Tehran: Vice-choncellor of Research of Tehran University of Medical Sciences, 1991, pp: 101 – 2.

Amori RE, Lau J, Pittas AG . "Efficacy and safety of incretin therapy in type 2 diabetes: systematic review and meta-analysis". JAMA. 2007. 298(2): 194–206.

Analava Mitra, Debaprasad Bhattacharya. Dose-dependent effects of Fenugreek composite in Diabetes with dislipidaemia.Internet Journal of Food Safety, Vol.8, 2006, p. 49-55.

Baqué S, Montell E, Guinovart JJ, Newgard CB, Gómez-Foix AM. Expression of glucokinase in cultured human muscle cells confers insulin-independent and glucose concentration-dependent increases in glucose disposal and storage. Diabetes. 1998; 47(9):1392-8.

Baicy K, London ED, Monterosso J, Wong ML, Delibasi T, Sharma A, Licinio J . "Leptin replacement alters brain response to food cues in genetically leptin-deficient adults". Proc. Natl. Acad. Sci. November 2007 104 (46): 18276–9

Basch E, Ulbricht C, Kuo G, Szapary Ph, Smith M. Therapeutic applications of Fenugreek. Altern. Med. Rev. 2003. 8 (1): 20 – 7.

Bartel DP, “MicroRNAs: genomics, biogenesis, mechanism, and function,” Cell. 2004. vol. 116, no. 2, pp. 281–297.

Belfiore A, Frasca F. "Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.". Endocr. 2009. Rev 30 (6): 586–623.

Bell G, Kayano T, Buse J, Burant C, Takeda J, Lin D, Fukumoto H, Seino S. "Molecular biology of mammalian glucose transporters". Diabetes Care. 1990. 13 (3): 198–208.

Berger J, Moller DE. The mechanisms of action of PPARs. Annu Rev Med. 2002. 53: 409–435.

Bernardi P, Paradisi V, Pozzan T, Azzone GF. Pathway for uncoupler-induced calcium efflux in rat liver mitochondria: inhibition by ruthenium red. Biochemistry. 1984. 10;23(8):1645-51.

Biddinger, S.B., and Kahn, C.R. From Mice to Men: Insights into the insulin resistance syndromes. Annu. Rev. Physiol. 2006. 68, 1–36.

Billings LK, Florez JC. The genetics of type 2 diabetes: what have we learned from GWAS?Ann N Y Acad Sci. 2010 Nov;1212:59-77.

Bluestone, J. A.; Herold, K.; Eisenbarth, G. "Genetics, pathogenesis and clinical interventions in type 1 diabetes". Nature. 2010. 464 (7293): 1293-1300.

Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A. The L-type calcium channel in the heart: the beat goes on. J. Clin. Invest. 2005; 115: 3306-17.

Boone AN, Chan A, Kulpa JE, Brownsey RW. "Bimodal Activation of Acetyl-CoA Carboxylase by Glutamate". J Biol Chem. 2000. 275 (15): 10819–25.

Brennan AM, Mantzoros CS. "Drug Insight: the role of leptin in human physiology and pathophysiology--emerging clinical applications". Nat Clin Pract Endocrinol Metab . 2006. 2(6): 318–327.

Brethauer SA, Aminian A, Romero-Talamás H, Batayyah E, Mackey J, Kennedy L, Kashyap SR, Kirwan JP, Rogula T, Kroh M, Chand B, Schauer PR. Can diabetes be surgically cured? Long-term metabolic effects of bariatric surgery in obese patients with type 2 diabetes mellitus. Ann Surg. 2013 ;258(4):628-36.

Brinchmann-Hansen O, Dahl-Jørgensen K, Hanssen KF, Sandvik L . "The response of diabetic retinopathy to 41 months of multiple insulin injections, insulin pumps, and conventional insulin therapy". Arch. Ophthalmol. 1988. 106 (9): 1242–6.

Broca C, Gross R, Petit P, Sauvaire Y, Manteghetti M, Tournier M, Masiello P, Gomis R, Ribes G. 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties. Am J Physiol. 1999. 277(4 Pt 1):E617-23.

Broca C, Manteghetti M, Gross R, Baissac Y, Jacob M, Petit P, Sauvaire Y, Ribes G. 4-Hydroxyisoleucine: effects of synthetic and natural analogues on insulin secretion. Eur J Pharmacol. 2000. 3;390(3):339-45.

Broca C, Breil V, Cruciani-Guglielmacci C, Manteghetti M, Rouault C, Derouet M, Rizkalla S, Pau B, Petit P, Ribes G, Ktorza A, Gross R, Reach G, Taouis M. Insulinotropic agent ID-1101 (4-hydroxyisoleucine) activates insulin signaling in rat. Am. J. Physiol. Endocrinol. Metab. 2004. 287 (3): E463 – E471.

Brozinick, J. T. Jr & Birnbaum, M. J. Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. J. Biol. Chem. 1998. 273, 14679–14682.

Brunton LL, Chabner BA, Knollmann BC: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th Edition:The McGraw-Hill Companies, 2011.

Brunetti A, Manfi oletti G, Chiefari E, Goldfi ne I D and Foti D. Transcriptional regulation of human insulin receptor gene by the high-mobility group protein HMGI(Y); FASEB J. 2001. 15 492–500.

Bryan T. Hennessy, Debra L. Smith, Prahlad T. Ram, Yiling Lu & Gordon B. Mills. Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. Nature Reviews Drug Discovery, 2005. 4, 988-1004.

Buller CL, Loberg RD, Fan MH, Zhu Q, Park JL, Vesely E, Inoki K, Guan KL, Brosius FC 3rd. A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. Am J Physiol Cell Physiol. 2008 Sep;295(3):C836-43.

Cappuccio FP, Strazzullo P, Farinaro E, Trevisan M. "Uric acid metabolism and tubular sodium handling. Results from a population-based study". JAMA.1993. 270 (3): 354–9.

Cawston, Erin E; Miller, Laurence J. "Therapeutic potential for novel drugs targeting the type 1 cholecystokinin receptor".British Journal of Pharmacology. 2010. 159 (5): 1009–1021.

Cerf ME. Beta cell dysfunction and insulin resistance. Front Endocrinol (Lausanne). 2013. 27;4:37.

Chan DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Banh A, Reynolds GE, Chi JT, Wu J, Solow-Cordero DE, Bonnet M, Flanagan JU, Bouley DM, Graves EE,Denny WA, Hay MP, Giaccia AJ. Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. Sci Transl Med. 2011 Aug 3;3(94):94ra70.

Chavez J A, Knotts T A, Wang L P, Li G, Dobrowsky R T, Florant G L and Summers S A. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids; J. Biol. Chem. 2003. 278 10297–10303.

Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, Saltiel AR. Insulin stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature. 2001. 19;410(6831):944-8.

Cingolani E, Ramirez Correa GA, Kizana E, Murata M, Cheol Cho H, Marban E. Gene therapy to inhibit the calcium channel b subunit. Physiological consequences and pathophysiological effects in models of cardiac hypertrophy. Circ. Res. 2007; 101: 166-75.

Clarke J.F., et al., Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. Biochem J, 1994. 300: 631-5.

Cook DG, A. G. Shaper, D. S. Thelle, and T. P. Whitehead. Serum uric acid, serum glucose and diabetes: relationships in a population study. Postgrad Med J. 1986 November; 62(733): 1001–1006.

Corradetti M.N. and K. Guan, Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene, 2006. 25: 6347-6360.

Dean L, McEntyre J. The Genetic Landscape of Diabetes. National Center for Biotechnology Information (US); 2004.

Da-Silva WS, Harney JW, Kim BW, Li J, Bianco SD, Crescenzi A, Christoffolete MA, Huang SA, BiancoAC. The small polyphenolic molecule kaempferol increases cellular energy expenditure and thyroid hormoneactivation. Diabetes. 2007; 56(3):767-76.

Davidson, M.B., Kaplan, S.A.. Increased insulin binding by hepatic plasma membranes from diabetic rats. Normalization by insulin therapy. J. Clin. Invest.1977; 59, 22–30.

Dehghan A, van Hoek M, Sijbrands EJ, Hofman A, Witteman JC. "High serum uric acid as a novel risk factor for type 2 diabetes". Diabetes Care. 2008. 31 (2): 361–2.

Denton RM, McCormack JG. Ca^{2+}transport by mammalian mitochondria and its role in hormone action. Am. J. Physiol. 1985; 249: E543-54.

Dinger ME, Mercer TR, Mattick JS. RNAs as extracellular signaling molecules. J Mol Endocrinol 2008. 40:151–159.

Dipolo R, Beaugé L. "Sodium/calcium exchanger: Influence of metabolic regulation on ion carrier interactions". Physiological Reviews 2006; 86 (1): 155–203.

Divakaruni AS, Wiley SE, Rogers GW, Andreyev AY, Petrosyan S, Loviscach M, Wall EA, Yadava N, Heuck AP, Ferrick DA, Henry RR, McDonald WG, Colca JR,Simon MI, Ciaraldi TP, Murphy AN. Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. Proc Natl Acad Sci U S A. 2013; 110(14):5422-7.

Doi M, Yamaoka I, Fukunaga T, Nakayama M. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. Biochem Biophys Res Commun. 2003 . 26;312(4):1111-7.

Doi M, Yamaoka I, Nakayama M, Mochizuki S, Sugahara K, Yoshizawa F. Isoleucine, a blood glucose-lowering amino acid, increases glucose uptake in rat skeletal muscle in the absence of increases in AMP-activated protein kinase activity.J Nutr. 2005. 135(9):2103-8.

Doi M, Yamaoka I, Nakayama M, Sugahara K, Yoshizawa F . Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. Am J Physiol Endocrinol Metab. 2007. 292:E1683-E169.

Edward J McManus, Kei Sakamoto, Laura J Armit, Leah Ronaldson, Natalia Shpiro, Rodolfo Marquez and Dario R Alessi. "Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis". The EMBO Journal. 2005. 24 (8): 1571–1583.

Efrat S. "Making sense of glucose sensing". Nat. Genet. 1997. 17 (3): 249–50.

Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ. Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr. 2002 Nov 76(5):911-22.

Elvira Isganaitis; Robert H. Lustig. "Fast Food, Central Nervous System Insulin Resistance, and Obesity". Arteriosclerosis, Thrombosis, and Vascular Biology. 2005. 25 (12): 2451–2462.

Emilyn U. Alejandro, Tatyana B. Kalynyak, Farnaz Taghizadeh, Kamila S. Gwiazda, Erin K. Rawstron, Karen J. Jacob, and James D. Johnson. Acute Insulin Signaling in Pancreatic Beta-Cells Is Mediated by Multiple Raf-1 Dependent Pathways. Endocrinology. 2010; 151(2):502-12.

Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 2010.79:351–379.

Fang XK, Gao J, Zhu DN. Kaempferol and quercetin isolated from Euonymus alatus improve glucose uptake of 3T3-L1 cells without adipogenesis activity. Life Sci. 2008; 82(11-12):615-22.

Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, Hughes IA, McCamish MA, O'Rahilly S. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. N Engl J Med. 1999. 341: 879-884.

Fenugreek seed bioactive compositions and methods for extracting same. United States Patent 7338675.

Flammang AM, Cifone MA, Erexson GL, Stankowski LF Jr. Genotoxicity testing of a fenugreek extract. Food Chem Toxicol. 2004. 42(11):1769-75.

García-Sáinz JA, Piña E, Chagoya de Sánchez V. Stimulatory action of cycloheximide on glucose metabolism in the rat epididymal fat pad. J Lipid Res. 1977 Jan;18(1):93-8.

García-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SC, Chresta CM, Alessi DR. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem J. 2009; 421(1):29-42.

Grant SF, Hakonarson H. Genome-wide association studies in type 1 diabetes. Curr Diab Rep. 2009 ;9(2):157-63.

Greene M W, Morrice N, Garofalo R S and Roth R A. Modulation of human receptor substrate 1 tyrosine phosphorylation by protein kinase C δ; Biochem. J. 2004. 378 105–116.

Guillam MT, Hümmler E, Schaerer E, Yeh JI, Birnbaum MJ, Beermann F, Schmidt A, Dériaz N, Thorens B, Wu JY . "Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2". Nat. Genet. 1997; 17 (3): 327–30.

Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. Am. J. Physiol. 1990; 258: C755-86.

Gupta A, Gupta R, Lal B. Effect of Trigonella foenum-graecum (fenugreek) seeds on glycaemic control and insulin resistance in type 2 diabetes mellitus: a double blind placebo controlled study.J Assoc Physicians India. 2001. 49:1057-61.

Haeri MR, Izaddoost M, Ardekani MR, Nobar MR, White KN. The effect of fenugreek 4-hydroxyisoleucine on liver function biomarkers and glucose in diabetic and fructose-fed rats. Phytother Res. 2009. 23(1):61-4.

Haeri MR, Limaki HK, White CJ, White KN. Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (Trigonella foenum graecum) in streptozotocin-induced type I diabetic rats. Phytomedicine. 2012;19(7):571-4.

Halestrap AP. Pyruvate and ketone-body transport across the mitochondrial membrane. Exchange properties, pH-dependence and mechanism of the carrier. Biochem J. 1978; 172(3):377-87.

Handa T, Yamaguchi K, Sono Y, Yazawa K. Effects of fenugreek seed extract in obese mice fed a high-fat diet. Biosci Biotechnol Biochem. 2005. 69(6):1186-8.

Harrison's Principles of internal medicine, 18th Edition. The McGraw-Hill Companies.

Hardman R, Abu-Al-Futuh IM. The detection of isomers of 4-hydroxyisoleucine by the Jeol Amino Acid Analyser and by TLC. The detection of isomers of 4-hydroxyisoleucine by the Jeol Amino Acid Analyser and by TLC. Planta Med. 1979. 36(1):79-84.

Heather Basciano, Lisa Federico and Khosrow Adeli. "Fructose, insulin resistance, and metabolic dyslipidemia". Nutrition & Metabolism. 2005. 2 (1): 5.

Herder, C; Roden, M. "Genetics of type 2 diabetes: pathophysiologic and clinical relevance". European journal of clinical investigation. 2011. 41 (6): 679–92.

Herzig, S., E. Raemy, S. Montessuit, J.L. Veuthey, N. Zamboni, B. Westermann, E.R. Kunji, and J.C. Martinou. . Identification and functional expression of the mitochondrial pyruvate carrier. Science. 2012; 337: 93-96.

Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab. 2007. 5:237–252.

Hutton JC. Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases. Diabetologia. 1994. 37 (Suppl.2): S48-56.

Hruz P, Mueckler M. "Structural analysis of the GLUT1 facilitative glucose transporter (review)". Mol. Membr. Biol. 2001. 18 (3): 183–93.

Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-: direct role in obesity-linked insulin resistance. Science. 1993. 259: 87–91.

Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor inhibits signaling from the insulin receptor. Proc Natl Acad Sci USA. 1994. 91: 4854–4858.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor- in human obesity and insulin resistance. J Clin Invest. 1995. 95: 2409–2415.

Hotamisligil GS. Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord. 2003. 27, Suppl 3: S53–S55.

Ikeda Y, Olsen G S, Ziv E, Hansen L L, Busch A K, Hansen Bo F, Shafrir E and Mostaf-Seedorf L . Cellular mechanism of nutritionally induced insulin resistance in Psammomys obesus; Diabetes . 2001. 50 584–592.

Ikehara O, Kawasaki N, Maezono K, Komatsu M, Konishi A. Acute and chronic treatment of L-isoleucine ameliorates glucose metabolism in glucose-intolerant and diabetic mice. Biol Pharm Bull. 2008. 31(3):469-72.

Islam, M.S. Effects of the aqueous extract of white tea (Camellia sinensis) in a streptozotocin-induced diabetes model of rats. Phytomedicine.2011; 19, 25–31.

Israelson A, Zaid H, Abu-Hamad S, Nahon E, Shoshan-Barmatz V. Mapping the ruthenium red-binding site of the voltage-dependent anion channel-1. Cell Calcium. 2008; 43(2):196-204.

Ito H, Abe M, Mifune M, Oshikiri K, Antoku S, Takeuchi Y, Togane M. Hyperuricemia is independently associated with coronary heart disease and renal dysfunction in patients with type 2 diabetes mellitus. PLoS One. 2011; 6 (11): e27817.

Jaiswal N, Maurya CK, Venkateswarlu K, Sukanya P, Srivastava AK, Narender T, Tamrakar AK. 4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway.Eur J Nutr. 2012. 51(7):893-8.

Jennifer Mayfield. Diagnosis and Classification of Diabetes Mellitus: New Criteria. American Academy of Family Physicians. 1998. Retrieved September 26, 2009.

Jette, L., Harvey, L., Eugen, K., Levens, N., 4-Hydroxyisoleucine: a plant-derived treatment for metabolic syndrome. Curr. Opin. Investig. Drugs. 2009; 10, 353–358.

Joost HG & Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Molecular Membrane Biology. 2001.18, 247–256.

Kim, K. H. Regulation of mammalian acetyl-coenzymeAcarboxylase. Annu. Rev. Nutr. 1997. 17: 77–99.

King AJ. The use of animal models in diabetes research. Br J Pharmacol. 2012 ;166(3):877-94.

Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs,W.H., 3rd,Wright, C.V., White, M.F., Arden, K.C., and Accili, D. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic b cell growth. J. Clin. Invest. 2002 .110, 1839–1847.

Kirpichnikov D, McFarlane SI, Sowers JR.Metformin: an updateAnn Intern Med. 2002. 137(1):25–33.

Kjoller, L. & Hall, A. Signaling to rho GTPases. Exp Cell Res. 1999. 25;253(1):166-79.

Koistinen, H. A., D. Galuska, A. V. Chibalin, J. Yang, J. R. Zierath, G. D. Holman, and H. Wallberg-Henriksson.5-Amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. Diabetes. 2003. 52: 1066–1072.

Lagos-Quintana M, R. Rauhut, W. Lendeckel, and T. Tuschl, “Identification of novel genes coding for small expressed RNAs,” Science. 2001. vol. 294, no. 5543. pp. 853–858.

Layden, B. T., Durai, V. & Lowe, Jr., W. L. G-Protein-Coupled Receptors, Pancreatic Islets, and Diabetes. Nature Education. 2010. 3(9):13.

Liang Y, Cushman SM, Whitesell RR, Matschinsky FM. GLUT1 is adequate for glucose uptake in GLUT2-deficient insulin-releasing beta-cells. Horm Metab Res. 1997; 29(6):255-60.

Lovejoy J and M DiGirolamo. "Habitual dietary intake and insulin sensitivity in lean and obese adults". American Journal of Clinical Nutrition.1992. 55 (6): 1174–9.

Louise Fritsche, Cora Weigert, Hans-Ulrich Häring and Rainer Lehmann. How Insulin Receptor Substrate Proteins Regulate the Metabolic Capacity of the Liver - Implications for Health and Disease.Current Medicinal Chemistry. 2008. 15, 1316-1329.

Malécot CO, Bito V, Argibay JA. Ruthenium red as an effective blocker of calcium and sodium currents in guinea-pig isolated ventricular heart cells. Br J Pharmacol. 1998 ;124(3):465-72.

Mallilankaraman K., Doonan P., Cardenas C., Chandramoorthy H.C., Muller M., Miller R., Hoffman N.E., Gandhirajan R.K., Molgo J., Birnbaum M.J., Rothberg B.S., Mak D.O., Foskett J.K., Madesh M. MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. Cell 2012,151:630-644.

Masako Doi, Ippei Yamaoka, Mitsuo Nakayama, Kunio Sugahara and Fumiaki Yoshizawa. Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. Am J Physiol Endocrinol Metab 2007. 292:E1683-E1693.

McAuley KA, Williams SM, Mann JI, Walker RJ, Ledwis-Barned NJ, Temple LA, Duncan AS: Diagnosing insulin resistance in the general population. Diabetes Care. 2001. 24:460–464.

McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanston-Flatt SK, Flatt PR., 1996. Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. Diabetes. 45(8):1132-40.

Malik, VS; Popkin, BM, Bray, GA, Després, JP, Hu, FB. "Sugar Sweetened Beverages, Obesity, Type 2 Diabetes and Cardiovascular Disease risk". Circulation. 2010. 121 (11): 1356–64.

Mard-Soltani M, Dayer MR, Ataie G, Moazedi AA, Dayer MS, Alavi SMR. "Coagulation Factors Evaluation in NIDDM Patients". American Journal of Biochemistry and Molecular Biology. 2011. 1 (3): 244–254.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985 Jul;28(7):412-9.

Matschinsky FM. "Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm". Diabetes.1996. 45 (2): 223–41.

Mascia F, Denning M, Kopan R, Yuspa SH. The black box illuminated: signals and signaling. J Invest Dermatol. 2012;132(3 Pt 2):811-9.

Martin DB, Vagelos PR."The Mechanism of Tricarboxylic Acid Cycle Regulation of Fatty Acid Synthesis". J Biol Chem. 1962. 237: 1787–92.

Mayo Clinic. Type 2 diabetes: Risk factors.. Retrieved 21 December 2011.

McGarry, Banting lecture. Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes. 2001. 51: 7-18.

Miao, Y., Ottenbros, S.A., Laverman, G.D., Brenner, B.M., Cooper, M.E., Parving, H.H., Grobbee, D.E., Shahinfar, S., de Zeeuw, D., Lambers Heerspink, H.J., 2011. Effect of a reduction in uric acid on renal outcomes during losartan treatment: a post hoc analysis of the reduction endpoints in non-insulin-dependent diabetes mellitus with the angiotensin II antagonist losartan trial. Hypertension 58, 2–7.

Michael D.Williams, Geraldine M.Mitchell. MicroRNAs in Insulin Resistance and Obesity.Exp Diabetes Res. 2012. 2012:484696.

Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, Spiegelman BM. Restoration of insulinsensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. Proc Natl Acad Sci USA. 2001. 98: 3820-3825.

Miller, P.J., Steele, C., Kerr, K. 2004. Food supplements containing 4-hydroxyisoleucine and creatine. US Patent 2003/0224062 A1.

Moller D E. New drug targets for type 2 diabetes and the metabolic syndrome; Nature (London) .2001. 414 821–827.

Montero M, Lobatón CD, Hernández-Sanmiguel E, Santodomingo J, Vay L, Moreno A, Alvarez J. Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. Biochem J. 2004 15;384(Pt 1):19-24.

Motyl, K., McCabe, L.R., 2009. Streptozotocin, type I diabetes severity and bone. Biol Proced. Online 11, 296–315.

Moon B, Kwan JJ, Duddy N, Sweeney G, Begum N. Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. Am J Physiol Endocrinol Metab. 2003. 285: E106-E115.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency is associated with severe earlyonset obesity in humans. Nature. 1997. 387: 903-908.

Morcos SR, Elhawary Z, Gabrial GN.Protein-rich food mixtures for feeding the young in egipt. 1. Formulation. Z. Ernahrungswiss. 1981. 20: 275 – 82.

Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest. 2005. 115: 3587-3593.

Mudaliar, S. "Serum glucose control in diabetic patients with cardiovascular disease: should we be less aggressive?".Current atherosclerosis reports. 2009. 11 (5): 384–90.

Mueckler M. Facilitative glucose transporters. European Journal of Biochemistry.1994. 219, 713–725.

Musi N, Hirshman MF, Nygren J, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes.Diabetes. 2002. 51(7):2074–81.

Nakae, J., Biggs, W.H., 3rd, Kitamura, T., Cavenee, W.K., Wright, C.V., Arden, K.C., and Accili, D. Regulation of insulin action and pancreatic b-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat. Genet. 2002. 32, 245–253.

Najjar S. "Insulin Action: Molecular Basis of Diabetes".Encyclopedia of Life Sciences (John Wiley & Sons). 2001. ISBN 0470016175.

Narender, T., Puri, A., Shweta, Khaliq, T., Saxena, R., Bhatia, G., Chandra, R., 4-Hydroxyisoleucine an unusual amino acid as antidyslipidemic and antihyper-glycemic agent. Bioorg. Med. Chem. Lett.2006; 16, 293–296.

Nelson, David L.; Cox, Michael M. Lehninger, Principles of Biochemistry. W. H. Freeman and Company. 2008 .ISBN 978-0-7167-7108-1.

Nishimura J, Masaki T, Arakawa M, Seike M, Yoshimatsu H.Isoleucine prevents the accumulation of tissue triglycerides and upregulates the expression of PPARalpha and uncoupling protein in diet-induced obese mice. J Nutr. 2010. 140(3):496-500.

Norris AW, Chen L, Fisher SJ, Szanto I, Ristow M, Jozsi AC, Hirshman MF, Rosen ED, Goodyear LJ, Gonzalez FJ, Spiegelman BM, Kahn CR. Muscle-specific PPAR-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. J Clin Invest. 2003. 112: 608–618.

Nugent C, Prins JB, Whitehead JP, Savage D, Wentworth JM, Chatterjee VK, O'Rahilly S. Potentiation of glucose uptake in 3T3-L1 adipocytes by PPAR- agonists is maintained in cells expressing a PPAR- dominantnegative mutant: evidence for selectivity in the downstream responses to PPAR- activation. Mol Endocrinol. 2001. 15: 1729–1738.

Nunoi K, Yasuda K, Tanaka H, Kubota A, Okamoto Y, Adachi T, Shihara N, Uno M, Xu LM, Kagimoto S, Seino Y, Yamada Y, Tsuda K. Wortmannin, a PI3-kinase inhibitor: promoting effect on insulin secretion from pancreatic beta cells through a cAMP – dependent pathway. Biochem Biophys Res Commun. 2000; 270(3):798-805.

Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem. 1971;246(1):174-81.

Ohtsubo K, Chen MZ, Olefsky JM, Marth JD. Pathway to diabetes through attenuation of pancreatic beta cell glycosylation and glucose transport. Nat Med; 2011.17: 1067–U1162.

Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes: studies with a selective inhibitor wortmannin. J. Biol. Chem. 1994; 269:3568-73.

Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, Winder WW. "Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle". J. Appl. Physiol. 2002. 92 (6): 2475–82.

Pravenec M, Kazdová L, Landa V, Zidek V, Mlejnek P, Jansa P, Wang J, Qi N, Kurtz TW. Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. J Biol Chem. 2003. 278: 45209-45215.

Peraldi P, Xu M, Spiegelman BM. Thiazolidinediones block tumor necrosis factor-α -induced inhibition of insulin signaling. J Clin Invest. 1997. 100: 1863–1869.

Picard F, Auwerx J. PPAR(gamma) and glucose homeostasis. Annu Rev Nutr. 2002. 22: 167–197.

Ploug, T. et al. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. J. Cell Biol. 1998. 142, 1429–1446.

Prentki, M., and B. E. Corkey. Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? Diabetes. 1996. 45: 273–283.

Quintanilla RA, Porras OH, Castro J, Barros LF. Cytosolic [Ca(2+)] modulates basal GLUT1 activity and plays a permissive role in its activation by metabolic stress and insulin in rat epithelial cells. Cell Calcium. 2000;28(2):97-106.

Rabuazzo AM, Buscema M, Vinci C, Caltabiano V, Anello M, Vigneri R, Purrello F. Inhibition of the high-affinity glucose transporter GLUT 1 affects the sensitivity to glucose in a hamster-derived pancreatic beta cell line (HIT). Diabetologia. 1993;36(11):1204-7.

Rayner DV, Thomas ME & Trayhurn P. Glucose transporters (GLUTs 1–4) and their mRNAs in regions of the rat brain: insulin-sensitive transporter expression in the cerebellum. Canadian Journal of Physiology and Pharmacology. 1994. 72, 476–479.

Reddy S, Wu D, Elliott RB . Low dose streptozotocin causes diabetes in severe combined immunodeficient (SCID) mice without immune cell infiltration of the pancreatic islets. Autoimmunity 1995. 20: 83–92.

Rees, D.A., Alcolado, J.C. Animal models of diabetes mellitus. Diabet. Med. 2005; 22, 359–370.

Regazzi R. Diabetes mellitus reveals its micro-signature. Circ Res. 2010. 107: 686–688.

Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature. 1998. 391: 79–82.

Ripsin CM, Kang H, Urban RJ. "Management of blood glucose in type 2 diabetes mellitus". Am Fam Physician. 2009. 79 (1): 29–36.

Robles-Cervantes JA, M. G. Ramos-Zavala, M. González-Ortiz, E. Martínez-Abundis, C. Valencia-Sandoval, A. Torres-Chávez, C. Espinel-Bermúdez, N. J. Santiago-Hernández, and S. O. Hernández-González . Relationship between Serum Concentration of Uric Acid and Insulin Secretion among Adults with Type 2 Diabetes Mellitus. International Journal of Endocrinology. 2011, Article ID 107904.

Rodrigo M Lago, Premranjan P Singh and Richard W Nesto. “Diabetes and hypertension”.Nature Clinical Practice Endocrinology & Metabolism. 2007. 3, 667.

Rolland-Fulcrand V, Rolland M, Roumestant ML, Martinez J. Chemoenzymatic synthesis of Enantiomerically pure (2S,3R,4S)-4 -hydroxyisoleucine, an insulinotropic amino acid isolated from fenugreek seeds. Eur. J. Org Chem. 2004. 873 – 7.

Rosolowsky, E.T., Ficociello, L.H., Maselli, N.J., Niewczas, M.A., Binns, A.L., Roshan, B., Warram, J.H., Krolewski, A.S. High-normal serum uric acid is associated with impaired glomerular filtration rate in nonproteinuric patients with type 1 diabetes. Clin. J. Am. Soc. Nephrol.2008. 3, 706–713.

Ruderman, N., and M. Prentki. AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. Nat. Rev. Drug Discov. 2004. 3: 340–351.

Ruan H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-. Cytokine Growth Factor Rev. 2003. 14: 447–455.

Ruan H, Pownall HJ, Lodish HF. Troglitazone antagonizes tumor necrosis factor-α -induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-KB. J Biol Chem. 2003. 278: 28181–28192.

Ruby BC, Gaskill SE, Slivka D, Harger SG. The addition of fenugreek extract (Trigonella foenum-graecum) to glucose feeding increases muscle glycogen resynthesis after exercise. Amino Acids. 2005.28(1):71-6.

Samir Bhattacharya, Debleena Dey , Sib Sankar Roy. Molecular mechanism of insulin resistance. J. Biosci. 2007. 32(2) 405–413.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005. 307, 1098–1101.

Sauvaire Y, Petit P, Broca C, Manteghetti M, Baissac Y, Fernandez-Alvarez J, Gross R, Roye M, Leconte A, Gomis R, Ribes G. 4-hydroxyisileucine: a novel amino acid potentiator of insulin secretion. Diabetes. 1998. 47 (2): 206 – 10.

Savage, D. B., C. S. Choi, V. T. Samuel, Z. X. Liu, D. Zhang, A. Wang, X. M. Zhang, G. W. Cline, X. X. Yu, J. G. Geisler, et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. J. Clin. Invest. 2006. 116: 817–824.

Schinner, S.; Scherbaum, W. A.; Bornstein, S. R.; Barthel, A. "Molecular mechanisms of insulin resistance". Diabetic Medicine. 2005. 22 (6): 674–682.

Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. Insulin receptors and insulin action in the brain: review and clinical implications. Neurosci Biobehav Rev. 2000;24(8):855-72.

Scott LJ, et al. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science. 2007;316:1341–1345.

Sener A, Somers G, Devis G, Malaisse WJ. The stimulus-secretion coupling of amino acid-induced insulin release. Biosynthetic and secretory responses of rat pancreatic islet to L-leucine and L-glutamine.Diabetologia. 1981. 21(2):135-42.

Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ. "The facilitative glucose transporter GLUT3: 20 years of distinction". Am. J. Physiol. Endocrinol. Metab. 2008. 295 (2): E242–53.

Sinagra D, A. M. Scarpitta, V. Bonaventura et al., “Serum uric acid and insulin secretion in diabetes mellitus,” European Review for Medical and Pharmacological Sciences, vol. 18, no. 4, pp. 173–177, 1996.

Singh AB, Tamarkar AK, Narender T, Srivastava AK. Antihyperglycaemic effect of an unusual amino acid (4-hydroxyisoleucine) in C57BL/KsJ-db/db mice. Nat Prod Res. 2010. 24(3):258-65.

Sharma, R. D.; Raghuram, T. C.; Rao, N. S. "Effect of fenugreek seeds on blood glucose and serum lipids in type I diabetes".European journal of clinical nutrition. 1990. 44(4): 301–306.

Sheshala R, Peh KK, Darwis Y (2009). Preparation, characterization, and in vivo evaluation of insulin-loaded PLA-PEG microspheres for controlled parenteral drug delivery. Drug Dev Ind Pharm 35:1364–1374.

Shepherd PR & Kahn BB. Glucose transporters and insulin action – implications for insulin resistance and diabetes mellitus. New England Journal of Medicine. 1999. 341, 248–257.

Slivka D, Cuddy J, Hailes W, Harger S, Ruby B. Glycogen resynthesis and exercise performance with the addition of fenugreek extract (4-hydroxyisoleucine) to post-exercise carbohydrate feeding. Amino Acids. 2008. 35(2):439-44.

Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA. Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. Am J Physiol Endocrinol Metab. 2002; 283(4):E784-93.

Stenbit, A. E. et al. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. Nature Med. 1997. 3, 1096–1101.

Stefano Del Prato, Piero Marchetti, and Riccardo C. Bonadonna, Phasic Insulin Release and Metabolic Regulation in Type 2 Diabetes, Diabetes. 2002. 51 Suppl 1:S109-16.

Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW. "Influence of dietary fat composition on development of insulin resistance in rats". Diabetes.1991. 40 (2): 280–289.

Stuart Wood I, Paul Trayhurn. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. British Journal of Nutrition. 2003. 89, 3–9.

Stumvoll M, Häring H. Resistin and adiponectin--of mice and men. Obes Res. 2002. 10: 1197-1199.

Sugimoto, K; Baba, M; Suda, T; Yasujima, M; Yagihashi, S. "Peripheral neuropathy and microangiopathy in rats with insulinoma: association with chronic hyperinsulinemia".Diabetes/metabolism research and reviews. 2003.19 (5): 392–400.

Sweeney, G. et al. An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. J. Biol. Chem. 1999. 274, 10071–10078.

Szallasi A, Blumberg PM. Vanilloid (Capsaicin) receptors and mechanisms. Pharmacol Rev. 1999 ;51(2):159-212.

Szkudelski, T., "The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas.". Physiol Res. 2001; 50 (6): 537–546.

Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006. 7:85–96.

Tal M, Liang Y, Najafi H, Lodish HF, Matschinsky FM. Expression and function of GLUT-1 and GLUT-2 glucose transporter isoforms in cells of cultured rat pancreatic islets. J Biol Chem. 1992 Aug 25;267(24):17241-7.

Tappy L, Acheson K, Normand S, Schneeberger D, Thélin A, Pachiaudi C, Riou JP, Jéquier E. Effects of infused amino acids on glucose production and utilization in healthy human subjects. Am J Physiol. 1992. 262(6 Pt 1):E826-33.

Tarasov AI, Semplici F, Ravier MA, Bellomo EA, Pullen TJ, Gilon P, Sekler I, Rizzuto R, Rutter GA.The mitochondrial Ca2+ uniporter MCU is essential for glucose-induced ATP increases in pancreatic β-cells. PLoS One. 2012 ;7(7):e39722.

Taubes G. "Diabetes. Paradoxical effects of tightly controlled blood sugar". Science. 2008. 322 (5900): 365–7.

Thorens B. "Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes". Am. J. Physiol. 1996. 270 (4 Pt 1): G541–53.

Thorens B, Mueckler M. Glucose transporters in the 21st Century. American Journal of Physiology - Endocrinology and Metabolism 2010;298(2):E141-E145.

Thorburn AW. "Fructose-induced in vivo insulin resistance and elevated plasma triglyceride levels in rats". American Journal of Clinical Nutrition. 1989.

Tong L. "Acetyl-coenzyme A carboxylase: crucial metabolic enzyme and attractive target for drug discovery". Cell. Mol. Life Sci. 2005. 62 (16): 1784–803.

Tranzer A and P. F. Stadler, “Evolution of microRNAs,” Methods in Molecular Biology, vol. 342, pp. 335–350, 2006.

Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997. 389: 610-614.

Uysal KT, Wiesbrock SM, Hotamisligil GS. Functional analysis of tumor necrosis factor (TNF) receptors in TNF--mediated insulin resistance in genetic obesity. Endocrinology. 1998. 139: 4832–4838.

Vannucci SJ, Maher F, Simpson IA. "Glucose transporter proteins in brain: delivery of glucose to neurons and glia". Glia. 1997. 21 (1): 2–21.

Vay L, Hernández-Sanmiguel E, Santo-Domingo J, Lobatón CD, Moreno A, Montero M, Alvarez J. Modulation of Ca(2+) release and Ca(2+) oscillations in HeLa cells and fibroblasts by mitochondrial Ca(2+) uniporter stimulation. J Physiol. 2007;580 (Pt 1):39-49.

Wang MY, Zhou YT, Newgard CB, Unger RH. "A novel leptin receptor isoform in rat". FEBS Lett. August 1996 392 (2): 87–90.

Wang Z, Gleichmann H.. "GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice". Diabetes.1998; 47 (1): 50–6.

Wieman H.L., J.A. Wofford, and J.C. Rathmell, Mol Biol Cell. Cytokine stimulation promotes glucose uptake via PI3K/Akt regulation of Glut1 activity and trafficking, 2007.Mol Biol Cell. 2007;18(4):1437-46.

Williams textbook of endocrinology.(12th ed.). Philadelphia: Elsevier/Saunders. pp. 1371–1435.ISBN 978-1-4377-0324-5.

Witters, L. A., T. D. Watts, D. L. Daniels, and J. L. Evans. Insulin stimulates the dephosphorylation and activation of acetyl CoA carboxylase. Proc. Natl. Acad. Sci. USA. 1988. 85: 5473– 5477.

World Health Organisation."Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications" (PDF). 1999.

Wright EM. Renal Na+ -glucose cotransporters. American Journal of Physiology. 2001. 280, F10–F18.

Xianjun Fang, Shuang Xing Yu, Yiling Lu, Robert C. Bast, Jr. James R. Woodgett, and Gordon B. Mills. "Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A". Proceedings of the National Academy of Sciences of the United States of America . 2000. 97 (22): 11960–11965.

Xu G, Chen J, Jing G, Shalev A. Preventing β-cell loss and diabetes with calcium channel blockers. Diabetes. 2012;61(4):848-56.

Xu L, Tripathy A, Pasek DA, Meissner G. Ruthenium red modifies the cardiac and skeletal muscle Ca(2+) release channels (ryanodine receptors) by multiple mechanisms. J Biol Chem. 1999; 12;274(46):32680-91.

Ying SY, D. C. Chang, J. D. Miller, and S. L. Lin, “The microRNA: overview of the RNA gene that modulates gene functions,” Methods in Molecular Biology. 2006. vol. 342, pp. 1–18.

Yoshikawa M, Murakami T, Komatsu H, Murakami N, Yamahara J, Matsuda H. Medicinal foodstuffs. IV. Fenugreek seed. (1): Structures of trigoneosides Ia, Ib, IIa, IIb and IIIb, new furostanol saponins from the seeds of Indian Trigonella foenum-graecum L. Chem. Pharm. Bull (Tokyo). 1997. 45: 81 – 7.

Youl E, Bardy G, Magous R, Cros G, Sejalon F, Virsolvy A, Richard S, Quignard JF, Gross R, Petit P, Bataille D, Oiry C., 2010. Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative damage via the ERK1/2 pathway.Br J Pharmacol. 161(4):799-814.

Youn JH, Gulve EA, Holloszy JO. Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. Am J Physiol. 1991;260(3 Pt 1):C555-61.

Yu C, Chen Y, Cline G W, Zhang D, Zong H, Wang Y, Bergeron R, Kim J K, Cushman S W, Cooney G J, Atcheson B, White M F, Kraegen E W and Shulman GI. Mechanisms by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3 kinase activity in muscle; J. Biol. Chem. 2002. 27 50230–50236.

Yu SP, Choi DW. "Na+/Ca2+ exchange currents in cortical neurons: concomitant forward and reverse operation and effect of glutamate". European Journal of Neuroscience 1997; 9(6): 1273–81.

Zaid H, Antonescu CN, Randhawa VK, Klip A. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem J. 2008. 413:201–215.

Zanatta L, Rosso A, Folador P, Figueiredo MS, Pizzolatti MG, Leite LD, Silva FR. Insulinomimetic effect of kaempferol 3-neohesperidoside on the rat soleus muscle. J Nat Prod. 2008; 71(4):532-5.

Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling revealsloss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010. 107:810–817.

Zawalich WS, Tesz GJ, Zawalich KC. Inhibitors of phosphatidylinositol 3-kinase amplify insulin release from islets of lean but not obese mice. J Endocrinol. 2002;174(2):247-58.

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes. 2007. 56(6):1647-54.

Zhang Y, Liu D, Chen X, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. Mol Cell. 2010. 39:133–144.

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms.Diabetes. 2007; 56(6):1647-54.

Appendix I

Animal study publication

(Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (Trigonella foenum graecum) in streptozotocin-induced type I diabetic rats)

G Model
PHYMED-51174; No. of Pages 4
ARTICLE IN PRESS

Phytomedicine xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats

Mohammed R. Haeri[a], Hamidreza Khalatbari Limaki[b,*], Christopher J. Branford White[b], Kenneth N. White[b]

[a] *Qom University of Medical Science, Qom, Iran*
[b] *Institute for Health Research and Policy, Faculty of Life Sciences, 166-220 Holloway Road, London Metropolitan University, London N7 8DB, UK*

ARTICLE INFO

Keywords:
Fenugreek
Trigonella foenum graecum
4-Hydroxyisoleucine
Diabetes
Streptozotocin
Rat

ABSTRACT

The seeds of fenugreek, *Trigonella foenum graecum*, commonly used as a spice in Middle Eastern countries and widely used in south Asia and Europe, are known to have anti-diabetic properties. They contain an unusual amino acid (2S, 3R, 4S) 4-hydroxyisoleucine (4HO-Ile), so far found only in fenugreek, which has anti-diabetic properties of enhancing insulin secretion under hyperglycaemic conditions, and increasing insulin sensitivity. Here we describe for the first time the anti-diabetic activity of 4HO-Ile in a model of type I diabetes, streptozotocin-treated rats, where levels of insulin are much reduced, by 65%, compared to normal animals. Treatment of diabetic rats with daily doses of 4HO-Ile at 50 mg/kg/day for four weeks could reduce plasma glucose in the diabetic group. Moreover the high levels of lipids (cholesterol, HDL, LDL and triglycerides) and uric acid in the diabetic rats, could be restored to levels found in non-diabetic controls by the treatment with 4HO-Ile. These results demonstrate that 4HO-Ile has significant anti-diabetic activities that are independent of insulin and suggest the potential of 4HO-Ile as an adjunct to diabetes treatment and for type 1 as well as type 2 diabetes.

Introduction

Fenugreek, *Trigonella foenum graecum*, has long been used in traditional treatments of diabetes (Al-Habori and Raman 1998) and is widely cultivated in India, the Mediterranean and China. Its long history has prompted a number of small clinical trials to assess the efficacy and safety of fenugreek seed powder in the treatment of type 1 and type 2 diabetes (reviewed in Basch et al. 2003), with variable but promising results. Analysis of active, soluble components of fenugreek seed revealed an unusual amino acid, 4-hydroxyisoleucine (4HO-Ile), that had anti-diabetic potential through its ability to stimulate secretion of insulin from rat pancreatic islet cells (Sauvaire et al. 1998). 4HO-Ile comprises 80% of the free amino acid content, and 0.6% (w/w), of defatted fenugreek seeds (Sauvaire et al. 1984, 1998). The molecule has three chiral centres and 90% is found in the form with stereochemistry (2S, 3R, 4S) and 10% with stereochemistry (2R, 3R, 4S) (Sauvaire et al. 1984; Alcock et al. 1989). A comparison of the activity of the (2S, 3R, 4S) stereoisomer with the (2R, 3R, 4S) isomer and another 10 congeners revealed the (2S, 3R, 4S) isomer to be the most potent form tried, when measuring insulin release from isolated rat pancreatic islets (Broca et al. 2000). So far 4HO-Ile has only been reported to be found in fenugreek seed, and all subsequent discussion is limited to the active 2S, 3R, 4S stereoisomer that is the predominant form in this plant species.

In the rat islet insulin secretion assay treatment with 4HO-Ile was found to be 15 to 25 times more potent than the branched chain amino acids L-leucine and L-isoleucine (Broca et al. 2000). Under these conditions the stimulated release of insulin from isolated human islets and perfused rat pancreas was also noted (Sauvaire et al. 1998; Broca et al. 2000). Furthermore 4HO-Ile could also improve glucose control in oral glucose tolerance tests in normal rats and dogs which was attributed to the increase in circulating insulin after 4HO-Ile treatment (Broca et al. 1999). Other studies on normal, type 2 diabetic, or obese Zucker *fa/fa* rats indicated that 4HO-Ile could have another anti-diabetic mode of action, by improving insulin sensitivity (Broca et al. 1999, 2004). This mechanism could explain the improvement in glucose clearance and lipidemia in dyslipidemic hamsters (Narender et al. 2006), fructose-fed rats (Haeri et al. 2009), and in *db/db* mice (Singh et al. 2010), after treatment with 4HO-Ile.

* Corresponding author. Tel.: +44 02071332245; fax: +44 02071334149.
E-mail address: h.khalatbarilimaki@londonmet.ac.uk (H.K. Limaki).

0944-7113/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2012.01.004

Please cite this article in press as: Haeri, M.R., et al., Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats. Phytomedicine (2012), doi:10.1016/j.phymed.2012.01.004

To date there have been no studies examining the effect of 4HO-Ile on models of type I diabetes since all the studies cited above are on normal animals or tissue, or animal models of type 2 diabetes. To investigate the mode of action of 4HO-Ile further we used a rat model of type 1 diabetes induced by streptozotocin in which levels of insulin are much reduced, allowing an examination of the hypoglycaemic and lipid modulating properties of 4HO-Ile independent of insulin sensitization. We found that 4HO-Ile had significant hypoglycaemic activity in this model and could also induce a significant reduction of serum triglyceride, LDL and uric acid close to levels found in non-diabetic control rats. The data show that 4-OH-Ile can ameliorate metabolic syndrome conditions independently of insulin and strengthen the case for assessment of 4HO-Ile in clinical trials.

Materials and methods

Animals

Male Wistar rats were purchased from the Pharmacological Research Center of Tehran University of Medical Sciences. Eight week-old rats weighing between 220 and 250 g were used at the start of each treatment protocol. Rats were housed in separate cages in an animal room kept at constant temperature (25 °C) with a 12 h light–dark cycle. A standard rat chow pellet and water were provided *ad libitum* throughout the experimental period. The animals were maintained in accordance with the Animal Ethics Committee of the University of Medical Science, Qom, Iran, following.

Induction of diabetes

Rats were divided into three groups each of six, normal controls (NC), diabetic controls (D) and diabetic rats treated with 4HO-Ile (D4H). Rats were rendered type 1 diabetic by intraperitoneal injection of streptozotocin (60 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) for five days consecutively (Motyl and McCabe 2009). After one week blood glucose concentration was measured with a Glucometer on a drop of blood from the tail. Rats were considered to be diabetic if blood glucose levels were greater than 300 mg/dl. The treatment group of diabetic rats were intubated daily with a solution of 4HO-Ile at a dose equivalent to 50 mg/kg/day for 4 weeks (Haeri et al. 2009). Normal control rats and diabetic control rats were intubated with saline alone.

Serum lipid profile, insulin, glucose and uric acid

At the end of the experiment rats were anaesthetized with ether and blood samples were collected through cardiac puncture in heparinized tubes and immediately centrifuged at 1000 × *g* for 15 min. Plasma was removed and stored at −20 °C. Glucose, triglycerides, cholesterol and LDL concentration were measured on an autoanalyser (Biosystem, Spain). Plasma insulin was measured by ELISA (DRG International, NJ, USA). Plasma uric acid was measured using a timed end point method on a Beckman Coulter Synchron LX20 (Rosolowsky et al. 2008).

Statistical analysis

Data were analysed using SPAW version 18.0 and are expressed as mean ± SD. ANOVA with Tukey and Bonferroni *post hoc* tests were used to determine significance of differences between groups. $p < 0.05$ was considered to be statistically significant.

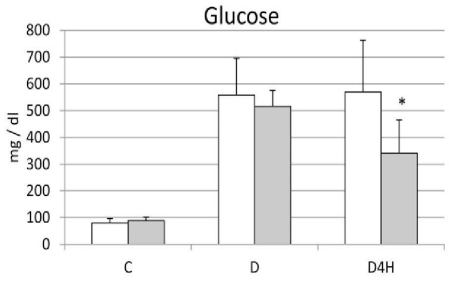


Fig. 1. Plasma glucose at the beginning and end of the 4HO-Ile treatment. Mean and SD plasma glucose of five or six rats per group. Control rats (C). Diabetic (D) and treatment (D4H) groups were made diabetic by repeated doses of STZ. Treatment with 4HO-Ile started one week after the STZ treatment and continued for four weeks. Glucose was measured at the start of the 4HO-Ile treatment (□) and at the end of four weeks treatment (■). 4HO-Ile induced a significant (*$p < 0.05$) decrease in plasma glucose within the treatment group after four weeks.

Results and discussion

Repeated dose STZ model of diabetes

STZ has been used for several decades to induce a type 1 diabetic state in rodents, usually administered as a single intravenous (i.v.) dose (Davidson and Kaplan 1977; Rees and Alcolado 2005). We compared three protocols for administering STZ through the intraperitoneal (i.p.) route as an alternative, to avoid difficulties and losses though i.v. injection. Single injections i.p. of STZ at doses of 60 mg/kg or 150 mg/kg were compared with the procedure of Motyl and McCabe (2009) of single injections i.p. of STZ at 60 mg/kg for five consecutive days. Insulin and glucose levels were monitored at two weeks after treatment and compared with control rats which received vehicle (0.1 M citrate pH 4.5) only. Single doses of STZ induced increases in glucose levels, but not to levels greater than 300 mg/dl, the threshold for diabetic phenotype (data not shown). Repeated doses of STZ induced glucose levels of 520 ± 13 mg/dl compared with 79 ± 3 mg/dl for controls ($n = 2$), and insulin levels were below the limit of detection (0.2 μg/l) compared with 1.79 μg/l in controls. On this basis the repeated doses of STZ were used to induce a type 1 diabetic state in rats.

After dividing the rats into three treatment groups the animals were treated with STZ or vehicle for five days, left for seven days and then intubated with 4HO-Ile (50 mg/kg/day) or saline vehicle daily for a further four weeks. Rats treated with STZ had a markedly elevated plasma glucose compared with controls one week after STZ administration (Fig. 1), that was sustained for a further four weeks (Fig. 1, groups D and D4H). Insulin levels in the diabetic groups were significantly lower than normal controls with 60–70% decreased levels (Fig. 2). The diabetic rats had a markedly higher intake of food and water compared with controls (Fig. 3), but the hyperphagia did not cause any increase in body weight in the diabetic rats compared with controls. Body weights (mean ± SD) at the end of the study were 289 ± 31 g (C), 269 ± 15 g (D) and 267 ± 5 (D4H). There was no significant difference between groups. The lipid profile of the diabetic rats was also consistent with a diabetic phenotype, with significantly elevated TG, cholesterol, HDL and LDL compared with the control group (Fig. 4). The changes in glucose and lipids coupled with the marked decrease in insulin indicate a type 1 diabetic phenotype is induced by the repeated i.p. doses of STZ.

Treatment of diabetic rats with 4HO-Ile

Generally the diabetic animals treated with 4HO-Ile had an improved appearance and it was noticeable that the heavy ocular

Please cite this article in press as: Haeri, M.R., et al., Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats. Phytomedicine (2012), doi:10.1016/j.phymed.2012.01.004

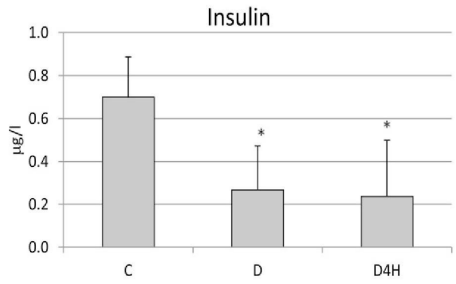


Fig. 2. Plasma insulin after treatment with 4HO-Ile. Data are mean + SD of five or six rats per group at the end of the treatment period. Insulin levels were significantly lower in diabetic groups [D, D4H] compared with control [C] (*$p < 0.05$).

vascularization induced by STZ was being reversed as the treatment with 4HO-Ile progressed (Haeri et al. unpublished data). Treatment of diabetic rats with 4HO-Ile induced a reduction in blood glucose from 500 mg/dl to 330 mg/dl after four weeks (Fig. 1). More strikingly the 4HO-Ile treatment also resulted in significant decreases in all lipid markers compared with untreated diabetic rats. The level of TG, LDL and HDL in the treated diabetic animals were not significantly different to those of control, non-diabetic rats and total cholesterol was reduced to near control levels (Fig. 4) indicating that treatment with 4HO-Ile had restored the diabetic lipid profile to an almost normal one.

In type 1 diabetes levels of HDL typically decrease but in our model the STZ-treated animals had elevated HDL. The reason for this is unclear but similar behaviour in STZ-rats has been found by others (Islam 2011). In our study the treatment with 4HO-Ile could reverse the changes in HDL induced by STZ and restore levels close to that of control non-diabetic animals (Fig. 4).

Similarly the increased hyperuricemia of the diabetic rats (Fig. 5) was restored to levels found in normal rat controls after treatment with 4HO-Ile. Elevated levels of uric acid are directly linked to damage to the kidney, which is one of the major causes of morbidity and mortality in diabetes (Rosolowsky et al. 2008). It is notable in this study that the reduction in hyperuricaemia caused by treatment

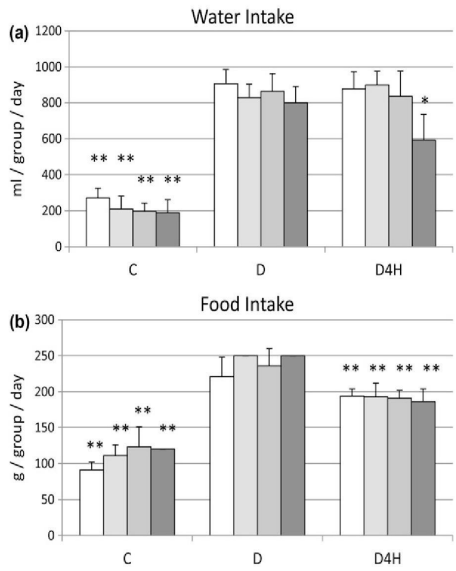


Fig. 3. Water and food intake over treatment period. Intake of water (a) and food (b) over the four week period of 4HO-Ile treatment. Data are mean + SD daily intake per animal over weekly periods for control (C), diabetic (D) and diabetic rats treated with 4HO-Ile (D4H). Data are for the four consecutive weeks of the period of treatment with 4HO-Ile: week 1 (□), 2 (□), 3 (□), 4 (□). Significant differences between means of group D compared with groups C or D4H, for a given week, are indicated where $p < 0.05$ (*) or $p < 0.01$ (**).

with 4HO-Ile is more pronounced than the reduction in hyperglycaemia, in that uric acid levels return to those of normal controls (Fig. 5 compares groups C and D4H) whereas levels of blood glucose remain three times higher than normal controls (Fig. 1 compare groups C and D4H). It remains to be seen whether or not the effects of 4HO-Ile on serum uric acid are mediated through improved, but not fully restored glucose levels, or by another mechanism. The latter idea is supported by increasing evidence from clinical data that

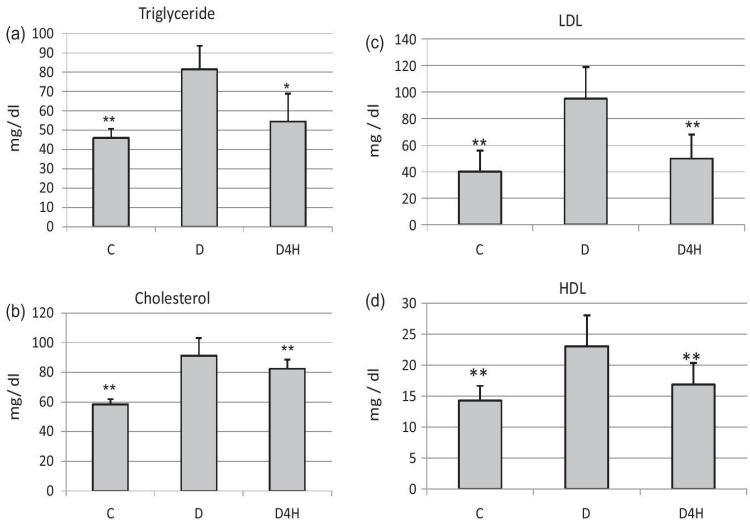


Fig. 4. Serum lipid profile of rats after four weeks treatment with 4HO-Ile. Triglyceride (a), cholesterol (b), LDL (c) and HDL (d) measured at the end of the treatment with 4HO-Ile are shown as the mean + SD of five or six rats per group. Levels were significantly lower in control (C) and treated diabetic (D4H) groups compared with untreated diabetic animals (D) (*$p < 0.05$, **$p < 0.01$).

Please cite this article in press as: Haeri, M.R., et al., Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats. Phytomedicine (2012), doi:10.1016/j.phymed.2012.01.004

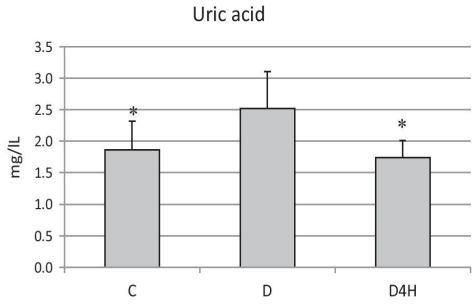


Fig. 5. Serum uric acid after four weeks treatment with 4HO-Ile. Data are mean + SD serum uric acid in rats at the end of the period of treatment with 4HO-Ile. Levels were significantly lower in control (C) and treated diabetic (D4H) groups compared with untreated diabetic animals (D) (*$p < 0.05$).

high levels of uric acid are an independent risk factor for kidney disease (Miao et al. 2011).

The effective correction by 4HO-Ile of the dyslipidaemia is similar to results of 4HO-Ile treatment of rats or mice with a type 2 diabetes phenotype, in which 4HO-Ile induced improvements in both lipid profile and hyperglycaemia (Haeri et al. 2009; Singh et al. 2010). Anti-dyslipidaemic activity of 4HO-Ile has also been demonstrated in a hamster model of dyslipidaemia (Narender et al. 2006).

It is notable that 4HO-Ile did not induce an increase in insulin levels in diabetic rats compared with untreated diabetic controls (Fig. 2). Both groups had insulin levels of about 0.3 μg/l, which were approximately 65% lower than the non-diabetic control group (Fig. 2). These data show that 4HO-Ile has no insulinotropic activity in this model of type 1 diabetes, despite the high levels of glucose in the diabetic rats. Thus, even though insulin levels were low, indicative of a small amount of pancreatic activity, they were unchanged by treatment with 4HO-Ile.

The improvement by treatment with 4HO-Ile in metabolic parameters of glucose, lipid profile and uric acid suggest that 4HO-Ile has a systemic effect on metabolically active tissues, including liver and muscle, that is independent of insulin. There have been no reports of any adverse effects of 4HO-Ile in any animal study indicating it is well tolerated and not toxic, and 4HO-Ile has been patented as one component of a food supplement (Miller et al. 2004). Others have suggested the use of 4HO-Ile for treatment of type 2 diabetes and metabolic syndrome (Jette et al. 2009) and the data from our study suggest that 4HO-Ile could also be used as a treatment for type1 diabetes, either alone or in combination with other protocols. Thus from our data it is postulated that 4HO-Ile could be a promising candidate for assessment in a clinical trial of diabetes treatment, but such a trial would require availability of very large amounts of pure 4HO-Ile that would be prohibitively costly. As an alternative to sourcing 4HO-Ile from fenugreek seeds, promising methodology is being developed that utilizes genetically modified bacteria to biosynthesize 4HO-Ile (Smirnov et al. 2010). The new method appears to produce good yields of the correct stereoisomer of 4HO-Ile in the bacteria culture medium, although purification of pure compound was not reported.

Conflict of interest

The authors declare no conflicting interests related to the study.

References

Alcock, N.W., Crout, D.H.G., Gregorio, M.V.M., Lee, E., Pike, G., Samuel, C.J., 1989. Stereochemistry of the 4-hydroxyisoleucine from *Trigonella foenum graecum*. Phytochemistry 28, 1835–1841.
Al-Habori, M., Raman, A., 1998. Antidiabetic and hypocholesterolaemic effects of fenugreek. Phythother. Res. 12, 233–242.
Basch, E., Ulbricht, C., Kuo, G., Szapary, P., Smith, M., 2003. Therapeutic applications of fenugreek. Altern. Rev. Med. 7, 20–27.
Broca, C., Breil, V., Cruciani-Guglielmacci, C., Manteghetti, M., Rouault, C., Derouet, M., 2004. Insulinotropic agent ID-1101 (4-hydroxyisoleucine) activates insulin signaling in rat. Am. J. Physiol. Endocrinol. Metab. 287, E463–E471.
Broca, C., Gross, R., Petit, P., Sauvaire, Y., Manteghetti, M., Tournier, M., Masiello, P., Gomis, R., Ribes, G., 1999. 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties. Am. J. Physiol. Endocrinol. Metab. 277, E617–E623.
Broca, C., Manteghetti, M., Gross, R., Baissac, Y., Jacob, M., Petit, P., Sauvaire, Y., Ribes, G., 2000. 4-Hydroxyisoleucine: effects of synthetic and natural analogues on insulin secretion. Eur. J. Pharmacol. 390, 339–345.
Davidson, M.B., Kaplan, S.A., 1977. Increased insulin binding by hepatic plasma membranes from diabetic rats. Normalization by insulin therapy. J. Clin. Invest. 59, 22–30.
Haeri, M.R., Rahbani, M., Ardekani, M.R.S., White, K.N., 2009. The effect of fenugreek 4-hydroxyisoleucine on liver function biomarkers and glucose in diabetic and fructose-fed rats. Phytother. Res. 23, 61–64.
Islam, M.S., 2011. Effects of the aqueous extract of white tea (Camellia sinensis) in a streptozotocin-induced diabetes model of rats. Phytomedicine 19, 25–31.
Jette, L., Harvey, L., Eugen, K., Levens, N., 2009. 4-Hydroxyisoleucine: a plant-derived treatment for metabolic syndrome. Curr. Opin. Investig. Drugs 10, 353–358.
Miller, P.J., Steele, C., Kerr, K. 2004. Food supplements containing 4-hydroxyisoleucine and creatine. US Patent 2003/0224062 A1.
Miao, Y., Ottenbros, S.A., Laverman, G.D., Brenner, B.M., Cooper, M.E., Parving, H.H., Grobbee, D.E., Shahinfar, S., de Zeeuw, D., Lambers Heerspink, H.J., 2011. Effect of a reduction in uric acid on renal outcomes during losartan treatment: a post hoc analysis of the reduction endpoints in non-insulin-dependent diabetes mellitus with the angiotensin II antagonist losartan trial. Hypertension 58, 2–7.
Motyl, K., McCabe, L.R., 2009. Streptozotocin, type I diabetes severity and bone. Biol Proced. Online 11, 296–315.
Narender, T., Puri, A., Shweta, Khaliq, T., Saxena, R., Bhatia, G., Chandra, R., 2006. 4-Hydroxyisoleucine an unusual amino acid as antidyslipidemic and antihyperglycemic agent. Bioorg. Med. Chem. Lett. 16, 293–296.
Rees, D.A., Alcolado, J.C., 2005. Animal models of diabetes mellitus. Diabet. Med. 22, 359–370.
Rosolowsky, E.T., Ficociello, L.H., Maselli, N.J., Niewczas, M.A., Binns, A.L., Roshan, B., Warram, J.H., Krolewski, A.S., 2008. High-normal serum uric acid is associated with impaired glomerular filtration rate in nonproteinuric patients with type 1 diabetes. Clin. J. Am. Soc. Nephrol. 3, 706–713.
Sauvaire, Y., Girardon, P., Baccou, J.C., Risterucci, A.M., 1984. Changes in growth, proteins and free amino acids of developing seed and pod of fenugreek. Phytochemistry 23, 479–486.
Sauvaire, Y., Petit, P., Broca, C., Manteghetti, M., Baissac, Y., Fernandez-Alvarez, J., Gross, R., Roye, M., Leconte, A., Gomis, R., Ribes, G., 1998. 4-Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion. Diabetes 47, 206–210.
Singh, A.B., Tamarkar, A.K., Narender, T., Srivastava, A.K., 2010. Antihyperglycaemic effect of an unusual amino acid (4-hydroxyisoleucine) in C57BL/KsJ-db/db mice. Nat. Prod. Res. 24, 258–265.
Smirnov, S.V., Kodera, T., Samsononva, N.N., Kotlyarova, V.A., Rushkevich, N.Y., Kivero, A.D., Sokolov, P.M., Hibi, M., Ogawa, J., Shimizu, S., 2010. Metabolic engineering of *Escherichia coli* to produce (2S, 3R, 4S)-4-hydroxyisoleucine. Appl. Microbiol. Biotechnol. 88, 719–726.

Please cite this article in press as: Haeri, M.R., et al., Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats. Phytomedicine (2012), doi:10.1016/j.phymed.2012.01.004

Appendix II

4-Hydroxyisoleucine certificate of analysis

Toronto Research Chemicals Inc.
Bringing you products for innovative research.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

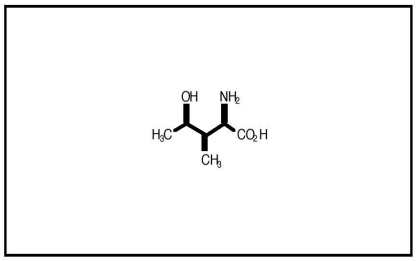
CAS Number:
781658-23-9

Catalogue Number:
H943570

Product:
4-Hydroxyisoleucine

Synonyms:
2-Amino-2,3,5-trideoxy-3-methyl-D-xylonic Acid, (2S,3R,4S)-4-Hydroxyisoleucine Major Isomer and (2R,3R,4S)-4-Hydroxyisoleucine Minor Isomer

Structure:



Molecular Formula:
$C_6H_{13}NO_3$

Molecular Weight:
147.13

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-AMC-88-3

Melting Point:
184-185°C

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Off-White Solid

Solubility
Water

Method for Determining Identity:
^{1}H NMR Spectroscopic and Mass Spectrometric Analysis

Stability
Hygroscopic

Purity:
98%

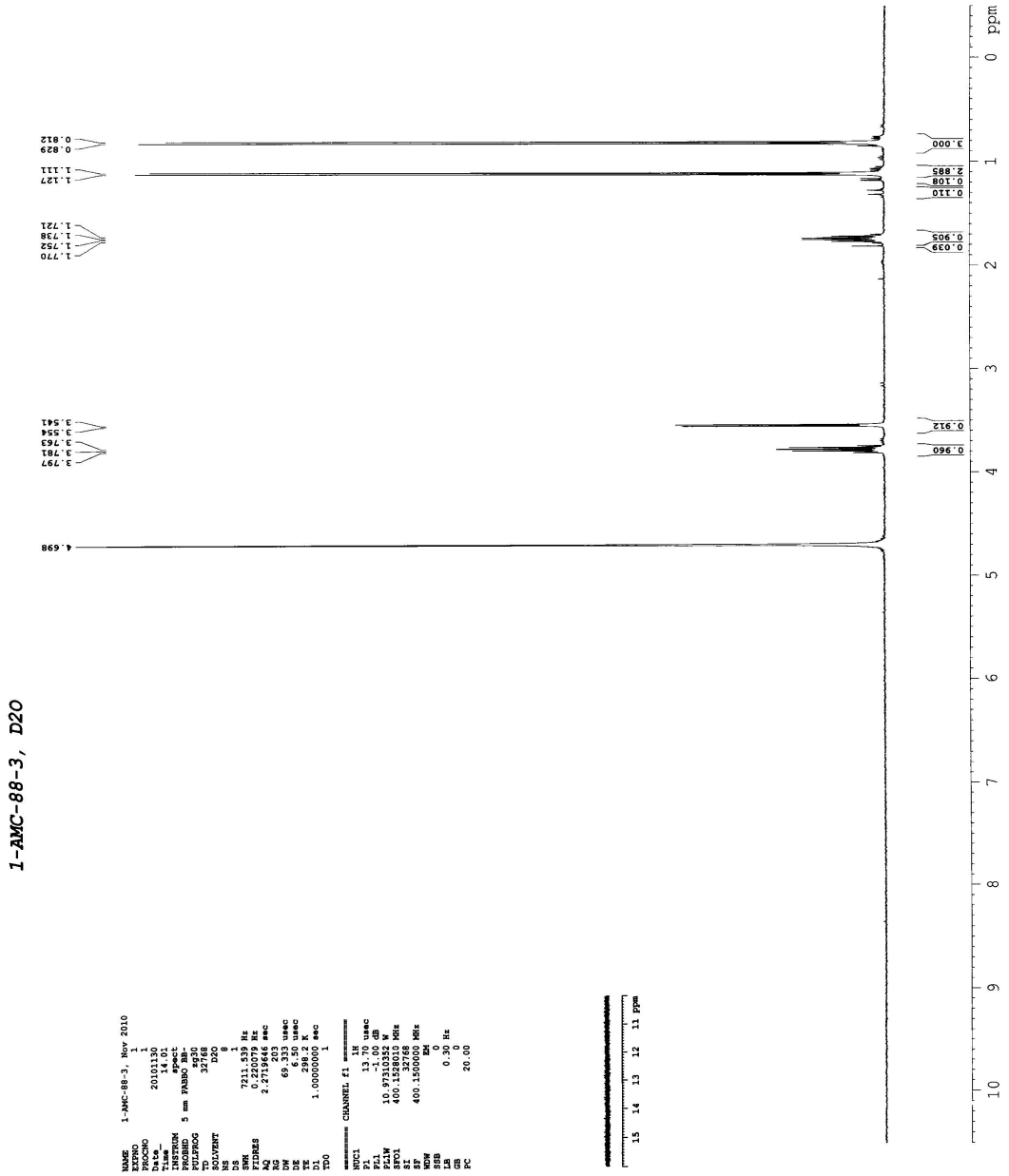
Long Term Storage Condition:
Hygroscopic, -20˚C Freezer, Under Inert Atmosphere

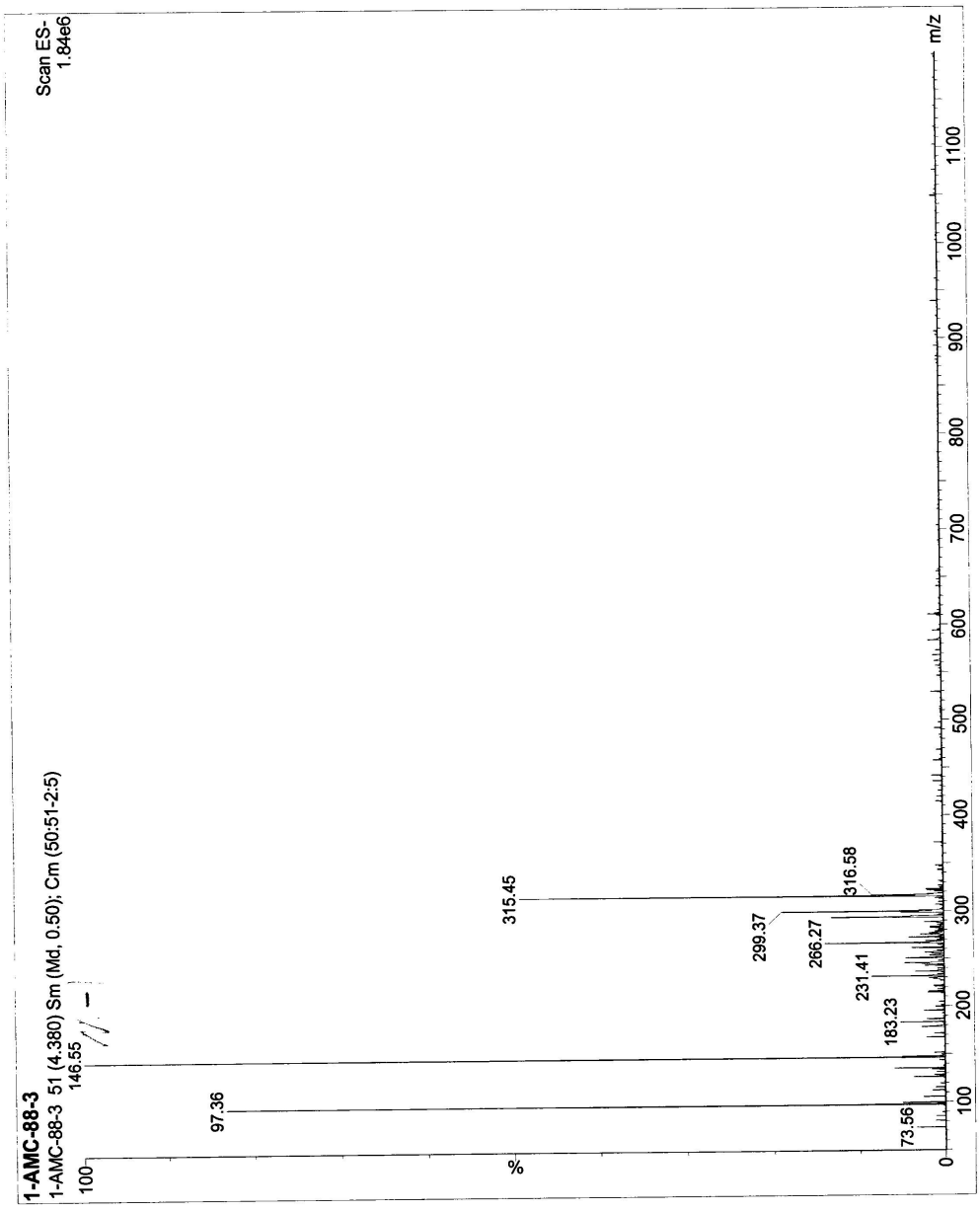
Additional Information:
TLC Conditions: C18; Methanol: Water: Ammonium Hydroxide; 9ml: 1ml: 10 drops; Visualized with Ninhydrin ; Single spot; Rf=0.70.
^{1}H NMR and mass spectra conform to structure.
Specific rotation: +24.7° (c=0.19, Water)

Philip Chan, Head of Quality Assurance

QC Test Date
July 20, 2012

Retest Date
November 30, 2013


1-AMC-88-3, D2O
4.698
3.797
3.781
3.763
3.554
3.541
1.770
1.752
1.738
1.721
1.127
1.111
0.829
0.812
3.000
2.885
0.912
0.960
0.903
0.039
0.110
0.108
NAME 1-AMC-88-3, Nov 2010
EXPNO 1
PROCNO 1
Date_ 20101130
Time 14.01
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 32768
SOLVENT D2O
NS 8
DS 8
SWH 7211.539 Hz
FIDRES 0.220079 Hz
AQ 2.2719646 sec
RG 203
DW 69.333 usec
DE 6.50 usec
TE 298.2 K
D1 1.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 13.70 usec
PL1 -1.00 dB
PL1W 10.97310352 W
SFO1 400.1528010 MHz
SI 32768
SF 400.1500000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 20.00
ppm


1-AMC-88-3
1-AMC-88-3 51 (4.380) Sm (Md, 0.50); Cm (50:51-2:5)
Scan ES-
1.84e6
100
%
0
146.55
97.36
73.56
315.45
316.58
299.37
266.27
231.41
183.23
100
200
300
400
500
600
700
800
900
1000
1100
m/z

1-AMC-88-3
1-AMC-88-3 44 (3.780) Sm (Md, 0.50); Cm (44:45-9:10)
Scan ES+
1.95e7
202.58
170.60
148.56
224.56
317.59
301.57
498.74
514.64
530.59
100
%
0
100
200
300
400
500
600
700
800
900
1000
1100
m/z

Appendix III

Glucometer accuracy measurements table

Sample (RPMI1640)	11mM Glucose	22mM Glucose
1	12.2	23
2	11.7	23.1
3	11.8	22.9
4	11.9	23.1
5	12.1	22.9
6	11.7	22.9
7	11.8	22.8
8	12.2	23.1
9	11.7	23.1
10	11.7	22.8
11	11.9	23.1
12	12.1	23.2
13	11.8	23.1
14	11.8	22.8
15	11.9	23.1
16	12	22.9
17	11.8	22.8
18	12.2	23.1
19	11.8	23
20	12.1	22.9
21	11.7	23
22	11.9	22.8
23	11.7	22.8
24	11.9	23
25	12.1	22.9
26	12.1	22.9
27	12.3	22.8
28	11.9	23
29	11.8	23.1
30	11.9	22.9
31	11.7	22.8
32	11.9	22.8
33	11.8	23.1
34	12.1	23
35	12.1	22.9
36	11.7	22.9
37	11.7	23
38	11.8	22.8
39	12.2	22.9
40	12	22.8
41	12.2	23.1
42	11.8	22.9
43	11.9	22.8
44	12.3	23
45	11.9	22.9
46	11.8	22.8
47	11.8	23
48	11.7	22.8
49	11.9	22.9
50	12.1	23.1
Mean	11.918	22.944
Uncertainty Intervals	0.5	0.3
Relative deviation	8.34%	4.29%