ANTI-DIABETIC EFFECTS OF
OLEANOLIC ACID AND MATRINE

A Thesis Presented in Total Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

Xiaoyi Zeng
BSc (Adv)(Hons)

Supervisors:
Professor Jiming Ye, PhD
Professor Aimin Xu, PhD

School of Health Science
College of Science, Engineering and Health
RMIT University
July 2014
DECLARATION

I, the candidate, Xiaoyi Zeng, certify that:

a) the work is that of the candidate alone, except where due acknowledgement has been made;
b) the work has not been submitted previously, in whole or in part, to qualify for any other academic award;
c) the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program;
d) any editorial work, paid or unpaid, carried out by a third party is acknowledged;
e) ethics procedures and guidelines have been followed.

Signature of the candidate

Xiaoyi Zeng
Molecular Pharmacology for Diabetes Group
School of Health Science
RMIT University
Bundoora Victoria 3083
Australia
PUBLICATIONS

Publications that have arisen as a direct result of this thesis:


Publications that have arisen in conjunction with this thesis:


Conference Abstracts that have arisen as a direct result of this thesis:


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Finally, I dedicate this thesis to my beloved grandma, my Liang Po, for her unconditional love and care for the past three decades.
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LIST OF ABBREVIATIONS

2DG: 2-deoxy-D-glucose
ACC: Acetyl-CoA carboxylase
ACS: Acyl-CoA synthetase
AGPAT: sn-1-Acyl-glycerol-3-phosphate acyltransferase
AMPK: AMP-activated protein kinase
Akt/PKB: Protein kinase B
ALT: Alanine transaminase
AMPK: AMP-activated protein kinase
AST: Aspartate transaminase
AUC: Area under the curve
BUN: Blood urea nitrogen
BW: Body weight
cDNA: Complementary DNA
CH: Chow diet
ChREBP: Carbohydrate-responsive element-binding protein
CPT: Carnitine palmitoyltransferase
Cr: Creatinine
CS: Citrate synthase
D&D: Drug discovery and development
DAG: Diacylglycerol
DGAT: Diacylglycerol acyltransferase
DHAP: Dihydroxyacetone phosphate
DHAPAT: Dihydroxyacetone-phosphate acyltransferase
DTT: DL-dithiothreitol
HDL: High-density lipoprotein
FA: Fatty acid
FAS: Fatty acid synthase
Feno: Fenofibrate
G6Pase: Glucose-6-phosphatase
GPAT: sn-1-Glycerol-3-phosphate acyltransferase
GPD1: Glycerol-3-phosphate dehydrogenase 1
GS: Glycogen synthase
GSK3: Glycogen synthase kinase 3
GTT: Glucose tolerance test
H&E: Hematoxylin and eosin
HF: High-fat diet
HFC: High-fat high-cholesterol diet
HSF1: Heat shock factor 1
HSP72: Heat shock protein 72
HSP90: Heat shock protein 90
HTS: High-throughput screening
iAUC: Incremental area under the curve
IDF: International Diabetes Federation
IKKβ: IκB kinase β
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrates</td>
</tr>
<tr>
<td>KRBH</td>
<td>Krebs-Ringer buffer containing HEPES</td>
</tr>
<tr>
<td>LCACoA</td>
<td>Long chain acyl-CoA</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Met</td>
<td>Metformin</td>
</tr>
<tr>
<td>Mtr</td>
<td>Matrine</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OA</td>
<td>Oleanolic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphohydrolase</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositol-dependent kinase 1</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PFK1</td>
<td>Phosphofructokinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>Phosphatase 2A</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element-binding protein 1c</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>VO2</td>
<td>Volume of oxygen consumed</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>β-HAD</td>
<td>β-Hydroxyacyl-CoA dehydrogenase</td>
</tr>
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</table>
ABSTRACT

Type 2 diabetes is a metabolic disease characterised by insulin resistance (reduced response of insulin-targeted tissues to insulin action) and β-cell failure (the inability of β-cells to secrete adequate levels of insulin) with hyperglycaemia. The chronic nature and fast-growing prevalence of type 2 diabetes, and many different forms of related complications, render this disease a heavy burden for both individuals and society. Long-term effective glycaemic control for type 2 diabetes remains a challenge as many anti-diabetic therapeutics have treatment-limiting side effects. Therefore, there is an urgent need for the development of novel drugs for this metabolic disease.

A common approach of drug discovery and development, widely used by the pharmaceutical industry in the past three decades, involves target-based high-throughput screenings of large synthetic compound libraries. While this approach is able to dramatically increase the number of screening candidates and capacity, the pharmaceutical industry is facing a high attrition rate and unimproved new-drug output despite an increasing commitment of resources.

In view of these challenges, this thesis aims to 1) identify leading compounds of interest from natural products using an in vivo phenotypic screening in combination with data mining of biological and chemical data; 2) evaluate the anti-diabetic effects of the identified investigational compounds, oleanolic acid and matrine, and investigate the corresponding mechanisms.

To address the first aim, the identification of investigational compounds of interest was facilitated by a number of established criteria (Chapter 3). These criteria include drug-likeliness, chemical structures in favour of modification, sufficient amount for in vivo studies and efficacy on functional readouts (e.g. basal blood glucose, plasma and liver triglyceride, and adiposity) in a type 2 diabetes mouse model. This thesis first confined the source of
investigational compounds to an in-house chemical library of natural products, which are known to be more structurally diverse and biochemically specific than synthetic compounds. A structural analysis of compounds with known chemical structures revealed that oleanolic acid (OA) and matrine (Mtr) are compliant with the Lipinski's rule of five, indicating they might have good oral availability. Literature search showed that the chemical structure of OA and Mtr can be modified to generate derivatives. The efficacy of test compounds to reduce basal blood glucose, plasma and liver triglyceride and adiposity, was assessed in a type 2 diabetes mouse model induced by high-fat feeding and streptozotocin. Type 2 diabetic mice had increased blood glucose, and plasma and liver triglyceride levels, compared to chow mice. OA, Mtr and THA-E (compound code for confidentiality reasons) suppressed the increase of blood glucose and plasma triglyceride levels in the type 2 diabetic mice. Furthermore, the elevated liver triglyceride level was normalised by Mtr and THA-E. However, THA-E induced a two-fold increase of adiposity in the type 2 diabetic mice. The forth compound, QH (compound code for confidentiality reasons), had no effect on blood glucose, and plasma and liver triglyceride levels. Based on these results, OA and Mtr were selected for further detailed investigations.

In Chapter 4, the chronic efficacy of oleanolic acid (100 mg/kg/day) on hyperglycaemia was investigated in type 2 diabetic mice induced by a similar method as in the first study. High-fat feeding and streptozotocin generated a steady hyperglycaemia (21.2 ± 1.1 mM) but OA administration reversed the hyperglycaemia by ~60%. Interestingly, after the cessation of OA administration, the reversed hyperglycaemia was sustained for the entire post-treatment period of the study (four weeks) despite the reoccurrence of dyslipidaemia. Examination of insulin secretion and pancreas morphology did not indicate improved β-cell function as a likely mechanism. Urine glucose loss was decreased with substantial improvement of diabetic nephropathy after the OA treatment. Pair-feeding the OA-treated mice to an untreated group
ruled out food intake as a main factor attributable for this sustained reduction in hyperglycaemia. Studies with the use of glucose tracers revealed no increase in glucose influx into muscle, adipose tissue or liver in the OA-treated mice. Finally, key regulators of gluconeogenesis in the liver were analysed and significant increases in the phosphorylation of both Akt and FoxO1 was found after treatment with OA. Importantly, these increases were significantly correlated with a down-regulation of glucose-6-phosphatase expression. These findings suggest triterpenoids are a potential source of new efficacious drugs for sustained control of hyperglycaemia. The liver appears to be a major site of action, possibly by the suppression of hepatic glucose production via the Akt/FoxO1 axis.

The study in Chapter 5 demonstrated the therapeutic efficacy of matrine, a safe drug for the treatment of chronic viral infections and tumours in the liver, for insulin resistance and hepatic steatosis in high fat-fed mice in a distinct manner from the commonly used anti-diabetic drug metformin. The results first showed that matrine reduced glucose intolerance and plasma insulin level, hepatic triglyceride content and adiposity without affecting caloric intake. The effect to reduce hepatosteatosis was attributed to a suppressed lipogenic pathway and increased fatty acid oxidation as suggested by ex vivo palmitate oxidation assay. Matrine neither suppressed mitochondrial respiration nor activated of AMPK in the liver. A computational docking simulation revealed HSP90, a negative regulator of HSP72, as a potential binding target of matrine. Consistent with the simulation results, matrine increased the hepatic protein level of HSP72, which inversely correlated with both liver triglyceride level and glucose intolerance. These results together suggested that matrine may be used for the treatment of T2D and hepatic steatosis, and the molecular action of this hepatoprotective drug might involve an activation of HSP72 in the liver.

In summary, the results from this thesis support the notion that phenotypic screenings of natural products in combination with data mining of biological and chemical data are a viable
approach for the discovery of new promising compounds for drug development. Results from the OA study provide a proof of concept for the potential of triterpenoids as a promising source to explore new drugs for the long-term control or cure of hyperglycaemia and diabetic kidney complications. The Mtr study suggests Mtr is a promising therapeutic to be used in humans for the metabolic syndrome with favourable effects on the associated non-alcoholic fatty liver disease.
Chapter 1
Introduction and Literature Review
1.1 DIABETES

Diabetes is a metabolic disease characterised by increased blood glucose levels [1, 2]. According to the recommendations from the World Health Organization (WHO) 2006 [1] and America Diabetes Association 2014 [2], the diagnostic criteria for diabetes and intermediate hyperglycaemia are summarised in Table 1.1.

**Table 1.1 Diagnostic criteria for diabetes and intermediate hyperglycaemia.**

<table>
<thead>
<tr>
<th>Diseases</th>
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<tr>
<td>Impaired fasting glucose</td>
<td>Fasting plasma glucose and 2-hr plasma glucose</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td>Fasting plasma glucose and 2-hr plasma glucose</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Fasting plasma glucose or 2-hr plasma glucose</td>
</tr>
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</table>

Type 1 and type 2 diabetes are the two major types of diabetes according to the etiologic classification, accounting for 5-10% and 90-95% of all cases of diabetes, respectively [2].

Type 1 diabetes (T1D) is characterised by an absolute deficiency in insulin production. Despite the exact cause of T1D still being unknown, it is widely believed that T1D is a chronic autoimmune disease with genetic predisposition, and it can be triggered by environmental factors such as viral infections [3]. The onset of T1D typically happens in people younger than 30 and it, therefore, is also termed juvenile- or childhood-onset diabetes.

Type 2 diabetes (T2D) is also known as noninsulin-dependent diabetes mellitus, maturity-onset or adult-onset diabetes. It is characterised by insulin resistance and β-cell failure.
Similar to T1D, both genetic predisposition and environmental factors (such as a sedentary lifestyle and overeating) play a role in the onset of T2D. The development of T2D is the net result of an imbalance between insulin action and insulin secretion. A decreased responsiveness of these tissues to the action of insulin can occur due to different mechanisms, such as excess lipid accumulation in non-adipose tissues (see Section 1.2.2). At the early stage of T2D, β-cells in the pancreas usually secrete more insulin to compensate for the decreased insulin sensitivity in tissues involved in the regulation of blood glucose levels. However, the ability of β-cells to secrete adequate levels of insulin deteriorates over time as a result of decreased β-cell functions (expression and secretion of insulin) and mass induced by glucotoxicity and lipotoxicity [4]. Hyperglycaemia eventually occurs when the insulin secretion from the β-cells is inadequate to compensate for insulin resistance (Figure 1.1) [5].

**Figure 1.1 Natural history of type 2 diabetes.** Adapted from Henry et al. [6]

In addition to these two major categories of diabetes, other specific types of diabetes also exist resulting from genetic defects in the β-cell or insulin action, diseases of the exocrine pancreas, endocrinopathies (e.g. excess amount of growth hormone, cortisol, glucagon or epinephrine), drug usage (e.g. Vacor, pentamidine, nicotinic acid), infections and gestation [1].
1.1.1 Prevalence of Diabetes

In the past two decades, the number of people with diabetes and the global or regional prevalence of diabetes have been estimated and projected a number of times by several independent research groups (Table 1.2). In 1993, King et al. [7], the WHO Ad Hoc Diabetes Reporting Group, estimated the prevalence of diabetes in 75 communities from 32 countries, based on surveys conducted by WHO during 1976-1991. One year later, the International Diabetes Federation (IDF) estimated that the global incidence of diabetes was 100 million, based on estimates of T1D and T2D incidence in its member nations [8], while McCarty et al. reported a similar figure (110 million in 1994) using data from population-based epidemiological studies [9]. Using a similar strategy as McCarty et al. [9], Amos et al. [10] estimated the age-distribution and global/regional incidence of diabetes during 1995-1997 (118 and 124 million, respectively) and projected this number would rise to 151 and 221 million in 2000 and 2010, respectively. As follow-up studies of King et al. [7], King et al. [11] and Wild et al.[12] estimated the global burden of diabetes during 1995-2025 (135-300 million) and 2000-2030 (171 - 366 million), respectively, using data from updated prevalence studies and demographic estimates and projections from the United Nations. IDF have published the *IDF Diabetes Atlas* with updated estimations of incidence and prevalence of diabetes since 2000 [13-18]. In the most recent *IDF Diabetes Atlas*, it was estimated that the global number of people with diabetes was 382 million (8.3% of the adult population) in 2013 and would rise to 592 million (10.1%) by 2035 [18].

Despite different sources of data and methodologies utilised in these estimates and projections, a clear increasing trend of the incidence and prevalence of diabetes is observed across all of these studies. Furthermore, the incidence of diabetes is growing at an increasing rate as indicated by steeper slopes in later projections shown in Figure 1.2.
1.1.2 Complications of Diabetes

Chronic elevation of blood glucose (resulting from inadequate or delayed treatment of diabetes), together with common conditions in diabetes, such as hypertension and increased cholesterol levels, can lead to a wide range of complications in different organs, especially the heart, kidneys, eyes and nerves [18]. These complications severely affect patients’ quality of life, and can result in disability and even death.

**Cardiovascular disease** Among the different diabetes-related complications, cardiovascular disease is the most common cause of morbidity and mortality [18, 19]. Individuals with diabetes have a 2- to 4-fold increased risk of developing coronary artery disease (which can lead to myocardial infarction), peripheral arterial disease, stroke and congestive heart failure (diabetic cardiomyopathy) [19, 20].
Table 1.2 Estimates and projections of the incidence number and prevalence of diabetes.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Global estimates and projections</th>
<th>Data sources</th>
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<tbody>
<tr>
<td></td>
<td>Year</td>
<td>Incidence number (million)</td>
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<td>IDF 1994 [8]</td>
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<td>2010</td>
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<td>246</td>
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<td></td>
<td>2025</td>
<td>380</td>
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<td>Shaw et al. 2010 [21]</td>
<td>2030</td>
<td>438</td>
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<td>IDF 2011 [17]</td>
<td>2011</td>
<td>366</td>
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<tr>
<td>Whiting et al. 2011 [22]</td>
<td>2030</td>
<td>552</td>
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<tr>
<td>IDF 2013 [18]</td>
<td>2013</td>
<td>382</td>
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<td></td>
<td>2035</td>
<td>592</td>
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</table>
Nephropathy Chronic elevation of blood glucose can dampen the ability of the kidney to filter waste products and recycle useful substrates, resulting in the development of chronic kidney disease [18]. Diabetes has become the leading cause of end-stage renal disease affecting 37.8% of all diagnosed cases of end-stage renal disease in the USA in 2011 [23]. While kidney failure is life-threatening, chronic kidney disease can also accelerate cardiovascular disease leading to further increased morbidity and mortality [24]. In 2011, the cost for the treatment of diabetes-related nephropathy reached US$24.6 billion in the USA [23].

Retinopathy High blood glucose levels not only damage renal tubules and affect their filtering ability, but also reduces the coverage of retinal capillaries and increases vascular leakage in the eyes [25]. Such damage can eventually lead to complete loss of eye vision. The prevalence of retinopathy varies greatly among countries, ranging from 10% to 61% in patients with diabetes and from 1.5% to 31% in patients with newly diagnosed diabetes [26]. In Australia, it was reported that approximately 17% of men and 12% of women with diabetes had eyesight problems in 2008 [27].

Neuropathy Nerve injury is the most common complication of diabetes. Depending on the nerve affected, diabetic neuropathy can lead to pain, tingling and numbness (peripheral neuropathy), and affect the function of a wide range of organs including the heart, eyes, gastrointestinal tract, bladder, sex organs and sweat glands (autonomic neuropathy) [28]. Furthermore, patients with diabetes have a 25-fold increase in the risk of amputation due to infection and ulceration resulted from the diabetic neuropathy and damage to the peripheral blood vessels [18]. The treatment for diabetic neuropathy is challenging as it might involve intensive and comprehensive intervention targeting glucose, blood pressure, lipids and different lifestyle factors, and there is no approved drug that can achieve complete relief of pain [28].
Furthermore, diabetes also leads to pregnancy complications, sleep apnoea, depression, anxiety, and oral health problems [18].

In 2013, there were 5.1 million deaths in people aged between 20 and 79 as a result of diabetes and its complications [18]. In Australia, diabetes was the sixth leading underlying cause of death in 2012, and contributes to 10% of total deaths [29].

### 1.1.3 Economic Burden of Diabetes

Despite the advances in diagnostic techniques for the early detection of diabetes-related complications and better interventions, the economic burden of diabetes has become heavier than ever due to the increasing prevalence of the disease. The global spending on the management of T2D and treatments of its related complications reached US$548 billion in 2013 and expected to rise to US$627 billion in 2035 [18]. In Australia alone, diabetes-related health-care expenditure rose from AUD$811 million in 2001 to AUD$1,507 million in 2009 accounting for 2.3% of the national health expenditure [30].

Due to the rapid increase of prevalence (see Section 1.1.1), severe health problems (Section 1.1.2) and enormous treatment costs involved (Section 1.1.3), diabetes has emerged as “a major threat to global development” [18] and warrants research for better management and treatments, especially for T2D, which accounts for approx. 90% of all cases of diabetes.

### 1.2 INSULIN

As mentioned in Section 1.1, insulin resistance is one of the fundamental defects of T2D. Insulin is an important hormone secreted by β-cells in the pancreas for the regulation of blood glucose and lipid metabolism. Major physiological roles of insulin, in terms of blood glucose regulation, include the stimulation of glucose uptake in peripheral tissues (such as muscle and adipose tissues) and inhibition of glucose production in the liver to lower the glucose level in the bloodstream.
1.2.1 Insulin Signalling Pathway

Insulin mediates the effects on glucose metabolism through the IRS-PI3K-Akt pathway. As shown in Figure 1.3, the insulin receptor (IR) is composed of two α subunits and two β subunits. Upon binding to the α subunits of the IR, insulin promotes the dimerisation of the β subunits of the IR and subsequent autophosphorylation of tyrosine residues (e.g. Tyr999). With the phosphorylation of tyrosine residues, the IR is activated and recruits insulin receptor substrates (IRS) and promotes their binding to SH2 domains on the regulatory p85 subunit of phosphoinositide 3-kinase (PI3K). The catalytic p110 subunit of PI3K then converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) on the plasma membrane. The resultant PIP3 recruits a subset of signalling proteins including phosphoinositide-dependent kinase 1 (PDK1) and Akt (also known as protein kinase B, PKB). PDK1 is also situated on the plasma membrane, and once activated by PIP3, it can phosphorylate Thr308 in the activation loop of Akt. The phosphorylation of Thr308 and Ser473 (by the sin1/raptor/mTOR complex) is essential for the function of Akt [31].

The activation of Akt initiates a wide range of cellular responses. This section mainly discusses its effects on the regulation of glucose metabolism. Activated Akt promotes the translocation of GLUT4 vesicles to the plasma membrane and stimulates glucose uptake in muscle and adipose tissue. Akt also indirectly activates phosphofructokinase 1 (PFK1), which is a rate-limiting enzyme converting fructose 6-phosphate to fructose 1,6-phosphate for glycolysis. Akt has been shown to phosphorylate and activate PFK2, leading to the formation of fructose 2,6-bisphosphate, which stimulates PFK1. Meanwhile, Akt facilitates glucose storage in the liver by inducing glycogen synthesis through inhibitory phosphorylation of glycogen synthase kinase 3 (GSK3), which phosphorylates and inhibits the activity of glycogen synthase (GS). While Akt facilitates the conversion of glucose to glycogen, it also indirectly suppresses the reverse reaction, glycogenolysis, through the activation of mTOR.
and phosphatase, and subsequent inhibition of glycogen phosphorylase. Furthermore, activation of Akt leads to the inhibitory phosphorylation and nuclear exclusion of FoxO1, a key transcription factor regulating *gluconeogenesis*, suppressing the glucose production from the liver [31].

![Diagram of insulin signalling cascade](image)

Figure 1.3 Schematic diagram of a simplistic insulin signalling cascade. Diagram drawn according to the description and abbreviations in the text of Section 1.2.1. Akt/PKB, protein kinase B; FoxO1, forkhead box protein O1; G6Pase, glucose-6-phosphatase; GLUT 4, glucose transporter type 4; GS, glycogen synthase; GSK3, glycogen synthase kinase 3; IR, insulin receptor; IRS, insulin receptor substrates; PDK1, phosphoinositide-dependent kinase 1; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate.

1.2.2 Lipid-induced Insulin Resistance

Insulin resistance is a complex metabolic disorder, which is known to be induced by a wide range of physiological insults, including ectopic lipid accumulation (accumulation of lipid in non-adipose tissues), endoplasmic reticulum stress and inflammation. Ectopic lipid accumulation is considered the most common mechanism leading to insulin resistance. A
number of lipid intermediates have been identified to have detrimental effects on the insulin signalling cascade.

**Fatty acids** (FAs) were suggested to compete with glucose as the substrate for oxidation and energy production in muscle by Randle et al. in the 1960s [32]. Increased levels of acetyl CoA, resulting from the oxidation of excess FAs, can stimulate pyruvate dehydrogenase kinase (PDK), which in turn induces the inhibitory phosphorylation of pyruvate dehydrogenase (PDH) [33]. As PDH is a key enzyme regulating the conversion of pyruvate (a product of glycolysis) to acetyl CoA, excess FAs thus inhibits insulin-stimulated glycolysis. Furthermore, oxidation of excess FAs can also increase the level of citrate, which inhibits PFK1 and blocks the formation of fructose 1,6-phosphate in glycolysis [34].

**Long Chain Acyl-CoAs** (LCACoAs) are the esterification product of FAs with CoA catalysed by acyl-CoA synthetase. LCACoA can enter mitochondria though CPT-1 for β-oxidation, which generates acetyl-CoA for the tricarboxylic acid cycle (TCA cycle, or citric acid cycle), or be used as the substrate for the production of triglyceride. The accumulation of LCACoA has been shown to associate with decreased insulin-mediated glucose uptake in soleus muscle *ex vivo* [35], impaired glycogen synthesis in the muscle of rats infused with intralipid [36] and a reduction of whole-body insulin action in both rats and human [37]. Furthermore, the increase of intracellular C18:2 CoA level is associated with the activation of protein kinase C (PKC) θ and inhibition of insulin-stimulated IRS-1 and PI3K activation. [38]. The impairment of insulin signalling transduction by LCACoA is attributed to its ability to activate novel PKCs directly or indirectly [39].

**Diacylglycerols** (DAGs) are intermediate lipid species during the synthesis or lipolysis of triglyceride or phospholipids. Accumulation of DAGs has been suggested to link to insulin resistance with the involvement of PKCs activation [40]. For example, insulin resistance is
associated with the activation of PKC ε and θ in high fat-fed rats [41], and PKC θ/δ in both rats and humans with lipid infusion [38, 42].

**Ceramides** are the product of the hydrolysis of sphingomyelin, condensation of palmitate and serine, and degradation of sphingolipids and glycosphingolipids. In addition to its structural role in the plasma membrane, ceramide has been shown to stimulate phosphatase 2A (PP2A) and leads to subsequent dephosphorylation and inhibition of Akt, impairing the insulin signalling transduction [43, 44]. Furthermore, it has been shown that inhibition of ceramide synthesis can improve insulin resistance induced by different insults including glucocorticoid, saturated-fat and obesity [45].

**Triglycerides** are composed of one glycerol molecule and three FAs. As triglycerides are the major storage form of lipids, it is commonly used as an indicator of ectopic lipid accumulation. Indeed, while triglycerides *per se* are generally considered benign to insulin resistance, it has been shown that the intracellular level of triglyceride in the muscle is inversely associated with whole-body insulin action in humans [46].

In addition to these lipid intermediates, other insults to the insulin signalling, such as ER stress and inflammation, are tightly intertwined with lipid metabolism (such as fatty acid uptake, lipogenesis and fatty acid oxidation) and often lead to ectopic lipid accumulation. For example, during ER stress, activation of IRE1 leads to the splicing of X-box-binding protein 1, which can promote lipogenesis by up-regulating the transcription of a number of lipogenic proteins [47]. A number of inflammation inducers, e.g. lipopolysaccharide (LPS), interleukin-1 beta, tumor necrosis factor α (TNFα) and IκB kinase β (IKKβ), are reported to increase lipogenesis and suppress fatty acid oxidation. IKKβ, a kinase that activates the inflammatory NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, has been shown to be implicated in the biogenesis of ceramide [48]. These findings suggest ectopic
lipid accumulation might be a convergence of several pathogenetic mechanisms, at least partly involved in these physiological insults to the insulin signalling pathway.
Figure 1.4 Schematic diagram of lipid metabolism in the liver. Pathways are explained in Section 1.3. ACC: acetyl-CoA carboxylase; ACS: acyl-CoA synthetase; CPT-1: carnitine-palmitoyl transferase 1; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; DHAP: dihydroxyacetone phosphate; GPAT: sn-1-glycerol-3-phosphate acyltransferase; GPD1: glycerol-3-phosphate dehydrogenase 1; LCACoAs: Long chain acyl-CoAs; LPA: lysophosphatidic acid; PA: phosphatidic acid; PAP: phosphatidic acid phosphohydrolase; PDH: pyruvate dehydrogenase; SCD1: stearoyl-CoA desaturase 1; TCA: tricarboxylic acid cycle; TG: triglyceride.


1.3 LIPID METABOLISM

Depending on the tissue of interest, ectopic lipid accumulation might result from different metabolic perturbations in lipid synthesis, mobilisation and oxidation. This section will mainly focus on the aspects of synthesis and oxidation (Figure 1.4).

1.3.1 Lipid Synthesis

Fatty acid is an essential substrate for the synthesis of triglycerides. In the liver, increased intracellular levels of FAs might be a result of increased dietary intake, lipolysis of adipose triglyceride, and/or hepatic de novo lipogenesis [49].

The process of synthesising FAs from simple carbohydrates is termed de novo lipogenesis. As noted in Section 1.2.2, the formation of malonyl-CoA is the first committed step in the process of de novo lipogenesis. Acetyl-CoA, generated from the dehydrogenation of pyruvate (product of glycolysis) or β-oxidation of fatty acid, is carboxylated with a molecule of bicarbonate catalysed by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. Multiple acetyl-CoAs are added to malonyl-CoA by a highly structured multi-enzyme complex called fatty acid synthase (FAS) to form long-chain fatty acids, such as 16C palmitic acid and 18C stearic acid.

These saturated FAs, resulting from de novo lipogenesis, increased dietary intake or lipolysis of adipose triglyceride, have to be esterified and activated with CoA by acyl-CoA synthetase (i.e. formation of Long chain acyl-CoAs, LCACoAs) before participating in other reactions, such as oxidation, elongation, desaturation, or synthesis of glycerolipids or cholesterol esters. These activated saturated FAs are then desaturated by stearoyl-CoA desaturase (SCD) and converted to monounsaturated FAs.

There are two main biosynthetic pathways for triglycerides, the sn-glycerol-3-phosphate pathway (predominantly in liver and adipose tissue) and the monoacylglycerol pathway (in
the intestines). The sn-glycerol-3-phosphate pathway is discussed here. In the sn-glycerol-3-phosphate pathway, the substrates for triglyceride synthesis are monounsaturated FAs (formed by the process discussed above) and glycerol 3-phosphate, which forms the backbone of triglyceride molecules. Glycerol 3-phosphate mainly results from the reduction of the glycolytic intermediate dihydroxyacetone phosphate (DHAP) catalysed by glycerol-3-phosphate dehydrogenase 1 (GPD1) or, to a lesser extent, ATP-dependent phosphorylation of glycerol catalysed by glycerol kinase. Glycerol 3-phosphate is acylated with fatty acyl-CoAs to form lysophosphatidic acid (LPA, or 1-acylglycerol-3-phosphate) catalysed by sn-1-glycerol-3-phosphate acyltransferase (GPAT). LPA can also form from the acylation of DHAP catalysed by dihydroxyacetone-phosphate acyltransferase (DHAPAT), followed by reduction of acyl-DHAP catalysed by alkyl-DHAP reductase. LPA is then further acylated by sn-1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) to form phosphatidic acid (PA, or 1,2-diacylglycerol-3-phosphate). PA is hydrolysed by phosphatidic acid phosphohydrolase (PAP) to form DAG. TG is synthesised by further esterification of DAG with unsaturated FAs catalysed by diacylglycerol acyltransferase (DGAT) [50, 51].

1.3.1.1 Regulation of Lipid Synthesis
Sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) are two critical transcription factors regulating lipid synthesis.

SREBP-1c regulates triglyceride synthesis, while its isoform SREBP-2 targets genes involved in cholesterol biosynthesis [52]. Inactive form of SREBP-1c is synthesised and resided in a SREBP-1c/SCAP/Insig complex, which is retained in the ER membrane by Insig. Under stimulation, SCAP dissociate from Insig and assists the transportation of SREBP-1c to the Golgi apparatus, in which inactive SREBP-1c undergoes proteolytic cleavage by S1P and S2P [52]. The expression and/or maturation of SREBP-1c is regulated by LXR [53], mTOR1 [54]
and insulin [52], in addition to autoregulation [55]. Activated SREBP-1c has been shown to regulate the expression of lipogenic enzymes including FAS [56], ACC [57] and SCD1 [58], which are rate-limiting enzymes for triglyceride synthesis. Inhibition of SREBP-1c by pharmaceutical agent was showed to reduce hyperlipidaemia and improve insulin resistance in western diet-fed C57BL/6J mice [59].

ChREBP has been shown to regulate triglyceride synthesis synergistically with SREBP-1c [60]. ChREBP is activated by high glucose and inhibited by cAMP [61]. In addition to the glucose-mediated regulation of expression, the translocation of ChREBP into the nucleus has been shown to be regulated by (de)phosphorylation events at Ser-196 and Thr-666 [62]. Furthermore, mutated ChREBP lacking these residues, which are inhibited by PKA-mediated phosphorylation, is not constitutively active, suggesting the activation of ChREBP might involve a second mechanism independent of PKA phosphorylation [63]. In addition to the regulation by (de)phosphorylation, the activity of ChREBP is regulated by intramolecular inhibition, which is removed by high glucose level [64].

1.3.2 Fatty Acid Oxidation

Fatty acid oxidation can occur in different forms including α-, β- and ω-oxidation. Among these three forms, β-oxidation is the major process to oxidise FAs for energy generation [65]. β-oxidation can occur in both the mitochondria and peroxisome. It is a process of sequential removal of 2-carbon units by oxidation at the β-carbon position of fatty acyl-CoA molecules. Mitochondrial β-oxidation is a reaction that involves four steps: 1) Two hydrogen atoms between carbon 2 and 3 are removed by Acyl-CoA dehydrogenases, forming a double bond (i.e. a trans enoyl-CoA) and FADH$_2$; 2) Enoyl-CoA hydratase adds a water molecule to this double bond forming 3-L-hydroxyacyl-CoA; 3) 3-L-hydroxyacyl-CoA dehydrogenase removes hydrogen atoms on carbon 2 and 3 forming 3-ketoacyl CoA and NADH; 4) The terminal acetyl-CoA group on the 3-ketoacyl CoA molecule is cleaved by β-ketothiolase
forming a new acyl-CoA with two carbons shorter than the original one. The removal of one 2-carbon unit (i.e. acetyl-CoA) from a fatty acyl-CoA molecule in the mitochondria generates one NADH and one FADH$_2$, which enter the electron transport chain in mitochondria for the generation of ATPs. The acetyl-CoA cleaved from the original fatty acyl-CoA can be converted to citrate, which enters the tricarboxylic acid cycle (TCA cycle, or Krebs cycle) for the generation of more NADH and FADH$_2$.

1.3.2.1 Regulation of Fatty Acid Oxidation

A major rate-limiting step in β-oxidation is the transportation of acyl-CoA across the mitochondrial membrane through the carnitine palmitoyltransferase system [66]. The carnitine palmitoyltransferase (CPT) system consists of CPT1 and CPT2. CPT1, which locates on the outer mitochondrial membrane, has been shown to be inhibited by malonyl-CoA [66]. The removal of malonyl-CoA-mediated inhibition of CPT1, at least in part, accounts for the effect of AMP-activated protein kinase (AMPK) on fatty acid oxidation. Activated AMPK induces inhibitory phosphorylation of ACC, which converts acetyl-CoA to malonyl-CoA, and reduces the intracellular level of malonyl-CoA and facilitate the transportation of acyl-CoA into the mitochondria [67]. Furthermore, enzymes involved in the β-oxidation are inhibited by fatty acyl-CoA intermediates produced during this process [68].

In addition to the regulation by substrates, fatty acid oxidation is regulated at the transcriptional level. It is well known that peroxisome proliferator-activated receptors (including PPARα, PPARδ and PPARγ) regulate the expression of a number of proteins involved in fatty acid transportation and oxidation, e.g. CPT1, acetyl-CoA synthase and β-ketothiolase [69]. Transcription coactivator PGC-1α mediates the expression of a number of proteins involved in β-oxidation, the TCA cycle and the mitochondrial electron transport chain [70].
1.4 CURRENT TREATMENT OF TYPE 2 DIABETES

Lifestyle interventions, including increased physical activity and reductions in food intake and body weight, are a critical part of T2D management. It can effectively control hyperglycaemia and delay the progression of T2D in the early stages. However, when lifestyle interventions are unsuccessful or moderate hyperglycaemia occurs, patients of T2D are advised to promptly start treatments with anti-hyperglycaemic agents [71].

1.4.1 Sulfonylureas

Sulfonylureas have been commonly used as anti-hyperglycaemic drugs since the 1950s. They reduce blood glucose by increasing insulin secretion from pancreatic β-cells. Sulfonylureas bind and stimulate the closure of ATP-sensitive potassium channels on the membrane of β-cells, leading to a depolarisation across the cell membrane. Such depolarisation opens voltage-dependent calcium channels and increases the intracellular level of calcium ions, resulting in an increased fusion of insulin granule with the cell membrane and release of insulin [72, 73]. There are two generations of sulfonylureas: tolbutamide, tolazamide and chlorpropamide (first generation); glipizide, glyburide and glimepiride (second generation). The second generation of sulfonylurea is more potent compared to the first generation and have longer durations of action, thus they can be administered in lower doses and less frequently. Furthermore, due to the different way of binding to other proteins (non-ionic vs. ionic), there are less drug interactions for the second generation compared to the first [74]. Despite being effective to lower blood glucose levels, chronic increases of insulin secretion induced by sulfonylureas can lead to β-cell dysfunction and a reduction of β-cell mass [75]. Furthermore, the use of sulfonylureas is also associated with other side effects such as hypoglycaemia and weight gain [76, 77].

1.4.2 Biguanides (Metformin)
Metformin, a biguanide, is a first-line treatment for T2D. In 2011, it was ranked 7th of the most prescribed drugs in the US [78]. Metformin was first used clinically as a glucose lowering drug in the 1950s, long before its molecular mechanism of action was understood. It is now generally believed that the major target tissue of metformin is the liver and it exerts its anti-hyperglycaemic effects by inhibiting hepatic glucose production [71]. The most common adverse effect related to the use of metformin is gastrointestinal upset (including diarrhoea and abdominal cramping), lactic acidosis and vitamin B12 deficiency. Apart from these side effects, metformin displays an attractive drug safety profile as it is not associated with weight gain, hypoglycaemia and is likely to reduce cardiovascular disease events.

Early works in the 1960s and 1970s showed that a number of guanidine-based compounds inhibit oxygen consumption in the mitochondria, suggesting the major site of action of these compounds might be in this organelle [79-81]. Furthermore, biguanides were found to inhibit gluconeogenesis [82] resulting in reduced blood glucose levels. Two independent studies in 2000 reported that metformin inhibits complex 1 in the mitochondrial respiratory chain [83, 84], and such inhibitions correlate well with the inhibition of gluconeogenesis [84]. Following the discovery of the inhibitory effect of metformin on the complex 1 of the mitochondrial respiratory chain, Zhou et al. in 2001 used an AMPK inhibitor (compound C) and reported that AMPK activation is required in metformin-mediated inhibition of hepatic glucose production, and that metformin promotes glucose uptake in muscle and suppresses lipogenic gene expressions in primary hepatocytes [85]. A number of studies have been carried out since then to investigate underlying mechanisms of metformin-mediated AMPK activation and the role of AMPK signalling in the anti-diabetic effects of metformin. Studies by Hawley et al. employing isogenic cell lines with stable expression of AMPK complexes containing AMP-insensitive (R531G) γ2 variants showed that metformin activates AMPK via a AMP-dependent mechanism [86]. In a liver-specific Lkb1-knockout mouse model, metformin-
mediated AMPK activation was found to be blunted in \textit{Lkb1}-knockout liver and the glucose-lowering effect of metformin in high fat-fed mice was lost, suggesting metformin requires LKB1 in the liver to lower blood glucose levels. With the use of small hairpin RNA for TORC2 to reduced PGC-1\(\alpha\) expression level, the same study proposed the LKB1 signalling controls gluconeogenesis via the up-regulation of PGC-1\(\alpha\). However, the effect of metformin on gluconeogenesis was not accessed in LKB1-knockout mice \cite{87}. Further studies with two mouse models with AMPK\(\alpha1\) and \(\alpha2\) subunits- or LKB1-knocked out in hepatocytes showed that the inhibitory effect of metformin on glucose production was intact in both AMPK\(\alpha1/2\) subunits-knockout and LKB1-knockout mice, suggesting that the inhibitory effect of metformin on gluconeogenesis is AMPK- or LKB1-independent \cite{88}. Furthermore, it was found that AICAR (an AMP mimetic), but not an allosteric activator of AMPK, can significantly inhibit gluconeogenesis, suggesting that AMP might play a more critical role in inhibiting gluconeogenesis \cite{88, 89}. Recent studies suggest the physiological effect of metformin on gluconeogenesis might involve a suppressed cAMP-PKA signalling pathway as a result of decreased adenylate cyclase activity \cite{90}, or inhibitory phosphorylation of CRTC2 (hence dissociation of the CREB-CBP-complex) by PKC\(\iota/\lambda\) \cite{91}.

1.4.3 Thiazolidinediones

Thiazolidinediones, also known as glitazones, are potent insulin sensitisers introduced in the late 1990s. They exert their therapeutic effects via activating the nuclear transcription factor peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) \cite{92, 93}. PPAR\(\gamma\) is highly expressed in the adipose tissue, which is also believed to be the major acting site of PPAR\(\gamma\) activation \cite{69}. Activation of PPAR\(\gamma\) has been shown to facilitate the differentiation of fibroblasts into adipocytes by increasing the expression of \(\alpha\)P2 \cite{94}, and up-regulate a number of proteins involved in lipid metabolism (e.g. acyl-CoA synthase \cite{95}, and LPL \cite{96}, FATP-1 \cite{97} and CD36 \cite{98}). In addition to these genes, PPAR\(\gamma\) also regulates the expression of UCP-1, UCP-
2 and UCP-3 [99] and leptin [100], which are involved in energy homeostasis. PPARγ activation has been shown to improve insulin action, at least in part, by suppressing the expression of the pro-inflammatory cytokine TNFα [101, 102] and 11β-hydroxysteroid dehydrogenase 1 [103, 104]. Consistent with the effects of PPARγ activation shown in cells and small animals, a multicentre, randomised, double-blind, controlled clinical trial shows that thiazolidinediones have slower loss of β-cell function, greater improvement of insulin sensitivity and a longer durability of glycaemic control compared to metformin and sulfonylurea [77]. However, the use of thiazolidinediones can cause a number of side effects including fluid retention, weight gain, bone loss and congestive heart failure [93].

1.4.4 Other Anti-Hyperglycaemic Agents

In addition to biguanides, sulfonylurea and thiazolidinediones, there are seven additional classes of non-insulin, anti-diabetic drugs available for T2D treatment, and their properties are summarised in Table 1.3.
Table 1.3 Properties of currently available non-insulin anti-hyperglycaemic drugs. Adapted from Inzucchi et al. [71].

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Cellular mechanism</th>
<th>Primary physiological action(s)</th>
<th>Major side effect(s)</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanides</td>
<td>Metformin</td>
<td>Activates AMPK</td>
<td>↓ glucose production</td>
<td>Gastrointestinal side effects (diarrhoea, abdominal cramping)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactic acidosis risk</td>
<td></td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>Glyburide / Glibenclamide</td>
<td>Closes K\textsubscript{ATP} channels on β-cell plasma membranes</td>
<td>↑ insulin secretion</td>
<td>Hypoglycaemia</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Glipizide</td>
<td></td>
<td></td>
<td>Weight gain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gliclazide</td>
<td></td>
<td></td>
<td>Low durability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glimepirid</td>
<td></td>
<td></td>
<td>? Blunts myocardial ischemic preconditioning</td>
<td></td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Repaglinide</td>
<td>Closes K\textsubscript{ATP} channels on β-cell plasma membranes</td>
<td>↑ insulin secretion</td>
<td>Hypoglycaemia</td>
<td>High</td>
</tr>
<tr>
<td>(glinides)</td>
<td>Nateglinide</td>
<td></td>
<td></td>
<td>Weight gain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low durability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>? Blunts myocardial ischemic preconditioning</td>
<td></td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Pioglitazone</td>
<td>Activates PPAR-γ</td>
<td>↑ insulin sensitivity</td>
<td>Weight gain</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Rosiglitazone</td>
<td></td>
<td></td>
<td>Oedema/heart failure</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bone fractures</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ LDL-C (rosiglitazone)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>? ↑ Myocardial infarction (rosiglitazone)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>? ↑ Bladder cancer (pioglitazone)</td>
<td></td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>Acarbose</td>
<td>Inhibits intestinal α-glucosidase</td>
<td>Slows intestinal carbohydrate digestion/absorption</td>
<td>Generally modest HbA\textsubscript{1c} efficacy</td>
<td>Moderate</td>
</tr>
<tr>
<td>inhibitors</td>
<td>Miglitol</td>
<td></td>
<td></td>
<td>Gastrointestinal side effects (flatulence, diarrhoea)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Voglibose</td>
<td></td>
<td></td>
<td>Frequent dosing schedule</td>
<td></td>
</tr>
<tr>
<td>Class</td>
<td>Compound</td>
<td>Cellular mechanism</td>
<td>Primary physiological action(s)</td>
<td>Major side effect(s)</td>
<td>Cost</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------</td>
<td>-----------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>DPP-4 inhibitors</td>
<td>Sitagliptin</td>
<td>Inhibits DPP-4 activity, increasing postprandial active incretin (GLP-1, GIP)</td>
<td>↑ insulin secretion (glucose-dependent)</td>
<td>Generally modest HbA1c efficacy</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Vildagliptin</td>
<td></td>
<td>↓ Glucagon secretion (glucose-dependent)</td>
<td>Urticaria/angioedema</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saxagliptin</td>
<td></td>
<td></td>
<td>? ↑ Pancreatitis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Linagliptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alogliptin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile acid sequestrants</td>
<td>Colesevelam</td>
<td>Binds bile acids in intestinal tract, increasing hepatic bile acid production; ? activation of farnesoid X receptor in liver</td>
<td>Unknown</td>
<td>Generally modest HbA1c efficacy</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>? ↓ Hepatic glucose production</td>
<td>Constipation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Incretin levels</td>
<td>↑ Triglycerides</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>May ↓ absorption of other medications</td>
<td></td>
</tr>
<tr>
<td>Dopamine-2 agonists</td>
<td>Bromocriptine</td>
<td>Activates dopaminergic receptors</td>
<td>Modulates hypothalamic regulation of metabolism</td>
<td>Generally modest HbA1c efficacy</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Insulin sensitivity</td>
<td>Dizziness/syncope</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nausea, Fatigue, Rhinitis</td>
<td></td>
</tr>
<tr>
<td>GLP-1 receptor agonists</td>
<td>Exenatide</td>
<td>Activates GLP-1 receptors</td>
<td>↑ Insulin secretion (glucose-dependent)</td>
<td>Gastrointestinal side effects (nausea/vomiting)</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Liraglutide</td>
<td></td>
<td>↓ Glucagon secretion (glucose-dependent)</td>
<td>C-cell hyperplasia/medullary thyroid tumours in animals</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slows gastric emptying</td>
<td>Injectable; Training requirements</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Satiety</td>
<td>? Acute pancreatitis</td>
<td></td>
</tr>
<tr>
<td>Amylin mimetics</td>
<td>Pramlintide</td>
<td>Activates amylin receptors</td>
<td>↓ Glucagon secretion</td>
<td>Generally modest HbA1c efficacy</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slows gastric emptying</td>
<td>Gastrointestinal side effects (nausea/vomiting)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Satiety</td>
<td>Hypoglycaemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Injectable; Frequent dosing schedule</td>
<td></td>
</tr>
<tr>
<td>SGLT2 inhibitors</td>
<td>Canagliflozin</td>
<td>Inhibits renal glucose re-absorption</td>
<td>↑ urinal glucose excretion</td>
<td>Vaginal yeast infections and urinary tract infections</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Dapagliflozin</td>
<td></td>
<td></td>
<td>Increased desire to urinate</td>
<td></td>
</tr>
</tbody>
</table>
The overall aims of using anti-hyperglycaemic agents are to 1) Prevent acute osmotic symptoms of hyperglycaemia (such as diuresis caused by glycosuria); 2) Achieve stable blood glucose over time; 3) Prevent/delay the progression of T2D and the onset of related complications without adverse impacts on the patient’s quality of life. Therefore, the choice of therapeutic agents is personalised with considerations including the primary physiological effects, side effects and cost of specific drugs [71]. Despite these current anti-diabetic therapeutics being very effective in lowering blood glucose levels, treatment-limiting side effects exist in all of them, prompting further discovery and development of novel anti-diabetic agents with unique molecular modes of action.

1.5 NATURAL PRODUCTS AS A SOURCE FOR DRUG DISCOVERY

Natural products are defined as a large and diverse group of substances from a variety of sources. They are produced by marine organisms, bacteria, fungi and plants. The term encompasses complex extracts from these producers, but also the isolated compounds derived from those extracts. It also includes vitamins, minerals and probiotics. [105].

Natural products have long been an attractive and fruitful source for the discovery of novel therapeutic agents. A search on PubMed using keywords “natural products” and “drugs” shows an increasing trend of research activities on natural products (Figure 1.5). In the year 2013 alone, close to 10,000 international publications describing natural product-related research had been documented. In the Western pharmaceutical industry, natural products had been actively investigated during the 1940s to 1980s for the discovery of new drugs [106].
Examinations of all therapeutic agents approved during 1981 and 2010 for all diseases report that close to 40% of recently approved new chemical entities are developed from natural products directly or indirectly. Among these drugs, 4% are natural products, 22% are derivatives of natural products, 4% are totally synthetic drugs with a pharmacophore from a natural product and 20% are totally synthetic drugs mimicking natural products (Figure 1.6) [107]. For drugs related to diabetes treatment, 14 out of the 22 currently used anti-diabetic drugs listed in Table 1.3 were approved between 1981 and 2010 [107]. Among these 14 drugs, 10 (71%) are either natural products or derivatives/mimics of natural products (Natural products, N: voglibose; Natural products derivatives, ND: acarbose, Liraglutide and miglitol; Totally synthetic drug with a pharmacophore from a natural product, S*: nateglinide; Totally synthetic drug mimicking natural products, S/NM: rosiglitazone, sitagliptin, vildagliptin, Saxagliptin and alogliptin) while only 4 are totally synthetic (colesevelam, glimepiride, repaglinide and pioglitazone).

**Figure 1.5 Number of publications containing key words natural products and drug.** A PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) search was performed using keywords *natural products* and *drug*. Publications were sorted by year and the number of publications per year was summarised.
1.5.1 The Evolving Roles of Natural Products in Drug Discovery

The use of natural products as a source for the discovery of novel therapeutics in the Western pharmaceutical industry reached its peak at the 1980s, and started to slow down in the 1990s, despite numerous new chemical entities being successfully developed [107]. Two major factors accounted for this change in emphasis of the Western pharmaceutical industry.

Firstly, the introduction of high-throughput screening (HTS) dramatically increased the capacity of pharmaceutical companies to identify compounds against defined molecular targets. In addition, advances in molecular biology, cellular biology and genomics provide more molecular targets for HTS and further reduce drug discovery timelines [108]. However, the extraction and synthesis of natural products are relatively difficult to generate such quantities to match this rapidly increasing screening capacity [106]. Secondly, the
development of combinatorial chemistry offers a simpler method to efficiently generate libraries containing an enormous number of compounds for drug screening [109], while the construction and maintenance of a high-quality natural product library are technically demanding [110].

There is, however, renewed interest in the use of natural products for drug discovery in the past decade [107]. This is due to, firstly, natural products have higher chemical diversity compared to compounds generated by combinatorial chemistry, despite combinatorial chemistry at first provided a prospect of generating screening libraries of wide chemical diversity [111]. Secondly, natural products have greater biochemical specificity (the selective involvement of chemicals in biochemical reactions, e.g. D-glucose is, in most cases, preferred over the L-glucose the metabolism of mammals) as a result of evolutionary selection [108]. In contrast, initial compound libraries generated by combinatorial chemistry, which focused merely on achieving greater library sizes, were found to contain many compounds with properties that are not favourable for drug development (e.g. more flexible chemical structure, insoluble, lipophilic, or high molecular weight) [112]. It is estimated that natural products have approximately a 200- to 300-fold higher hit rate than synthetic compounds [113]. Thirdly, while natural products occupy different chemical space compared to current synthetic compounds, unique scaffold structures found in natural products can be used to facilitate the drug design in synthetic compounds [114]. Finally, there are still more than 90% of the world’s biodiversity that have not be explored for drug development [115].

In summary, natural products have historically been a rich source for the discovery of novel therapeutic compounds. With a greater understanding of their wide chemical diversity and extraordinary biochemical specificity, and advances in synthetic chemistry allowing the incorporation of their unique functional structures, natural products will continue to be an invaluable source for drug developments.
1.6 CONVENTIONAL DRUG DISCOVERY AND DEVELOPMENT

The discovery and development of new drugs is a lengthy process that often involves a timescale of more than 10 years [116]. Conventional drug discovery and development includes several different stages, which are summarised in Table 1.4.

Table 1.4 Key phases of drug discovery and development.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Approach</th>
<th>Aim</th>
<th>Duration (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target identification</td>
<td>Basic research to identify cellular and genetic target(s) for particular disease</td>
<td>Identify reliable targets for screening</td>
<td>~5</td>
</tr>
<tr>
<td>Hit discovery</td>
<td>Synthesis and isolation; Assay development; Library development; High-throughput screenings</td>
<td>Identify molecules (hits) with desired activity in a screening</td>
<td></td>
</tr>
<tr>
<td>Hit-to-lead, lead optimisation</td>
<td>Structure-activity relationship studies; <em>In Silico</em> screening; Chemical synthesis</td>
<td>Produce more potent and selective compounds (leads)</td>
<td></td>
</tr>
<tr>
<td>Pre-clinical studies</td>
<td><em>In vitro</em> studies; <em>In vivo</em> studies</td>
<td>Test the potency and safety for selecting drug candidates for clinical trials</td>
<td></td>
</tr>
<tr>
<td>Clinical development</td>
<td>Clinical trials (Phase I – III)</td>
<td>Phase I: examine if the drug is safe in humans; II: examine the short-term side effects and to optimise the dosage and schedules; III: determine the statistical efficacy and safety of the new drug candidate</td>
<td>~6</td>
</tr>
<tr>
<td>Application for approval</td>
<td>Government drug administrative authority filing</td>
<td>Request drug approvals</td>
<td>~2</td>
</tr>
<tr>
<td>Phase IV clinical trial</td>
<td>Clinical trials (Phase IV)</td>
<td>Monitor the long-term or low incident adverse events</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>
During the drug discovery stage, the conventional approach for the identification of hits relies on intensive *in vitro* screening of a large number of small molecules using different high-throughput screening techniques [116]. However, the attrition rate (a failure rate associated with pharmaceutical developments) for compounds entering the pre-clinical phase is as high as 50%, and only 70% of the rest of the compound can pass the *in vivo* pre-clinical test and enter Phase I clinical trial [117]. Such a high attrition rate is attributed to lack of efficacy *in vivo*, poor oral bioavailability and unexpected toxicity (poor biochemical specificity) [118]. In fact, despite the investments in drug discovery and development increasing dramatically (USD$50 billion in 2009), the current success rate of new drugs reaching the market is no greater than it was 50 years ago, suggesting there are limitations of the current drug discovery and development model [119].

**1.7 ALTERNATIVE APPROACH FOR NEW DRUG DISCOVERY**

In view of these limitations of random screening, an alternative approach is used in this project to select potential compounds for further investigation.

Firstly, test compounds are selected based on the following criteria to ensure that they have relatively good drug-like properties. 1) Compound selection was limited to ones with known efficacy on related metabolic disorders (see Section 4.1 and 5.1 for details). 2) Compounds of interest must have a chemical structure in favour of modification for chemical diversity. 3) Compounds need to have relatively good oral availability according to the Lipinski’s rule of five (Molecular weight < 500 Daltons; LogP, octanol-water partition coefficient, < 5; Number of hydrogen bond donors < 5; Number of hydrogen bond acceptors < 5 [120]). 4) Only compounds with sufficient quantities for *in vivo* studies were selected in this project.

Secondly, in contrast to the conventional approach with target-based *in vitro* screening as a starting point, an *in vivo* model with basal blood glucose, plasma and liver triglyceride, and
adiposity as functional readouts (phenotypic screening) was used as a second-line screening to select compounds for further detailed characterisation and mechanistic studies [121]. While this thesis focuses on using an in vivo model as a second-line phenotypic screening, a cell-based phenotypic screening was also developed in collaboration with my colleague during this PhD study [122].

**Conventional Approach**

| Target Identification/Validation | Hit Discovery (Cell-based) | Hit-to-Lead, Lead Optimisation | Pre-clinical (Animal-based) | Clinical (Human-based) |

**Alternative Approach**

- **First-line screening**

- **Second-line screening**
  - Lead Identification (Animal-based)

**Figure 1.7 Schematic diagram of different approaches in drug discovery and development.**

### 1.8 SUMMARY, AIMS AND THESIS FOCUS

Diabetes is a metabolic disease characterised by high blood glucose levels. It is becoming increasingly evident that diabetes, which can lead to serious health problems (Section 1.1.2), has become a pandemic (Section 1.1.1), placing a significant economic burden on both individuals and countries (Section 1.1.3). Among different forms of diabetes, T2D is the most common, affecting approximately 90% of people with diabetes around the world. One of the fundamental metabolic defects of T2D is insulin resistance, the inability of tissues to respond to insulin. It is now widely accepted that ectopic lipid accumulation can lead to insulin resistance (Section 1.2), and increased lipid accumulation can result from dysregulated lipid synthesis and/or utilisation (Chapter 1.3). In addition to lifestyle interventions, anti-diabetic
drugs form a significant part of T2D treatments. Despite current anti-diabetic drugs offering effective blood glucose management to T2D patients, their applications are often limited by various side-effects, durability of the treatment effect and/or high treatment-related costs (Section 1.4), suggesting that there is still an urgent need for the discovery of novel anti-diabetic drugs.

Thus, the **Overall Aim** of this thesis was to examine the anti-diabetic effects of two novel compounds, which were identified using an in vivo phenotypic screening, and investigate the underlying mechanism(s) of their therapeutic effects. The investigation presented in this thesis was based on a **Hypothesis** that an in vivo phenotypic screening is a feasible strategy for the discovery of novel anti-diabetic compounds from natural products.

As discussed in Section 1.6, drug discovery and development is a costly and lengthy process. The current approach involved in the phase of drug discovery relies heavily on high-throughput screening of chemical libraries prepared by combinatorial chemistry. However, compounds identified by this approach face a high attrition rate due to issues related to *in vivo* efficacy, bioavailability and drug safety. In view of these challenges, this project focuses on compounds isolated from natural products, which have been shown to have greater chemical diversity and biochemical specificity (Section 1.5).

The first aim, as discussed in Chapter 3, was to **identify compounds with potential therapeutic effects for type 2 diabetes** by data mining with pre-set selection criteria and an *in vivo* screening with glucose tolerance as a functional output. Subsequent studies were dedicated to **investigate the therapeutic efficacy and underlying molecular mechanisms of selected compounds, oleanolic acid (Chapter 4) and matrine (Chapter 5), in a type 2 diabetes (high-fat feeding and streptozotocin) or insulin resistance (high-fat feeding) mouse model.**
Chapter 2
Materials and Methods
2.1 INTRODUCTION

This chapter explains in detail all the common methods and techniques used for the studies presented in Chapters 3, 4 and 5. Special methods and techniques for a particular study are described separately in corresponding chapters. Measurements performed by an external collaborator are indicated with the appropriate acknowledgment.

2.2 ANIMALS

All experimental procedures were approved by the Animal Ethics Committee of the Garvan Institute (#0847) and RMIT University (#1012) in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

Male C57BL/6J mice (10-week old) were purchased from the Animal Resources Centre (Perth, Australia). The animals were kept in a temperature-controlled room (22 ± 1°C) on a 12-h light/dark cycle. Before any procedure was performed, mice were first allowed to acclimatise for one week, during which they were fed ad libitum with a standard lab chow diet.

2.3 PREPARATION OF DIETS

The standard chow diet (CH; Meat Free Rat and Mouse Diet) was purchased from Specialty Feeds, Western Australia. It contains 12% calories from fat, 23% from protein, and 65% from carbohydrate. The digestible energy for this diet is 3.34 kcal/kg.

The high fat diet was prepared in-house using the following recipe (Table 2.1). The digestible energy for this diet is 4.94 kcal/kg.

The high-fat high-cholesterol diet (HFC) was prepared in-house using the same recipe as the HF diet (Table 2.1) with additional 2.28 g (0.2 w/w) of cholesterol (Catalogue No.C75209, Sigma-Aldrich). The digestible energy for this diet is 4.94 kcal/kg.
### Table 2.1 Composition of the high-fat diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Manufacturer</th>
<th>Catalogue No.</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>MPD Dairy Products</td>
<td>ACIDCASEIN</td>
<td>215.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Hudson Pacific Corp.</td>
<td>-</td>
<td>294</td>
</tr>
<tr>
<td>Starch (cornflour)</td>
<td>Hudson Pacific Corp.</td>
<td>FLCO1</td>
<td>173</td>
</tr>
<tr>
<td>AIN-76 Mineral Mixture</td>
<td>MP Biomedicals</td>
<td>0290545502</td>
<td>51</td>
</tr>
<tr>
<td>Trace Minerals</td>
<td>MP Biomedicals</td>
<td>0296026401</td>
<td>14.8</td>
</tr>
<tr>
<td>Bran</td>
<td>Hudson Pacific Corp.</td>
<td>BC20KGA</td>
<td>63</td>
</tr>
<tr>
<td>Methionine</td>
<td>Sigma-Aldrich</td>
<td>M9500</td>
<td>2.8</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Hudson Pacific Corp.</td>
<td>GEPO1</td>
<td>19</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>Sigma-Aldrich</td>
<td>C1629</td>
<td>4.6</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>Stoney Creek Oil Products</td>
<td>SAFFLOWER</td>
<td>30</td>
</tr>
<tr>
<td>Lard</td>
<td>Allowrie</td>
<td>Prime Lard</td>
<td>250</td>
</tr>
<tr>
<td>AIN Vitamin Mix 76 - A</td>
<td>MP Biomedicals</td>
<td>0296009801</td>
<td>14.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>1134.9</strong></td>
</tr>
</tbody>
</table>

#### 2.4 MEASUREMENT OF BLOOD AND PLASMA PARAMETERS

Blood samples were collected using heparinised capillary tubes (SteriHealth Laboratory Products, Australia) to prevent clotting. Blood samples were then immediately mixed with the same volume of saline and stored on ice. Plasma separated by centrifugation (13,000 rpm, 1 min) was transferred to a new eppendorf tube and stored at -80°C for subsequent measurements.

After 5-7 hours of fasting, blood samples were collected from the tail tip and the blood glucose levels were analysed using a glucometer (Accu-Chek Performa Nano; Roche, Australia).
Plasma insulin levels were determined by radioimmunoassay (Merck Millipore; Catalogue No. SRI-13K). Blood insulin levels were determined by a mouse insulin ELISA kit (Linco/Millipore; Catalogue No. 90080).

Blood glucose levels were measured after 5-7 hours of fasting. The triglyceride level in plasma and liver extracts were determined by a Peridochrom triglyceride GPO-PAP kit (Roche Diagnostics), according to the manufacturer’s instructions (Roche Diagnostic, Australia). Briefly, 5 µl of plasma samples were mixed with 300 µl of triglyceride reagent and then incubated at 37°C for 10 min. The absorbance was measured at 485 nm using a FlexStation microplate reader (Molecular Devices, USA). Internal standards (glycerol solution, Sigma-Aldrich, Catalogue No.G5516) were included to create a standard curve ranging from 0 to 1.050 µg/well.

The body weight of mice was measured in the afternoon one day before the glucose tolerance test (GTT) for the calculation of glucose loads. On the day of testing, mice were first fasted for 5-7 hours before glucose was injected intraperitonially at a dose indicated in each study. Blood or plasma samples were obtained from the tail tip at described time points for the measurement of blood glucose or plasma insulin (Section 2.4).

**2.5 TISSUE TRIGLYCERIDE EXTRACTION AND DETERMINATION**

Mice were killed by cervical dislocation and liver samples were immediately freeze-clamped and stored in -80°C. To extract triglyceride, a pre-weighed sample of tissue (30-40 mg liver) was homogenised in 4 ml of chloroform/methanol (2:1) using a glass pestle tissue grinder. After transferring the homogenate to a clean 15 ml tube, the homogeniser was rinsed with another 2 ml of chloroform/methanol (2:1) and added to the homogenate. The tubes were tightly capped and rotated at room temperature overnight to ensure the complete solubilisation of the triglyceride. The next day, 2 ml of 0.6% NaCl was added to the tubes followed by
centrifugation at 2,000 rpm for 10 min to separate the aqueous from the organic phases. The lower chloroform layer contained triglycerides and was carefully transferred into a glass vial and dried completely under the nitrogen or air at 45°C. The extract was reconstituted in 500 µl absolute ethanol and the triglyceride concentration was determined by a Peridochrom triglyceride GPO-PAP reagent (Roche Diagnostics, Catalogue No. 11730711), as described in Section 2.4.

2.6 IMMUNOBLOTTING

Reagents / Buffers The preparation of all reagents and buffers for immunoblotting are summarised as follows (Table 2.2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA buffer</td>
<td>65 mM Trizma® base (Tris. Sigma-Aldrich, Catalogue No. T1503), 150 mM NaCl, 5mM EDTA, 1% Nonide-P 40 Substitute (NP-40. Sigma-Aldrich, Catalogue No. 74385), 0.05% sodium-deoxycholate (Sigma-Aldrich, Catalogue No. D6750), 0.1% (w/v) sodium dodecyl sulphate (SDS. Sigma-Aldrich, Catalogue No. L4390), 10% glycerol (Sigma-Aldrich, Catalogue No. 49770), pH 7.5 and stored at 4°C.</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>10 mM Sodium fluoride (NaF. Sigma-Aldrich, Catalogue No.S7920), 1 mM sodium orthovanadate (Na₃VO₄. Sigma-Aldrich, Catalogue No. S6508), 1 mM phenylmethanesulfonyl fluoride (dissolved in 100% ethanol. Sigma-Aldrich, Catalogue No. 78830), and 10 µl/ml protease/phosphatase inhibitor (Sigma-Aldrich, Catalogue No. P5726) in RIPA buffer.</td>
</tr>
<tr>
<td>4x Laemmli’s buffer</td>
<td>8.2 g SDS, 40ml glycerol, 50 ml 0.5 M Tris, 500 µl 1% bromo-phenol blue (Sigma-Aldrich, Catalogue No. 114391) in dH₂O, pH 6.8 and stored at -20°C. 6.2 mg DL-dithiothreitol (DTT, Sigma-Aldrich, Catalogue No. D9779) are added before use.</td>
</tr>
</tbody>
</table>
**Chapter Two – Methods**

<table>
<thead>
<tr>
<th>Name</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Running buffer</td>
<td>30 g Tris, 144 g glycine (Sigma-Aldrich, Catalogue No. G8898) and 10 g SDS in dH₂O, pH 8.8 and stored at room temperature.</td>
</tr>
<tr>
<td>(1 L)</td>
<td></td>
</tr>
<tr>
<td>10x Transfer buffer</td>
<td>30 g Tris, 144 g glycine in dH₂O and stored at room temperature.</td>
</tr>
<tr>
<td>(1L)</td>
<td></td>
</tr>
<tr>
<td>10x TBS (1 L)</td>
<td>24.2 g Tris and 80 g NaCl in dH₂O, pH 7.6 and stored at room temperature.</td>
</tr>
<tr>
<td>1x TBST (1 L)</td>
<td>100 ml 10x TBS buffer and 500 µl Tween® 20 (Sigma-Aldrich, Catalogue No. P9416) in 900 ml dH₂O and stored at room temperature.</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>3% (w/v) Bovine serum albumin (BSA. Sigma-Aldrich, Catalogue No. A9418) in 1x TBST and stored at 4°C.</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>6.25% 1 M Tris-HCl (pH 6.7), 10% (w/v) 20% SDS in dH₂O and stored at room temperature. 100 mM 2-mercaptoethanol (Sigma-Aldrich, Catalogue No. M7154) is added before use.</td>
</tr>
<tr>
<td>(1L)</td>
<td></td>
</tr>
</tbody>
</table>

**Tissue homogenisation** Freeze-clamped tissues were homogenised in ice-cold RIPA lysis buffer and solubilised for 2 hr at 4°C. After centrifugation at 15,000 rpm for 10 min at 4°C to eliminate debris, the supernatant from each homogenised sample was collected and stored at -80°C.

**Protein quantification** Protein concentrations were determined by a commercial colorimetric protein assay kit (Bicinchoninic acid kit, Sigma-Aldrich, Catalogue No.B9643). 5 µl of sample was mixed with 200 µl of reagent mix (reagent A:B = 50:1), and incubated at 37°C for 30 min prior to the determination of absorbance by spectrophotometry at 562 nm using a FlexStation microplate reader (Molecular Devices, USA). In each protein assay, a set of solution with different concentrations of BSA protein (0-2 µg/ml) was included as standards.
SDS-PAGE Tris-glycine based acrylamide gels with different percentages of polyacrylamide were prepared for separation of proteins according to their molecular weights (Table 2.3). Polymerisation of the gel was facilitated by the addition of APS and TEMED. After polymerisation of the running gel (Table 2.4), stacking gel (Table 2.5) was poured on top of the running gel and polymerised.

An aliquot of tissue lysate from each sample was diluted with dH2O to the same concentration (e.g. 2.5 μg/μl) containing 1x Laemmli’s buffer. The samples were heated at 37°C for 30 min prior to SDS-PAGE. Equal amounts of protein (20- 50 μg) were loaded into the stacking gels. A protein ladder (Bio-Rad Laboratories Inc., Australia; Catalogue No. 161-0374) was loaded as a reference for the estimation of molecular sizes. The gels were run at 100 V until the markers above or below the protein of interest were well resolved.

Table 2.3 Recommended Polyacrylamide % for Separation in Denaturing Gels

<table>
<thead>
<tr>
<th>Polyacrylamide %</th>
<th>Effective range of separation (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>25 - 200</td>
</tr>
<tr>
<td>10</td>
<td>15 - 100</td>
</tr>
<tr>
<td>12.5</td>
<td>10 - 70</td>
</tr>
<tr>
<td>15</td>
<td>12 - 45</td>
</tr>
<tr>
<td>20</td>
<td>4 - 40</td>
</tr>
</tbody>
</table>

Table 2.4 Composition of the running gel.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume for 2 gels with different polyacrylamide %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8%</td>
</tr>
<tr>
<td>1.5M Tris Buffer, pH 8.8</td>
<td>5</td>
</tr>
<tr>
<td>30% Acrylamide/Bis-acrylamide</td>
<td>5.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2</td>
</tr>
<tr>
<td>dH2O</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Table 2.5 Composition of the stacking gel.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume (ml) for 2 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris Buffer, pH 8.8</td>
<td>5</td>
</tr>
<tr>
<td>30% Acrylamide/Bis-acrylamide</td>
<td>5.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2</td>
</tr>
<tr>
<td>dH2O</td>
<td>9.5</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.2</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Immunoblotting** Following the separation of protein by SDS-PAGE, proteins were then transferred from the gels to methanol-activated PVDF membranes (Bio-Rad Laboratories Inc., USA) at 90 V for 90 min. Non-specific binding was blocked with blocking buffer for 1 hour at room temperature. The membranes were then incubated with specific primary antibodies (Table 2.6) for 2 hr at room temperature or overnight at 4°C.

After blocking, the membranes were washed with TBST 6 times (10 min each time), to remove any unbounded antibody. The membranes were subsequently incubated with an appropriate secondary antibody (1:3000 dilution in TBST) (Table 2.6) for 2 hr at room temperature. The membranes were washed again with TBST 6 times (10 min each time) to remove excess unbounded secondary antibody. The bound antibody was detected using a chemiluminescence system with Western Lighting Ultra Solution (Perkin Elmer, Catalogue No.NEL113001EA). The membranes were exposed in a ChemiDoc (Bio-Rad Laboratories
Inc., USA) for images capturing. Quantitative densitometry analysis of bands of interest was performed using Image Lab software (version 4.0. Bio-Rad Laboratories Inc., USA).

**Table 2.6 Antibody list.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA Carboxylase</td>
<td>Cell Signaling</td>
<td>3662</td>
</tr>
<tr>
<td>Acetyl-CoA Carboxylase (Phospho-Ser79)</td>
<td>Cell Signaling</td>
<td>3661</td>
</tr>
<tr>
<td>ACOX1 (H-140)</td>
<td>Santa Cruz</td>
<td>sc-98499</td>
</tr>
<tr>
<td>Akt</td>
<td>Cell Signaling</td>
<td>9272</td>
</tr>
<tr>
<td>Akt (Phospho-ser473)</td>
<td>Cell Signaling</td>
<td>9271</td>
</tr>
<tr>
<td>AMPKα</td>
<td>Cell Signaling</td>
<td>2532</td>
</tr>
<tr>
<td>AMPKα (Phospho-Thr172)</td>
<td>Cell Signaling</td>
<td>2535</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Santa Cruz</td>
<td>sc-47778</td>
</tr>
<tr>
<td>Fatty Acid Synthase (C20G5)</td>
<td>Cell Signaling</td>
<td>3180</td>
</tr>
<tr>
<td>FoxO1 (L27)</td>
<td>Cell Signaling</td>
<td>9454</td>
</tr>
<tr>
<td>FoxO1 (Phospho-Ser256)</td>
<td>Cell Signaling</td>
<td>9461</td>
</tr>
<tr>
<td>HSF1</td>
<td>Cell Signaling</td>
<td>4356</td>
</tr>
<tr>
<td>HSP70/72</td>
<td>Enzo</td>
<td>C92F3A-5</td>
</tr>
<tr>
<td>HSP90α, mAB (9D2) (HRP conjugate)</td>
<td>Enzo</td>
<td>ADI-SPA-840HRP</td>
</tr>
<tr>
<td>SAPK/JNK</td>
<td>Cell Signaling</td>
<td>9252</td>
</tr>
<tr>
<td>SAPK/JNK (Phospho-Thr183/Tyr185)</td>
<td>Cell Signaling</td>
<td>9251</td>
</tr>
<tr>
<td>SCD1 (C12H5)</td>
<td>Cell Signaling</td>
<td>2794</td>
</tr>
<tr>
<td>Name</td>
<td>Supplier</td>
<td>Catalogue No.</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>SP1</td>
<td>Cell Signaling</td>
<td>5931</td>
</tr>
<tr>
<td>SREBP-1 (K-10)</td>
<td>Santa Cruz</td>
<td>sc-367</td>
</tr>
<tr>
<td>α-Tubulin (DM1A)</td>
<td>Cell Signaling</td>
<td>3873</td>
</tr>
<tr>
<td>Mito Mix</td>
<td>MitoScience</td>
<td>MS604</td>
</tr>
<tr>
<td>Goat Anti Mouse</td>
<td>Santa Cruz</td>
<td>sc-2005</td>
</tr>
<tr>
<td>Goat Anti Rabbit</td>
<td>Santa Cruz</td>
<td>sc-2004</td>
</tr>
<tr>
<td>Donkey Anti Rabbit</td>
<td>Santa Cruz</td>
<td>sc-2020</td>
</tr>
</tbody>
</table>

2.7 REAL-TIME PCR

**Isolation of RNA from animal tissues** Liver tissues (20-30mg) were homogenised in 1 ml TRIZOL® reagent (Invitrogen, Catalogue No.15596026). The homogenate was mixed with 200 µl of Chloroform (VWR, Catalogue No.22711324) by inverting several times, and incubated at room temperature for 5 min. The homogenates were subsequently centrifuged at 13,000 rpm for 15 min at 4°C. The upper aqueous phase containing RNA was collected and mixed with 500 µl of isopropanol (Sigma-Aldrich, Catalogue No.I9516). After another centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant was removed. The remaining RNA precipitate was washed twice with 500 µl 75% ethanol with vortex. The ethanol was removed by centrifugation at 13,000 rpm for 5 min at 4°C. The air-dried RNA pellet was dissolved in 100 µl of DEPC-treated water (Invitrogen, Catalogue No.AM9916) for the measurement of RNA concentration.

**Measurement of RNA concentration** The RNA purity and concentrations were assessed using a NanoDrop Spectrophotometer (Eppendorf Thermo Scientific, Australia) at the
absorbance of 260 and 280 nm, with DEPC water as a blank. The absorption ratio of 260/280 nm is used to assess the purity of RNA samples (usually around 2).

**Complimentary DNA synthesis by reverse transcription** Purified RNA with known concentrations was used to generate the complementary DNA (cDNA) using a Reverse Transcription System (Bio-Rad Laboratories Inc., USA) with random primers according to the manufacturer’s instructions. The RNA concentration of each sample was normalised to 1 µg/8 µl with DEPC water on ice. To remove DNA contamination, 1 µg of RNA was mixed with 2 µl of DNase I (Invitrogen, Catalogue No.18068-015), and incubated at room temperature for 15 min. After the removal of DNA, 1 µl of 25 mM EDTA was added and incubated for 10 min at 65°C to inactivate DNase I. 2 µl of purified RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Life Technologies, Catalogue No. 4374967) (2 µl of reverse transcription buffer, 0.8 µl of dNTP mix, 2 µl of random primers, 1 µl of reverse transcriptase and 12.2 µl of DEPC water). Reverse transcription polymerase chain reaction was carried out using the following steps: equilibrated at 25°C for 10 min, 37°C for 2 hr, 85°C for 5 sec, and finally maintained at 4°C. The cDNA products from reverse transcription reactions were stored at 4°C to use for real time-PCR analysis.

**Real-time polymerase chain reaction** The cDNA samples were analysed for genes of interest by real-time polymerase chain reaction (rt-PCR) using the SYBR Green real-time PCR system (Bio-Rad Laboratories Inc., USA). A reaction master mixture (1x IQ SYBR Green Supermix (Bio-Rad Laboratories Inc., USA; Catalogue No.170-8882), 500 nM forward primers and 500 nM reverse primers, DEPC water to a final volume of 24 µl) for each gene of interest was prepared and added to each 1 µl cDNA sample in a sterile 96-well plate. The plate was placed in a controlled-temperature heat block equilibrated at 50°C for 2 min, 95°C for 3 min and 40-50 cycles of 95°C for 15 seconds, 72°C for 30 seconds. The gene expression from each sample was analysed in duplicates and normalised against the ribosomal
housekeeper gene 18S (GeneWorks, Australia). All reactions were performed on the iQ™ 5 Real-time PCR Detection System (Bio-Rad Laboratories Inc., USA). The results are expressed as relative gene expression using the ΔCt method. Primers used for specific genes are in Table 2.7.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Forward: 5’-CGCCGCTAGAGGTGAAATTCT</td>
</tr>
<tr>
<td></td>
<td>Reversed: 5’-CGAACCTCCGACTTTCGTCT</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Forward: 5’-CCACAGCTGCTGCAAGACA</td>
</tr>
<tr>
<td></td>
<td>Reversed: 5’-GAAGGGTCGATGGCAGAAA</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Forward: 5’-AACGCCTTCTATGTCTCTTTTC</td>
</tr>
<tr>
<td></td>
<td>Reversed: 5’-GTGTTGCCTAGTAGTGGGATG</td>
</tr>
<tr>
<td>TNFα</td>
<td>Forward: 5’-CACAAGATGCTGGGACAGTGA</td>
</tr>
<tr>
<td></td>
<td>Reversed: 5’-TCCTTGTGGTGTTGTCATGA</td>
</tr>
</tbody>
</table>

**2.8 STATISTICAL ANALYSES**

Data are presented as means ± SE in this thesis. Two-tailed Student's t-test was used for comparison of relevant groups. Pearson's two-sided correlation was used for the correlation analysis. The analysis was performed using GraphPad Prism software (5.0, GraphPad Software Inc, CA, USA). Differences at p < 0.05 were considered to be statistically significant.
Chapter 3
Identification of Investigational Compounds
3.1 INTRODUCTION

As discussed in Section 1.6, there are different screening approaches in drug discovery and development to identify compounds of interest. Target-based high-throughput screening is widely used in the pharmaceutical industry in combination with synthetic compound libraries. While this approach can rapidly identify compounds modulating a particular molecular target, in vitro and/or in vivo phenotypic screening is able to identify compounds with end-point biological responses. This thesis project employed the phenotypic screening strategy as it is more successful in producing first-in-class drugs [123] and is a strength of my laboratory [122, 124]. In parallel with the in vivo phenotypic screening described in this thesis, a lipogenesis-based in vitro high-throughput screening was developed in my laboratory [122].

The first study of this thesis project aims to identify two compounds from natural products with potential therapeutic effects for T2D. These test compounds were selected as described below from the compound library established at the Molecular Pharmacology of Diabetes group of RMIT University. Briefly, two lines of screening, involving structural analysis and an in vivo screening with functional responses as end-point readouts, were applied in the selection of potential compounds for further investigation as described in Section 1.7.

Compounds with known chemical structure are first subjected to a structural analysis to estimate their bioavailability and potential for chemical modification for the generation of derivatives. The abundance of their derivatives will be investigated by consulting published literature (e.g. PubMed).

In addition to the structural analysis and data mining in the literature, the suitability of test compounds for further investigation will be assessed in a pilot in vivo study with a T2D mouse model. As described previously (Chapter 1), insulin resistance and β-cell dysfunction are two fundamental defects of T2D [5]. An established animal model of T2D is to use high-
fat feeding and streptozotocin injection to induce hyperglycaemia with insulin resistance and β-cell failure [125, 126]. This animal model provides a valuable system for the investigation of potential therapeutic effects of test compounds for T2D at the whole body level. Excess lipid accumulation in non-adipose tissues involved in glucose regulation (e.g. liver and muscle) is the most common cause of insulin resistance as discussed in Section 1.2.2. Compounds that reduce ectopic lipid accumulation have been shown to improve glucose homeostasis by reversing lipid-induced insulin resistance [92, 127]. Therefore, the effect of test compounds on liver triglyceride (in addition to basal blood glucose levels) is used as a functional output for the selection of potential compounds for further detailed investigations.

3.2 MATERIAL AND METHODS

3.2.1 Test Compounds

Test compounds (purity > 99.5%) extracted from natural products were a gift from Prof. Yang Ye and Prof. Li-Hong Hu from Shanghai Institute of Material Medica. The natural sources and chemical structures of these compounds are summarised in Table 3.1.

Table 3.1 Origins and chemical structures of test compounds

<table>
<thead>
<tr>
<th>Name</th>
<th>Natural sources</th>
<th>Chemical structure and formula</th>
</tr>
</thead>
</table>
| Oleanolic acid (OA) | *Phytolacca americana*  
*Syzygium spp*  
*Olea europaea* L. | ![Chemical Structure](image)  
C_{30}H_{48}O_{3} |
<table>
<thead>
<tr>
<th>Name</th>
<th>Natural sources</th>
<th>Chemical structure and formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrine (Mtr)</td>
<td><em>Sophora flavescens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>THA-E (code name)</td>
<td></td>
<td>(Confidential)</td>
</tr>
<tr>
<td>QH (code name)</td>
<td></td>
<td>(Confidential)</td>
</tr>
</tbody>
</table>

### 3.2.2 Type 2 Diabetes in vivo Model

Male C57BL/6J mice (10-week old) were purchased and acclimatised as described in Section 2.2. After acclimatisation, mice were fed *ad libitum* for five weeks with a standard lab chow diet (CH. Section 2.3) or a high-fat diet (HF. Section 2.3) in order to induce insulin resistance [124, 128-130]. One group of CH- or HF-fed mice were then injected with either vehicle (saline) or a low dose of streptozotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce diabetes (fasting blood glucose > 12 mM) [125, 126]. CH-fed mice treated with STZ comprised a model of type-1 diabetes (T1D mice). HF-fed mice treated with STZ comprised a model of type-2 diabetes (T2D mice). One week after the last STZ injection (‘baseline’), a subset of T2D mice received test compounds as a food additive at indicated dosage (OA, Mtr, QH: 100 mg/kg/day; THA-E: 300 mg/kg/day) for four weeks, while the remaining T1D and T2D mice continued to receive their normal HF diet (T2D-Veh). Body weight, food intake and fasting blood glucose levels were monitored on a weekly basis.

### 3.2.3 Measurement of Blood Glucose, Plasma and Liver Triglyceride

Blood glucose levels were measured once a week using a glucometer as described in Section 2.4. Plasma and liver triglyceride were determined as described in Sections 2.4 and 2.5.
3.3 RESULTS

3.3.1 Assessment of Bioavailability

A structure-based analysis using PubChem Compound (ncbi.nlm.nih.gov/pccompound) suggested that both OA and Mtr are small molecules with a molecular weight less than 500. The number of H-bond donor or acceptor of these two compounds is within the range (<5 H-bond donor or acceptor) stated by Lipinski’s rule of 5 [131]. While Mtr has a favourable LogP value of 1.6, OA has a LogP value of 7.5, which falls outside the parameter cutoff (LogP < 5) (Table 3.2). Due to confidentiality reasons, the chemical structure of THA-E and QH were not disclosed and hence no structure-based analysis was performed.

Table 3.2 Drug likeness score for test compounds.

<table>
<thead>
<tr>
<th>Lipinski’s rule of five</th>
<th>OA</th>
<th>Mtr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>456.7</td>
<td>248.4</td>
</tr>
<tr>
<td>H-bond donor</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>H-bond acceptor</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>LogP</td>
<td>7.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>

OA, oleanolic acid; Mtr, matrine.

3.3.2 Induction of Type-1 and Type-2 Diabetes

A type 1 or 2 diabetes model of hyperglycaemia was first induced by STZ injections without or with HF feeding respectively based on previous reports [125, 126]. As shown in Table 3.3, HF feeding resulted in a ~50% increase of liver triglyceride compared to CH (Figure 3.1), despite no significant increase in body weight or adiposity. The lack of apparent increases in body weight was expected with the homemade HF diet. The circulating levels of glucose (Table 3.3) and triglyceride (Table 3.3) were similar between HF and CH mice. As expected,
CH-fed mice demonstrated moderate increases in blood glucose (~30%, $p < 0.05$ compared to CH mice) and plasma triglyceride (~20%, $p < 0.05$) after streptozotocin treatment. HF-fed mice treated with streptozotocin (T2D-Veh) recapitulated several major characteristic metabolic disorders in type-2 diabetes, namely hyperglycaemia (~80% increase, $p < 0.01$) and hepatic steatosis (50% increase) compared to CH mice. Compared with HF feeding alone, T2D mice displayed hyperglycaemia (100%, $p < 0.01$) and similar levels of plasma and liver triglyceride. Compared with T1D, T2D mice showed greater hyperglycaemia (~40%) (Table 3.3).
Table 3.3 Metabolic profiles before and after drug/vehicle treatments.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Blood glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0</td>
<td>Week 4</td>
</tr>
<tr>
<td>CH-Veh</td>
<td>28.9 ± 0.6</td>
<td>29.2 ± 0.6</td>
</tr>
<tr>
<td>T1D-Veh</td>
<td>27.9 ± 0.4</td>
<td>29.1 ± 0.6</td>
</tr>
<tr>
<td>HF-Veh</td>
<td>28.7 ± 0.7</td>
<td>30.5 ± 1.1</td>
</tr>
<tr>
<td>T2D-Veh</td>
<td>27.7 ± 0.5</td>
<td>27.3 ± 0.7</td>
</tr>
<tr>
<td>T2D-OA</td>
<td>27.3 ± 0.6</td>
<td>25.7 ± 0.8**</td>
</tr>
<tr>
<td>T2D-Mtr</td>
<td>25.7 ± 0.4**#</td>
<td>25.1 ± 0.4**#</td>
</tr>
<tr>
<td>T2D-THA-E</td>
<td>26.6 ± 0.4**</td>
<td>26.4 ± 0.4**</td>
</tr>
<tr>
<td>T2D-QH</td>
<td>25.7 ± 0.7*</td>
<td>24.0 ± 0.7**#</td>
</tr>
</tbody>
</table>
Table 3.3 Metabolic profiles before and after drug/vehicle treatments (continued).

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Intake (g/day/mouse)</th>
<th>Plasma TG Week 0 (mM)</th>
<th>EPI (g)</th>
<th>EPI/BW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-Veh</td>
<td>-</td>
<td>1.6 ± 0.1</td>
<td>0.43 ± 0.04</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>T1D-Veh</td>
<td>-</td>
<td>1.9 ± 0.1</td>
<td>0.36 ± 0.03</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>HF-Veh</td>
<td>2.9</td>
<td>1.4 ± 0.1</td>
<td>0.71 ± 0.29</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>T2D-Veh</td>
<td>2.8</td>
<td>1.9 ± 0.2</td>
<td>0.40 ± 0.05</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>T2D-OA</td>
<td>2.3</td>
<td>-</td>
<td>0.31 ± 0.03*</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>T2D-Mtr</td>
<td>2.5</td>
<td>-</td>
<td>0.29 ± 0.02**†</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>T2D-THA-E</td>
<td>2.5</td>
<td>-</td>
<td>1.16 ± 0.02**††</td>
<td>4.4 ± 0.1**††</td>
</tr>
<tr>
<td>T2D-QH</td>
<td>2.8</td>
<td>-</td>
<td>0.30 ± 0.04*</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

C57BL/6J mice were fed a chow diet and injected with saline (CH-Veh) or streptozotocin (STZ, T1D-Veh), or fed a HF diet and injected with saline (HF-Veh) or STZ (T2D-Veh). Blood samples were collected from mice in the 5-6 hour fasted state. One week after the final injection of STZ, a subset of T2D mice received test compounds as a food additive for four weeks. BW, body weight; EPI, epididymal fat pad; Mtr, matrine; OA, oleanolic acid; TG, triglyceride. Data are expressed as means ± SE. * p < 0.05, ** p < 0.01 vs. CH; † p < 0.05, †† p < 0.01 vs. HF; ## p < 0.01 vs. T2D. n = 5 - 8 per group.
Figure 3.1 Effects of test compounds on plasma and liver triglyceride. Plasma (A) or liver (B) triglyceride level was assessed after four weeks of drug treatments. Mice were euthanized following a 5-7 hour fast, and livers and plasma were collected for the measurement of triglyceride levels. CH, chow diet; HF, hig-fat diet; Mtr, matrine; OA, oleanolic acid; STZ, streptozotocin; T1D-Veh, CH-fed mice with STZ injections; T2D-Veh, HF-fed mice with STZ injections. n.d., not determined. * p < 0.05 vs. CH; # p < 0.05 vs. HF; † p < 0.01, †† p < 0.01 vs. T2D-Veh, n = 5 - 8 per group.

3.3.3 Effects of Test Compounds on Body Weight, Food Intake and Adiposity

As shown in Table 3.3, the control T2D mice maintained similar body weight before and after the four-week drug treatment (27.7 ± 0.5 vs. 27.3 ± 0.7 g). Compared to the T2D control mice, there was no significant body weight change in mice treated with Mtr, THA-E and QH. OA
treatment induced a significant body weight loss after two weeks of treatment (-6.4 ± 0.4 vs. 0.5 ± 0.3 g compared to T2D, p < 0.01) probably due to reduced food intake (2.0 vs. 2.8 g/mouse/day compared to T2D). The body weight of OA-treated mice recovered after OA was removed from the diet at the end of week 2, and the overall body weight change and adiposity was not different from the T2D mice. In contrast to Mtr, OA and QH, which had a neutral effect on the adiposity of T2D mice, THA-E induced a marked increase in the epididymal fat/body weight ratio (2-fold increase, p < 0.01). Due to the housing design (5 mice per cage, hence n = 1), no statistical analysis was performed for food intake. All drugs except QH seemingly reduced the food intake slightly. More careful planning of the housing design (e.g. to increase the total number of mice or reduce the number of mice per cage in order to increase the n number for statistical analysis) and monitoring of food intake should be considered in following experiments to avoid the impact of altered food intake on the interpretation of metabolic effects.

### 3.3.4 Effects of Test Compounds on Blood Glucose

During the four-week drug treatment period, there was a significant increase of blood glucose in T2D mice compared to both CH and HF mice (4.7 ± 0.8 vs. 0.5 ± 0.3 and 1.1 ± 1.2 mM respectively). All test compounds except QH suppressed the increase of blood glucose in T2D mice. This anti-hyperglycaemic effect was most profound in OA-treated mice (-8.7 ± 1.3 vs. 4.7 ± 0.8 compared to T2D mice) (Table 3.3).

### 3.3.5 Effects of Test Compounds on Plasma and Liver Triglyceride

There was a mild increase of plasma triglyceride in T2D mice at the beginning of the drug treatment (Table 3.3). At the end of the four-week drug treatment period, T2D mice had significantly higher levels of plasma (~50% increase) and liver (~50% increase) triglyceride compared to CH-fed mice. Both Mtr and THA-E treatments normalised both plasma (p =
triglyceride levels in T2D mice. While a trend of increased liver triglyceride level was observed in OA-treated T2D mice, OA normalised the triglyceride level in the plasma (p < 0.05). QH had no effect on either plasma or liver triglyceride level (Figure 3.1).

3.4 DISCUSSION

The study in this Chapter investigated the drug likeness of four natural products, namely Mtr, OA, THA-E and QH, and their metabolic effects on body weight, adiposity, hyperglycaemia, dyslipidaemia and hepatic lipid accumulation in a T2D mouse model. Results from this study showed that OA and Mtr might have good oral availability according to the Lipinski’s rule of five. Furthermore, these two compounds demonstrated exciting anti-hyperglycaemic effects in a diabetic mouse model resulting from high-fat feeding and streptozotocin treatment. Both OA and Mtr normalised plasma triglyceride level in T2D mice, and Mtr further alleviated ectopic lipid accumulation in the liver. While THA-E had similar effects of Mtr in reducing blood glucose, plasma and liver triglyceride levels, it resulted in a marked increase of adiposity. QH had no effect on body weight gain, blood glucose, and plasma and liver triglyceride levels. Collectively, these results suggest OA and Mtr are promising candidates for further investigations on their anti-diabetic effects and underlying mechanisms.

Lipinski’s rule of five, which was based on an analysis of the World Drug Index, identifies several key chemical properties that are important for the pharmacokinetics of a chemical compound in the human body [131]. It provides useful guidelines for the design of orally bioavailable small-molecule drugs, as a compound with more violations of these rules is more likely to be problematic in the drug development. For example, if the molecular weight of a lead compound is relatively large, the further increase in molecular weight, which often happens during the lead optimisation to increase the potency and selectivity, can lead to safety
and tolerability issues. While the chemical structure of THA-E and QH were not disclosed due to confidentiality reasons, a structure-based analysis showed that Mtr had full compliance with Lipinski’s rule of five and OA had one violation (LogP > 5). LogP, or octanol-water partition coefficient, is an indication of the ability of a compound to cross biological membranes. Lipinski et al. states explicitly that natural products or substrates with biological transporters are exceptions to the rule [131]. Therefore, Mtr and OA, as natural products, are likely to have good oral availability despite OA exceeding the cutoff of LogP value.

During the lead optimisation stage in drug discovery and development (see Section 1.6), the chemical structure of lead compounds is modified to improve deficiencies while maintaining favourable properties and generating potential back up compounds [116]. Therefore, compounds with reaction sites for chemical modification or a diverse range of derivatives would potentially facilitate this process, e.g. different derivatives of the same compound can provide useful information for structure-activity relationship studies. Examination of the chemical structure of OA shows that it has two O-H bonds, to which different functional groups can be attached [132]. Furthermore, a variety of chemical modifications can be applied to various sites on the pentacyclic structure of OA [133]. At least four reaction sites for chemical modifications have been reported for Mtr in the literature [134], and more than 15 Mtr-like alkaloids can be isolated from Sophora flavescens alone [135]. Therefore, OA and Mtr might be good candidates for drug development due to their favourable structural features and the existence of their many derivatives.

It is well established that there is a strong correlation between hyperglycaemia and the risk of macro- or micro-vascular complications in T2D [136]. Thus, reducing hyperglycaemia and maintaining stable blood glucose over time is one of the major goal of anti-diabetic agents as discussed in Section 1.4. In the study described in this Chapter, HF-feeding and streptozotocin were used to induce hyperglycaemia in a mouse model with the two fundamental defects of
T2D, namely insulin resistance and β-cell failure. Under this setting, OA, Mtr and THA-E treatment achieved a significant reduction of blood glucose at the basal level. While this result is encouraging, further careful characterisations (e.g. glucose tolerance, insulin secretion profile and food intake) are required to confirm their efficacy in achieving effective blood glucose control, and guide the investigation of their molecular mode of action.

Improving insulin resistance is one of the therapeutic approaches for the treatment of T2D. A number of current anti-diabetic drugs have been shown to improve insulin resistance, e.g. metformin [137], thiazolidinediones [138] and dopamine-2 receptor agonist [139]. It is widely accepted that ectopic lipid accumulation can contribute to insulin resistance (as discussed in Section 1.2.2). Importantly, a modest weight loss, which reversed hepatic steatosis, has been shown to improve hepatic insulin resistance and normalise fasting hyperglycaemia in patients with T2D [140]. In the current study, Mtr and THA-E effectively normalised both plasma and liver triglyceride levels, which were up-regulated along with the induction of hyperglycaemia in the T2D mouse model. These results indicated that the anti-hyperglycaemic effects of these compounds might involve an improvement of lipid metabolism in tissues involved in blood glucose regulation. However, while THA-E improved the ectopic lipid accumulation in the liver, it resulted in a marked increase of epididymal fat pad weight. This is similar to the known side-effects of PPARγ agonists, which improves insulin sensitivity in peripheral tissues by sequestering lipids in adipose tissue [92] resulting in increased adiposity.

In summary, OA and Mtr are two natural products that have good drug-likeness and chemical structures that favour derivatisation. They exhibited encouraging metabolic effects to reduce hyperglycaemia, dyslipidaemia and/or hepatic steatosis without increasing adiposity (in contrast to THA-E) in type 2 diabetic mice (Table 3.4). Therefore, OA and Mtr were selected for further detailed investigation on their anti-diabetic effects and molecular mechanism in this PhD thesis.
### Table 3.4 Summary of the drug-likeness and metabolic effects of test compounds.

<table>
<thead>
<tr>
<th></th>
<th>Oleanolic Acid</th>
<th>Matrine</th>
<th>THA-E</th>
<th>QH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug-likeness</td>
<td>Good</td>
<td>Good</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Derivatisation</td>
<td>Good</td>
<td>Good</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BW Gain</td>
<td>= or ↓</td>
<td>=</td>
<td>=</td>
<td>=  or ↓</td>
</tr>
<tr>
<td>Adiposity</td>
<td>=</td>
<td>=</td>
<td>↑↑</td>
<td>=</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>↓↓</td>
<td>↓</td>
<td>↓</td>
<td>=</td>
</tr>
<tr>
<td>Plasma TG</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>n.d.</td>
</tr>
<tr>
<td>Liver TG</td>
<td>=</td>
<td>↓</td>
<td>↓</td>
<td>=</td>
</tr>
</tbody>
</table>

The drug-likeness of different compounds was assessed according to the Lipinski’s rule of five. The metabolic effects on body weight (BW) gain, adiposity, basal blood glucose, and plasma and liver triglyceride (TG) was examined in a type 2 diabetes mouse model induced by high-fat feeding and streptozotocin treatment. TG, triglyceride. Detailed results are shown in Table 3.2, Table 3.3 and Figure 3.1.
Chapter 4
Sustained Glucose-lowering Effect of Oleanolic Acid
4.1 INTRODUCTION

Oleanolic acid (OA) is a member of the triterpenoid family, of which there are more than 20,000 members existing in nature [141]. A recent study showed that triterpenoid compounds isolated from bitter melon have potent efficacy in stimulating GLUT4 translocation in L6 myotubes and 3T3L1 adipocytes, along with activation of the AMPK pathway [124]. Acute studies in mice showed that triterpenoids are able to reduce glucose intolerance in insulin resistant HF-fed mice after a single injection [124]. These results collectively raised the attractive possibility that members of the triterpenoid family can be potential therapeutic agents for T2D.

Indeed, the in vivo study in Chapter 3 showed that two-week OA treatment significantly normalised hyperglycaemia in HF- and STZ-induced T2D mice. Furthermore, an OA analogue has been shown to alleviate diabetic nephropathy in type-2 diabetic patients [142]. OA and its analogues have been shown to lower hyperglycaemia in STZ-treated rodents [143], HF-fed or db/db mice [144], to protect against diabetic nephropathy [143] and to enhance the survival of pancreatic islets [145]. However, all of these studies were relatively short-term (mostly less than two weeks) and the sustainability of these therapeutic effects is not known. Thus, the aim of this study was to investigate of the long-term therapeutic efficacy of OA in reducing hyperglycaemia in a diabetic mouse model produced by chronic HF feeding combined with low doses of STZ.

The results presented in this Chapter demonstrate a potent glucose-lowering effect of OA that was sustained well beyond the treatment period and was accompanied by a significant improvement in renal structures. Furthermore, Akt/FoxO1-mediated suppression of glucose-6-phosphatase (G6Pase), a key regulator of hepatic glucose production, was identified as a likely mechanism underlying the improved glucose homeostasis.
4.2 MATERIALS AND METHODS

4.2.1 Animal Model

Male C57BL/6J mice (10-week old) were purchased and acclimatised as described in Section 2.2. After acclimatisation, mice were fed *ad libitum* for 10 weeks a standard lab chow diet (CH. Section 2.3) or a high-fat diet (HF. Section 2.3) in order to induce insulin resistance [124, 128-130]. Mice were then injected with either vehicle (saline) or a low dose of streptozotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce diabetes (fasting blood glucose > 12 mM) [125, 126]. CH-fed mice treated with STZ comprised a model of type-1 diabetes (T1D mice). HF-fed mice treated with STZ comprised a model of type-2 diabetes (T2D mice). One week after the last STZ injection (‘baseline’), a subset of T1D and T2D mice received OA as a food additive at 100 mg/kg/day for two weeks (T1D-OA and T2D-OA respectively). This dose of OA was selected on the basis of a previous study [124]. The remaining T1D and T2D mice received their normal CH or HF diet (T1D-Veh and T2D-Veh, respectively)(Figure 4.1).

**Figure 4.1** Experimental design of the oleanolic acid study. 10-week old male C57BL/6J mice were acclimatised for one week before receiving a chow (CH) or a high-fat (HF) diet for 10 weeks. Mice were then injected with either vehicle (saline) or a low dose of streptozotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce hyperglycaemia. One week after the last STZ injection, a subset of mice received OA as a food additive at 100 mg/kg/day for two weeks. Glucose tolerance test (GTT) was performed two weeks after the removal of OA.
4.2.2 Measurement of Metabolic Parameters and Glucose Tolerance

Blood glucose levels were measured once a week as described in Section 2.4. Plasma and liver triglyceride were determined as described in Section 2.4 and 2.5. Two weeks after the cessation of OA treatment, glucose tolerance tests (GTT; glucose load 1 g/kg BW, i.p.) were performed as described in Section 2.4. During the GTT, blood samples were taken at 0, 15, 30 and 60 min for the measurement of blood glucose levels. Plasma samples were collected at 0, 5, 30 and 60 min for the measurement of insulin levels using ELISA (Section 2.4).

4.2.3 Insulin Secretion Assays and Insulin Content Measurements

Islet isolation and ex vivo insulin secretion assays were performed as previously described [146]. Briefly, mice were killed by cervical dislocation and the pancreas was perfused with 2 ml of Liberase (Roche, Switzerland) solution (0.25 mg/ml in Krebs-Ringer buffer) via injection into the common bile duct. After pancreatic digestion at 37°C, islets were purified using a Ficoll-paque (GE Healthcare, U.K.) gradient. Islets were washed and immediately pre-incubated for 1 hr in Krebs-Ringer buffer containing HEPES (KRBH), 0.1% BSA and 2 mM glucose. Batches of five islets were incubated at 37°C for 1 hr in 130 μl KRBH containing 0.1% BSA and 2, 5.5, 11 or 20 mM glucose. For the measurement of pancreatic insulin content, the pancreas was weighed and then homogenised in ice-cold acid ethanol (0.15 M HCl in 75% ethanol) immediately after collection. Insulin concentrations in the incubation medium and pancreatic extracts were determined by methods described in Section 2.4. The islet isolation and ex vivo insulin secretion assays were performed by Dr James Cantley in the Garvan Institute of Medical Research in Sydney.
4.2.4 Pancreatic Histology

Quantification of β-cell area was performed based on previous methods [147]. Each pancreas was removed, cleared of fat and lymph nodes, fixed in 10% neutral buffered formalin and embedded in paraffin wax. 5 μm sections were cut and incubated for 30 min at room temperature with blocking solution (PBS buffer with 2% BSA and 5% chick serum) before incubation overnight at 4°C in blocking solution containing mouse anti-insulin antibody (Sigma-Aldrich, Catalogue No. I2018). Sections were then incubated with chicken anti-mouse IgG-Alexa Fluor 594 conjugate (Invitrogen) for 1 hr at room temperature in darkness. Transmitted light images were captured (magnification × 20). To quantitate β-cell area, the outline of the pancreas section and all insulin-positive cells were traced and scored using ImageJ image analysis software (ImageJ, NIH, Bethesda, MD). Results are expressed as the percentage of the total pancreatic area stained positive for insulin.

4.2.5 Assessment of Urine Glucose Secretion and Kidney Morphology

Urine samples were collected in the morning three weeks after the completion of OA treatment. The urinary glucose level was measured by the glucose oxidase assay using an automated glucose analyser (YSI 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, Ohio, USA).

For the kidney morphology study, kidneys were rapidly removed after cervical dislocation. Coronal sections of renal tissue were immersion-fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 5 μm thick were stained with periodic acid-Schiff and evaluated using methods described previously [148]. To quantitate tubular atrophy, the tubule cell height of an individual cortical tubule was measured using line morphometry (magnification × 200) by ImageJ. A total of 50 randomly-selected cortical tubules in 10 non-overlapping fields (magnification × 200) were measured, and the mean cross-sectional tubule cell height was determined for each section. The degree of glomerular hypertrophy was
measured quantitatively. The outline of the glomeruli and glomerular capillary tuft was traced, and the computed area was used as a measure of total glomerular area and tuft area. The mean value of 20 randomly selected glomeruli was determined for each section. The cortical interstitial volume included the tubular basement membrane and peritubular capillaries. To quantitate this area, cortical fields (magnification × 200) were viewed on a video screen, and the area of interstitial space was determined with image analysis software and expressed as a percentage of the total area of the field. The mean percentage area of five non-overlapping cortical fields was calculated for each section. The assessment of kidney morphology was performed in collaboration with Dr Yiping Wang from the Westmead Millennium Institute, Sydney.

4.2.6 Measurement of Glucose Flux in Key Tissues for Glucose Homeostasis

In one subset of mice, [³H] labelled 2-deoxy-D-glucose (2DG; PerkinElmer, USA) and D-[¹⁴C] glucose (PerkinElmer, USA) were used to measure glucose metabolism in skeletal muscle, fat and liver [129]. Briefly, four weeks after the cessation of OA treatment, a GTT was performed after 5-7 hours of fasting. Glucose (1 g/kg BW) containing [³H]-2DG (65 μCi/kg BW) and D-[¹⁴C] glucose (32 μCi/kg BW) was injected i.p.. Plasma samples were obtained from the tail tip at 10, 20, 30 and 40 min after glucose administration for estimation of plasma tracer concentration. At the completion of the GTT, mice were culled and tissue samples were immediately freeze-clamped for subsequent analysis.

4.2.7 Analysis of Gene Expression and Immunoblotting

Targeted genes in a complementary DNA library, generated from total RNA extracted from the liver, was amplified by real-time polymerase chain reaction using specific primer sets as described in Section 2.7. Western blotting was performed as described in Section 2.6.
4.2.8 Statistical Analyses

Data are presented as means ± SE. Two-tailed Student's t-test was used for comparison of relevant groups. Pearson’s two-sided correlation was used for the correlation analysis. Differences at p < 0.05 were considered to be statistically significant.

4.3 RESULTS

4.3.1 Induction of Type-1 and Type-2 Diabetes

Similar to the study described in Chapter 3, a T2D model of hyperglycaemia was first induced by chronic HF-feeding and low-dose STZ based on previous reports [125, 126]. As shown in Table 4.1, HF feeding caused a small increase in body weight and a 2.3 fold increase in epididymal fat compared to CH mice. HF mice also exhibited increased blood insulin (~40%) and liver triglyceride (2.4 fold) compared to CH mice, but the circulating levels of glucose and triglyceride were similar between HF and CH mice. CH-fed mice treated with STZ (T1D-Veh), demonstrated moderate increases in blood glucose and plasma triglyceride compared to CH mice (~50-60%, p < 0.05 vs. CH-fed mice), but liver triglyceride content was unchanged.

As expected, HF- and STZ-treated mice (T2D-Veh) recapitulated several major characteristic metabolic disorders in type-2 diabetes, namely hyperglycaemia (~2 fold), hyperinsulinaemia (by 20%), dyslipidaemia (80%) and liver steatosis (2.5 fold) compared to CH mice. Consistent with previous reports [126], T2D mice also displayed a lack of glucose response to insulin action during an insulin tolerance test - a typical feature of insulin resistance (data not shown). Compared with HF feeding alone, T2D mice displayed hyperglycaemia (> 2 fold, p < 0.01), hypertriglyceridemia (50%, p < 0.05) and reduced blood insulin level (50%, p < 0.01), while retaining a similar level of liver steatosis. Compared with T1D, T2D mice showed more epididymal fat (30%), greater hyperglycaemia (~30%) and significantly increased triglyceride content in the liver (2 fold).
Figure 4.2 Metabolic effects of oleanolic acid in T2D and T1D mice over time. HF-fed mice with streptozotocin (STZ) injections were treated with (T2D-OA) or without (T2D-Veh) OA in the diet for two weeks, at the end of which OA was removed from the diet. Blood glucose, food intake and body weight were monitored between 14:00 and 16:00 once a week (A, B and C). CH-fed mice with STZ injections were treated with (T1D-OA) or without (T1D-Veh) OA in the diet for two weeks, at the end of which OA was removed from the diet. Effects of OA on hyperglycaemia in T1D mice (D). CH, chow diet; T1D, type 1 diabetes; T2D, type 2 diabetes. Data are expressed as means ± SE. † p < 0.05, †† p < 0.01 vs. T2D-Veh group, n = 11 - 16 per group.
Table 4.1 Metabolic characteristics of the type-1 and type-2 diabetes mouse models.

<table>
<thead>
<tr>
<th></th>
<th>CH-Veh</th>
<th>HF-Veh</th>
<th>CH-STZ (T1D)</th>
<th>HF-STZ (T2D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.5 ± 0.4</td>
<td>31.7 ± 0.8†</td>
<td>28.8 ± 0.4†</td>
<td>27.8 ± 0.2†** #</td>
</tr>
<tr>
<td>EPI fat (% BW)</td>
<td>1.4 ± 0.1</td>
<td>3.2 ± 0.3††</td>
<td>1.2 ± 0.1†</td>
<td>1.6 ± 0.1** #</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>9.2 ± 0.2</td>
<td>8.3 ± 0.4</td>
<td>13.6 ± 0.6††</td>
<td>17.5 ± 1.2††**#</td>
</tr>
<tr>
<td>Blood insulin (pg/ml)</td>
<td>367 ± 29</td>
<td>503 ± 130†</td>
<td>300 ± 26</td>
<td>301 ± 63†*</td>
</tr>
<tr>
<td>Plasma triglyceride (mM)</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.1††</td>
<td>2.2 ± 0.2**</td>
</tr>
<tr>
<td>Liver triglyceride (µmol/g)</td>
<td>7.8 ± 0.8</td>
<td>18.6 ± 2.0††</td>
<td>9.4 ± 0.9</td>
<td>19.6 ± 1.1††**##</td>
</tr>
</tbody>
</table>

C57BL/6J mice were fed a chow diet (CH) and injected with saline (CH-Veh) or STZ (T1D-Veh), or fed a high-fat (HF) diet and injected with saline (HF-Veh) or STZ (T2D-Veh). Blood samples were collected from mice in the 5-6 hour fasted state. Blood glucose (n = 8-10) and insulin (n ≥ 5), and plasma triglyceride (n = 7-8) were measured as described in the methods. Body weight, epididymal weight and liver triglycerides were measured at the end of the study (n ≥ 10). BW, body weight; EPI fat, epididymal fat. Data are expressed as means ± SE. † p <0.05, †† p <0.01 vs. CH; * p < 0.05, ** p < 0.01 vs. HF; # p < 0.05, ## p < 0.01 vs. T1D.

4.3.2 Effects of OA on Hyperglycaemia and Glucose Tolerance

Oral administration of OA, at a dose of 100 mg/kg/day, dramatically reversed the hyperglycaemia of T2D mice by more than 60% (approaching the level of CH-fed mice) by the end of the two-week treatment (22.5 ± 0.6 vs. 13.2 ± 1.9 mM, p < 0.001). Intriguingly, following the removal of OA from the HF diet, the reversed hyperglycaemia evident in the T2D-OA group persisted for the rest of the study. Meanwhile the hyperglycaemia in the untreated T2D-Veh group remained high (Figure 4.2A). Under a similar treatment regime, OA showed no significant effect in lowering the hyperglycaemia evident in the T1D mouse.
model (Figure 4.2D). Along with a reduced food intake at the end of the two-week treatment (Figure 4.2B), T2D-OA group had a lower body weight than T2D-Veh group at week 3 and 4 (Figure 4.2C). However, food intake and body weight of T2D-OA mice was completely recovered by the end of the study (Figure 4.2B and C).

As the anti-hyperglycaemic effect of OA was only observed in T2D mice, subsequent studies were performed only in the T2D mouse model. OA significantly improved the glucose tolerance of T2D mice as evidenced by decreased blood glucose levels in T2D-OA compared to T2D-Veh at all time points (p < 0.01) during the GTT as well as a reduced area under the curve (Figure 4.3A and B). However, when expressed as the incremental area under the curve, there was no significant difference between the T2D-Veh and T2D-OA groups. During the GTT, blood insulin levels were significantly higher in T2D-OA compared to T2D-Veh mice at 30 min, with a similar trend at 5 min (p = 0.082) (Figure 4.3C). When the blood insulin level was expressed as the average value from 5 to 60 min, there was no statistical difference between the T2D-Veh and T2D-OA groups (p = 0.053, Figure 4.3D).
Figure 4.3 Effects of oleanolic acid on glucose tolerance and blood insulin. Studies were performed in mice two weeks after the removal of OA. A glucose tolerance test (GTT) was performed with an injection of glucose (1 g/kg, i.p.) after 5-7 hours of fasting. Blood glucose during the glucose tolerance test (GTT) was performed with an injection of glucose (1 g/kg, i.p.) after 5-7 hours of fasting. Blood glucose was performed with an injection of glucose (1 g/kg, i.p.) after 5-7 hours of fasting. Blood glucose during the glucose tolerance test (GTT) (A). GTT results were quantified by calculating the area under the blood glucose curve (AUC) and the incremental AUC (B). Insulin levels throughout the GTT (C). The average value of blood insulin levels from 5 to 60 min during the GTT (D). CH, chow diet; T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and oleanolic acid (OA) treatment. ** p < 0.01 vs. CH; †† p < 0.01 vs. T2D-Veh, n = 5 - 8 per group.

4.3.3 Insulin Secretion, β-cell Numbers and Pancreatic Insulin Content

Results in Figure 4.3C and D appeared to indicate a seeming improvement in β-cell function after treatment of T2D mice with OA, which could provide a mechanism for the improved blood glucose levels in these mice. Therefore, glucose-stimulated insulin secretion was further assessed in isolated islets. As shown in Figure 4.4A, islets isolated from T2D-OA mice showed an insulin secretion response to increasing glucose stimulation that was
indistinguishable from T2D-Veh mice. The examination of total pancreatic insulin level showed that the administration of OA had no effect on the total pancreatic insulin content in T2D mice (29.1 ± 10.6 vs. 26.6 ± 6.0 μg/g pancreas in the T2D-Veh group, p > 0.05) (Figure 4.4B). Finally, immunohistochemical staining of pancreatic sections showed that OA did not affect the total number of β-cells per pancreas in T2D mice (0.29 ± 0.11% vs. 0.27 ± 0.11%, p = 0.894) (Figure 4.4C and D).
Figure 4.4 Effects of oleanolic acid on insulin secretion and insulin in pancreatic β-cells.
Four weeks after the cessation of oleanolic acid (OA) treatment, fresh islets were isolated from T2D mice treated with or without OA, and insulin secretion in response to different glucose concentrations was measured (A). Pancreatic insulin content (B). β-cell area (expressed as a percentage of pancreatic area) (C). Representative images of immunohistochemical staining of β-cells (D). Scale bar: 50 µm. T2D, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. n = 4 - 6 per group.
4.3.4 Effects of OA on Glucose Loss in Urine and Nephropathy

In order to investigate if the reduced blood glucose level in T2D-OA mice was due to an increase in the urine excretion of glucose, the glucose level in the urine of T2D mice, before and after the treatment with OA, was measured. T2D mice excreted more glucose in the urine compared to CH-fed mice at baseline (Figure 4.5A). Whilst glucose excretion remained high in T2D mice when measured three weeks after the cessation of OA treatment, T2D-OA mice demonstrated a significant drop in urine glucose levels (11.8 ± 1.2 vs. 5.1 ± 0.8 mM before vs. after OA treatment, p < 0.01) (Figure 4.5A). Morphology studies revealed that the OA treatment dramatically improved kidney structure of T2D mice, as indicated by significant reductions in interstitial volume (13.2 ± 0.7 vs. 24.9 ± 0.8 % in untreated T2D, p < 0.01) and glomerular tuft area in T2D-OA mice (3251 ± 41 vs. 4248 ± 83 μm² in untreated T2D, p < 0.01) to levels similar to CH-fed mice (Figure 4.5B and C). Associated with the normalisation of interstitial volume and glomerular tuft area, the reduced tubular cell height evident in T2D mice was significantly ameliorated in T2D-OA mice (13.6 ± 0.1 vs. 12.4 ± 0.1 μm in untreated T2D, p < 0.01) (Figure 4.5B and C).
Figure 4.5 Effects of oleanolic acid on glucose levels in urine and kidney morphology. Urine glucose levels in CH, T2D-Veh and T2D-OA mice were measured before the oleanolic acid (OA) treatment began and at the end of the study (four weeks after cessation of OA treatment) (A). Kidneys were harvested and coronal sections of renal tissues were stained with periodic acid-Schiff for the quantification of interstitial volume, glomerular tuft area and tubular cell height (B). Representative images of stained kidney sections (C). Scale bar: 100 µm. T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. * p < 0.05, ** p < 0.01 vs. CH; †† p < 0.01 vs. T2D-Veh, n = 5 - 10 per group.

4.3.5 Effects of OA on Plasma and Liver Triglyceride Levels

T2D-Veh mice had significantly higher levels of plasma and liver triglyceride compared to CH-fed mice at the end of the two-week treatment period (Figure 4.6A) and at four-week
post-treatment period (Figure 4.6B). As expected, two weeks of OA treatment substantially reduced the triglyceride levels in plasma (Figure 4.6A) and liver (Figure 4.6B) of T2D mice. However, four weeks after the termination of OA administration, both plasma and liver triglycerides had returned to the levels of the T2D-Veh group (Figure 4.6A and B).

**Figure 4.6 Effects of oleanolic acid on triglyceride levels in plasma and liver.** Lipid accumulation in the liver and plasma was assessed both during oleanolic acid (OA) treatment (at week 2) and four weeks after the cessation of OA treatment. Mice were euthanized following a 5-7 hour fast and samples of plasma (A) and liver (B) were collected for the measurement of triglyceride levels. T2D-Veh, high fat-fed mice with streptozotocin (STZ) injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. ** p < 0.01 vs. CH; †† p < 0.01 vs. T2D-Veh, n = 5 - 8 per group.
4.3.6 Influence of Pair-feeding on Hyperglycaemia and Glucose Tolerance

Since a fluctuation in food intake was observed in the T2D-OA group during the initial experiment, an additional pair-feeding study was carried out to determine if the sustained reversal of hyperglycaemia during the period of post-OA treatment was due to the difference in food intake. The food intake in pair-fed T2D-Veh and T2D-OA groups (2.7 ± 0.1 vs. 2.5 ± 0.1 g/mouse/day, p > 0.05) was well matched (Figure 4.7A). T2D-Veh and T2D-OA groups were not different in body weight at the baseline nor at the end of the study (Figure 4.7B). The basal blood glucose of the T2D-OA group showed a significant decrease compared to the T2D-Veh group after two weeks of OA treatment despite matching of food intake, and this was maintained for three weeks post-treatment (Figure 4.7C). Similar to the previous observations, T2D-OA mice were more glucose tolerant than T2D-Veh mice in the GTT performed two weeks after the removal of OA (Figure 4.7D and E).
Figure 4.7 Comparisons between pair-fed T2D-Veh and T2D-OA mice. Food intake (A), body weight (B) and basal blood glucose (C) were monitored throughout the pair-feeding study. A glucose tolerance test (GTT) was performed on mice two weeks after the cessation of oleanolic acid (OA) treatment, with an injection of glucose (1 g/kg, i.p.) after 5-7 hours of fasting. Blood glucose was monitored at 0, 15, 30, and 60 min following the glucose injection (D). GTT results were quantified by calculating the area under the blood glucose curve (AUC) and the incremental AUC (E). T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. † p < 0.05, †† p < 0.01 vs. T2D-Veh, n = 5 - 10 per group.
4.3.7 Effects of OA on Glucose Flux in Muscle, Fat and Liver

To further investigate the mechanism underlying the sustained improvement in glycaemia of OA treated T2D mice, $[^3]$H-2DG and $[^14]$C-glucose tracers were employed to measure the glucose uptake in muscle and adipose tissue during a GTT conducted four weeks after the cessation of OA treatment. As expected, T2D mice demonstrated reduced glucose uptake in muscle compared to CH-fed mice (Figure 4.8A)(14.4 ± 2.9 vs. 7.0 ± 0.5 μmol/100g/min, p < 0.05). A similar tendency was evident in fat tissue (7.2 ± 2.3 vs. 3.3 ± 0.8 μmol/100g/min, p > 0.05; Figure 4.8B). However, OA treatment of T2D mice did not cause any improvement of glucose uptake in these two tissues (Figure 4.8A and B). Furthermore, measurement of glucose incorporation into glycogen and lipid in the liver (as a proxy of glucose influx) did not show any improvement in T2D-OA as compared to the T2D-Veh group (Figure 4.8C and D).
Figure 4.8 Changes in glucose flux into muscle, adipose tissue and liver. A glucose tolerance test (GTT. glucose load, 1 g/kg, *i.p.*) using 2-deoxy-D-[1,2-^3H] glucose and D-[^14C] glucose was performed four weeks after the cessation of OA treatment. At the end of the 40-min GTT, tissue samples were freeze-clamped immediately for the measurement of glucose uptake in quadriceps muscle (A) and epididymal fat (B), as well as the glucose incorporation into lipid (C) and glycogen (D) in the liver. CH, chow-fed mice; T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. * p < 0.05, ** p < 0.01 vs. CH, n = 6 - 12 per group.

4.3.8 Effects of OA on Key Regulators of Hepatic Glucose Production

As hepatic glucose production is a major factor affecting glucose homeostasis [149], the protein expression and post-translational modification of key molecules (Akt and FoxO1 [150]) that regulate hepatic gluconeogenesis and glucose production were investigated. As shown in Figure 4.9A-D, compared to CH-fed mice, T2D mice displayed a 60% reduction of p-/-t-Akt. The p-/-t-Akt ratio was similar in T2D-OA and T2D-Veh mice. However, the absolute p-Akt level was lower in T2D-Veh mice compared to CH, but restored to CH levels in T2D-OA mice. In the case of FoxO1, T2D-OA mice demonstrated substantially increased p-/-t-FoxO1 (200% increase) (p < 0.01) and a 50% reduction in total FoxO1 protein compared to both T2D-Veh and CH mice (p < 0.05) (Figure 4.9A and E-G).

The transcriptional expression levels of the key enzymes controlling gluconeogenesis, e.g. phosphoenolpyruvate carboxykinase (PEPCK), and glucose production, e.g. G6Pase, were next examined. As shown in Figure 4.10A and B, compared to CH-fed mice, T2D mice demonstrated an elevated expression of PEPCK (~80% increase, p < 0.05) and G6Pase (~50% increase, p > 0.05) at the transcriptional level. Interestingly, treatment of T2D mice with OA tended to reduce G6Pase RNA (~30%, p = 0.09, Figure 4.10B), while the level of PEPCK expression remained unchanged. Further analysis showed that there was a significant reversed
correlation of both phosphorylated Akt (p < 0.05) and phosphorylated FoxO1 (p < 0.05) to the transcriptional expression level of G6Pase (Figure 4.10C).

Figure 4.9 Changes in Akt and FoxO1 in the liver. Four weeks after the cessation of oleanolic acid (OA) treatment, mice were sacrificed following a 5-7 hour fast. Liver samples were freeze-clamped and stored at -80°C for subsequent Western blotting analysis. Representative Western blot images of phosphorylated and total Akt and FoxO1 (A). Quantification of p-Akt/GAPDH (B), t-Akt/GAPDH (C), p/t-Akt (D), p-FoxO1/GAPDH (E), t-FoxO1/GAPDH (F) and p/t-FoxO1 (G). CH, chow-fed mice; T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. * p < 0.05 vs. CH; † p < 0.05, †† p < 0.01 vs. T2D-Veh, n = 6 - 8 per group.
Figure 4.10 Changes in PEPCK and G6Pase expression in the liver. Four weeks after the cessation of oleanolic acid (OA) treatment, mice were sacrificed following a 5-7 hour fast. Liver samples were freeze-clamped and stored for subsequent analysis of PEPCK and G6Pase RNA expression. The expression levels of PEPCK and G6Pase mRNA relative to 18S (A and B). Correlation of p-Akt/GAPDH and FoxO1/GAPDH with G6Pase mRNA expression in T2D-Veh and T2D-OA groups by a best-fit regression analysis (C). CH, chow-fed mice; T2D-Veh, high fat-fed mice with STZ injections; T2D-OA, high fat-fed mice with STZ injections and OA treatment. * p < 0.05 vs. CH, n = 6 - 8 per group.
4.4 DISCUSSION

The study in this Chapter investigated the chronic effect of OA, a triterpenoid selected based on the structural analysis and pilot in vivo study described in Chapter 3, on hyperglycaemia in diabetic mice and the contributing mechanisms involved. Results from this study showed that OA did not affect hyperglycaemia in T1D mice, but was able to reverse hyperglycaemia in T2D mice. Intriguingly, this reversed hyperglycaemia was sustained well beyond the treatment period - for at least four weeks after cessation of OA treatment. Along with the correction of hyperglycaemia, elevated urine glucose loss in T2D mice was also completely reversed and damage to renal structures was markedly attenuated. Although OA has been reported to lower hyperglycaemia by counteracting insulin resistance within the period of its administration [151-153], the sustained reversal of hyperglycaemia for such a long duration after terminating the treatment has not been described before.

Since previous studies reported a hypoglycaemic effect in insulin resistant HF-fed [151-153] and db/db mice [144], the effects of OA on hyperglycaemia was first investigated in a T2D model generated by chronic HF feeding in combination with low doses of STZ. Chronic HF feeding in rodents is a widely used model of insulin resistance, associated with lipid accumulation in muscle and liver [128, 154]. However, HF feeding alone is insufficient to cause diabetes due to the capacity of pancreatic β-cells to increase insulin secretion in order to compensate for the insulin resistance [155]. Therefore, multiple low doses of STZ were administered to restrict the ability of pancreatic β-cells to increase insulin secretion, thereby generating hyperglycaemia as previously reported [125, 126]. As expected, the combination of HF feeding and low doses of STZ resulted in marked hyperglycaemia with the characteristics of overt type-2 diabetes, namely, failed compensatory increases in blood insulin levels with concurrent hypertriglyceridemia and fatty liver. Compared with CH-fed
mice injected with STZ (T1D-Veh), HF-fed mice injected with STZ (T2D-Veh) showed significantly more severe hyperglycaemia liver steatosis and visceral adiposity (Table 4.1).

Oral administration of OA to the T2D mouse model progressively reduced hyperglycaemia to a level similar to that of normal mice within two weeks. Interestingly, after the termination of OA treatment, the reversed hyperglycaemia was retained for the remaining four-week period of the study (Figure 4.2A). Along with the reduced blood glucose level, T2D-OA mice also displayed an improved glucose tolerance at the end of the study when measured with the total area of glucose under the curve (Figure 4.2A and B). While the hypoglycaemic effect of an anti-diabetic compound is expected during the period of treatment, few reports demonstrate similarly sustained correction of hyperglycaemia after cessation of drug treatment. This dramatic improvement of glucose homeostasis for such a long time post-OA treatment is particularly interesting because sustained control of glycaemia is an important target in the treatment of type-2 diabetes.

Possible mechanisms of the reversal of hyperglycaemia following the treatment of OA were investigated from several different perspectives. The half-life of OA in the circulation is reported to be less than 30 min in mice [156], suggesting that the reversal of hyperglycaemia post-OA in this study is unlikely to result from a prolonged presence of OA in the circulation. Therefore, it was first investigated whether OA may induce sustained improvement of pancreatic β-cell function to increase insulin secretion, given that acute treatment of OA was previously reported to enhance insulin secretion in isolated rat β-cells [157]. Such a possibility has been implicated in several previous studies using a T1D model induced by STZ in CH-fed rodents [143, 145]. Conceivably, the sustained reversal of hyperglycaemia may be achieved with a compensatory increase in insulin secretion as observed in the HF-fed mice in this study (Table 4.1) or improved β-cell function as suggested for incretins [158] (see Table 1.3). However, a two-week treatment of T2D mice with OA did not increase the
number or enhance the function of β-cells (pancreatic insulin content or insulin secretion of β-cells in response to glucose stimulation). Furthermore, the blood insulin profiles during the GTT were not different between T2D mice with/without OA treatment. These results are also consistent with the inability of OA to lower hyperglycaemia in STZ-injected CH-fed mice in this study. The reason for the discrepancy between the results in this study and previous reports in T1D rodent model [143, 145] is not clear. However, findings in T2D and T1D models in this study are internally consistent and collectively indicate that it is unlikely that OA exerts its beneficial effects on glycaemia through an improvement in β-cell function.

It has been proposed that the inhibition of SGLT2 (a transporter for glucose re-absorption in the kidney) can increase glucose excretion in the urine and thereby reduce hyperglycaemia for the treatment of type-2 diabetes [158, 159] (see Table 1.3). Therefore, it was examined whether the sustained anti-hyperglycaemic effect of OA was achieved by increasing urine glucose excretion. However, in contrast to an increase in the glucose excretion through the kidney, OA treatment significantly reduced the urine glucose level in T2D mice to the level of CH-fed mice. Furthermore, the diabetic nephropathy evident in T2D-Veh mice was abolished in T2D mice treated with OA, as indicated by increased tubular cell height, and decreased interstitial volume and glomerular tuft area. These results rule out increased urine excretion of glucose as a mechanism for the reduced glucose level following OA treatment. These findings are consistent with a recent clinical trial which demonstrated the beneficial effects of an OA derivative for nephropathy in type-2 diabetes patients [160]. As OA can improve renal functions in diabetic mice by inhibiting the formation of advanced glycation endproducts [143], it is possible that the reversal of hyperglycaemia following the treatment of OA contributed to the alleviated nephropathy.

The reduced food intake evident in T2D-OA mice during the period of OA treatment (which was also observed in the study in Chapter 3) presented another potential mechanism
underlying the sustained reduction in glycaemia. Reduced food intake has been shown to have effects on fasting blood glucose levels, glucose tolerance and insulin sensitivity in mice and rats [161, 162]. However, it is unknown whether such effect, especially on fasting blood glucose, can be maintained for days after food intake has normalised. To investigate possible influences of the different food intake pattern of T2D-Veh and T2D-OA groups, a pair-feeding study with an additional two groups of mice was conducted. Despite food intake of the pair-fed T2D-Veh and T2D-OA groups being matched throughout the study, T2D-Veh mice maintained significantly higher glycaemia than T2D-OA mice from the second week of OA treatment and through to the end of the post-treatment period (Figure 4.7A-C). Furthermore, pair-fed T2D-Veh mice were not as glucose tolerant as T2D-OA mice as evidenced by a GTT performed at the end of the study (Figure 4.7D and E). These data indicate that the effect of OA to improve glucose homeostasis in diabetic mice does not rely on its effect to reduce food intake. In support of this interpretation, a recent study has demonstrated that an OA analogue can lower blood glucose in both HF-fed and db/db mice without affecting food intake [144].

Several studies have shown that triterpenoids, including OA, can reverse insulin resistance and glucose intolerance during the treatment period [59, 144, 151, 152]. This study similarly observed that T2D-OA mice displayed significantly lower plasma glucose levels during GTT. Since OA treatment attenuates the glucose intolerance with similar blood insulin levels as T2D-Veh mice, the improved glucose tolerance and glycaemia in T2D-OA mice is probably due to improved insulin sensitivity of the peripheral tissues.

As discussed in Section 1.2.2, insulin resistance is closely related with increased accumulation of lipids in peripheral tissues [163] and a reduction in lipid content in these tissues is an effective means to improve insulin sensitivity [128]. The reversal of insulin resistance and glucose intolerance during OA treatment has been associated with a reduction of lipid
accumulation in muscle and liver [59, 144, 151, 152]. As triterpenoids can acutely activate AMPK and promote fat oxidation [124], one plausible mechanism of the sustained efficacy to reduce hyperglycaemia in this study could conceivably be due to improved insulin action as a result of reduced lipid accumulation in muscle and liver. Such a mechanism has been demonstrated with berberine [164], and Abbot compound A [165] during the period of treatment. To investigate if OA improves insulin sensitivity through a similar mechanism, the triglyceride levels in plasma and liver were measured because a reduction in hepatic steatosis has been shown to normalise glycaemia in T2D [140]. Indeed, associated with the corrected glycaemia, the triglyceride levels in plasma and liver were reduced to almost the normal levels of CH-mice during the period of OA administration. These results are similar to those recently reported in db/db mice within the treatment duration of an OA analogue, along with increased AMPK activity [144]. However, as the efficacy of OA on triglyceride levels in plasma and liver did not persist after cessation of OA treatment, the sustained reduction in glycaemia is likely to involve alternative/additional mechanisms rather than the improvement in dyslipidaemia and hepatic steatosis alone.

Glucose uptake into skeletal muscle and hepatic production of glucose are two major metabolic pathways responsible for glucose homeostasis. With the use of $[^3]$H-2DG and D-$[^{14}]$C glucose tracers, this study demonstrated a reduction of glucose uptake into muscle and adipose tissue in T2D-Veh mice and there was no improvement of glucose uptake in these two tissues in T2D-OA mice. The lack of an effect of OA upon glucose uptake into muscle and liver indicated that the liver may be the major site for the sustained improvement of glycaemia after the treatment with OA. To investigate the possible role of the liver, glucose influx into this organ was first assessed. However, no improvement in glucose incorporation into either glycogen or triglyceride was found (note that measurement of glucose uptake with 2DG in the liver is not valid, as 2DG is not trapped in the liver due to the presence of G6Pase).
In view of the unchanged glucose uptake into these tissues, indicators of hepatic glucose production (efflux) were examined. Interestingly, the insulin signalling transduction (indicated by the phosphorylation of Akt and FoxO1. See Figure 1.3) regulating gluconeogenesis was significantly improved in OA treated mice. FoxO1 is a key transcription factor regulating hepatic gluconeogenesis. The phosphorylation of FoxO1 by Akt leads to its expulsion from the nucleus for degradation [150, 166]. A recent study has indicated that Akt-FoxO1 signalling plays a key role in controlling the expression of G6Pase [166]. Indeed, a trend of reduced expression of G6Pase (p = 0.09) was observed. Importantly, the suppressed expression of G6Pase was significantly correlated with increased phosphorylation of Akt and FoxO1 (both p < 0.05). As G6Pase is the gate-keeping enzyme for hepatic glucose production, these results indicate that its down-regulation induced by the Akt/FoxO1 signalling pathway is a likely mechanism for the sustained improvement of glycaemia after the treatment with OA (Figure 4.11).

In summary, the study in this Chapter demonstrated a sustained effect of OA to reverse hyperglycaemia in T2D mice induced by HF-feeding and STZ. Results presented in this study indicate that OA, a triterpenoid abundantly present in natural products, may be a potential drug for the sustained control of hyperglycaemia in T2D and related kidney complications independent of lipid metabolism, insulin secretion and glucose disappearance into muscle and fat tissue. Furthermore, results from this study suggest that the sustained improvement of glucose homeostasis is due, at least in part, to a suppression of gluconeogenesis in the liver mediated by the Akt/FoxO1 axis.
Figure 4.11 A proposed mechanism for the anti-hyperglycaemia of oleanolic acid. Oleanolic acid (OA) treatment up-regulated Akt activity, which led to an increase inhibitory phosphorylation and FoxO1. Phosphorylated FoxO1 was excluded from the nucleus and degraded. A reduction of FoxO1 level caused in a suppressed gluconeogenic gene expression. As a result, hepatic glucose production was reduced and hyperglycaemia was normalised.
Chapter 5
Matrine Improves Fatty Liver and Insulin Resistance
5.1 INTRODUCTION

As described in Section 1.6, despite the current high-throughput screening approach for drug discovery is highly effective in identifying potential compounds for T2D, the vast majority of these identified compounds failed to translate into usable drugs in humans largely due to concerns about the safety. Alternatively, drugs with established safety profile are investigated for the treatment of a different disease (drug repurposing) [167].

Using a similar approach of drug repurposing, the study described in this Chapter investigates the potential of matrine (Mtr) for the treatment of T2D and hepatic steatosis. This study chose to target the liver because it is often the first site to develop insulin resistance [168], a fundamental abnormality of T2D and closely linked with hepatosteatosis [34]. Furthermore, Mtr is a small molecule of alkaloid (Table 3.1) used clinically for the treatment of viral hepatitis and hepatic tumours in the form of capsule or intravenous injection solution (e.g. China Food and Drug Administration approval no. H20010242 or H20044669) [169] with little adverse effects [170]. Interestingly, recent studies showed that both viral hepatitis and hepatocellular carcinoma are associated with increased lipogenesis [171, 172] and suppressed fatty acid oxidation [173], which together contribute to the development of hepatic steatosis [174, 175]. Furthermore, patients with hepatitis C also develop insulin resistance and associated metabolic syndrome [176], and treatments targeting disturbed lipid metabolism have been shown to inhibit the progression of hepatic viral infection [177]. Recent work in our laboratory showed that oxymatrine, which is converted to Mtr in vivo [178], can reduce lipid content in vitro [122].

In this study, the therapeutic efficacy of Mtr for insulin resistance and hepatic steatosis, and the underlying mechanism were investigated in high-fat high-cholesterol-fed mice, with metformin as a comparison.
5.2 MATERIALS AND METHODS

5.2.1 Animal Model

Male C57BL/6J mice (10-week old) were purchased and acclimatisation as described in Section 2.2. After one week of acclimatisation, mice were fed *ad libitum* for 10 weeks with a standard lab chow diet (CH. Gordon's Specialty Stock Feeds, Yanderra, Australia) or a high-fat high-cholesterol diet (HFC; 45% calories from fat, 0.2% w/w cholesterol). One group of CH or HFC mice then received Mtr (100 mg/kg/day) or metformin (250 mg/kg/day) as a food additive for four weeks while the rest of the mice remained on the CH or HFC diet (Figure 5.1). Body weight, food intake and fasting blood glucose levels were monitored on a weekly basis. Following 7 hours of fasting at the end of the study, plasma was extracted from blood samples collected from the tail tip and stored at -80°C. Mice were killed by cervical dislocation. Liver and epididymal fat mass were weighed using an analytical balance. Tissue samples were immediately freeze-clamped. All experiments were approved by the Animal Ethics Committee of RMIT University (#1012; #1208) in accordance with the guidelines of the National Health and Medical Research Council of Australia. Mtr (Purity > 99.5%) is a gift from Prof. Li-Hong Hu from Shanghai Institute of Materia Medica; metformin is purchased from Sigma-Aldrich.

5.2.2 Assessment of Metabolic Parameters and Immunoblotting

Blood glucose levels were measured once a week as described in Section 2.4. After two-week Mtr treatment, glucose tolerance tests (glucose load 1 g/kg BW, *i.p.* ) were performed as described in Section 2.4.. Plasma insulin levels at each time point during the glucose tolerance test were determined by a radioimmunoassay (Section 2.4). Plasma and liver triglyceride were determined as described in Section 2.4 and 2.5. Western blotting was performed as described in Section 2.6.
5.2.3 Whole-Body Metabolic Measurement

The acute effect of Mtr on whole body metabolic rate was assessed at 22°C using an indirect calorimeter (Oxymax, Columbus Instruments, USA) as described previously [124]. Briefly, 12-week old chow-fed mice were acclimated in the Oxymax system for 2 hrs. Volume of O₂ consumption and CO₂ production were continuously monitored for 7 hrs after mice received Mtr (100 mg/kg, dissolved in 0.5% methylcellulose) or metformin (250 mg/kg, dissolved in 0.5% methylcellulose) by oral gavage.

5.2.4 Citrate Synthase and β-Hydroxyacyl-CoA Dehydrogenase Activity.

Liver samples were homogenised in 175 mM KCl and 1.98 mM EDTA-containing buffer (pH 7.4) with a glass homogeniser before being subjected to three freeze-thaw cycles. Citrate synthase and β-hydroxyacyl-CoA dehydrogenase activities were determined as described previously [179] with a Flexstation 3 plate reader (Molecular Devices, USA).

5.2.5 Histological Analysis

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**Figure 5.1 Experimental design of the matrine study.** 10-week old male C57BL/6J mice were acclimatised for one week before receiving a chow (CH) or a high-fat high-cholesterol (HFC) diet for 14 weeks. One group of CH or HFC mice then received matrine (100 mg/kg/day) or metformin (250 mg/kg/day) as a food additive for the last four weeks while the rest of the mice remained on the CH or HFC diet. Glucose tolerance test (GTT) was performed at week 12.
Liver tissues fixed in 10% neutral-buffered formalin were embedded in paraffin, cut into 5-μm sections and stained with hematoxylin and eosin (H&E) for microscopic examination. Five random images from each section were taken (objective magnification ×40). Hepatocyte ballooning was quantified as described by Kleiner et al. [180] by an investigator blinded to the study design.

5.2.6 Measurement of Respiration in Isolated Mitochondria

Mitochondria were isolated from quadriceps muscle of 10-week old C57BL/6J mice with an isolation medium consisted of 100 mM Sucrose, 50 mM Tris, 100 mM KCl, 1 mM KH$_2$PO$_4$, 0.1 mM EGTA, 0.2% fatty acid-free BSA, pH 7.0. Respiration of isolated mitochondria was measured at 37°C with a Clark-type oxygen electrode (Strathkelvin Instruments, Motherwell, Scotland) in a respiration medium contains 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, 10 mM KH$_2$PO$_4$, 10 mM KCl, 0.8 mM MgCl$_2$, 0.1 mM EDTA, 0.3% fatty acid-free BSA, pH 7.0. Dose-response effects of the different compounds on mitochondrial respiration were determined in the presence of excess ADP (2.4 mM), using substrate combinations targeting either Complex I (5 mM pyruvate plus 2 mM malate) or Complex II (10 mM succinate plus 4 μM rotenone) of the respiratory chain.

5.2.7 Determination of Fatty Acid Oxidation in Liver

Palmitate oxidation was measured in liver homogenates using methods described previously [181]. Briefly, the liver was collected from 10-week old CH-fed mice and stored in ice-cold isolation buffer (pH 7.4) containing (in mM) 250 sucrose, 10 Tris-HCl and 1 EGTA. The liver homogenate was prepared using a glass Dounce homogeniser with a loose-fit pestle and then incubated at 30°C for 90 min in a reaction mixture (pH 7.4) containing (in mM) 100 sucrose, 80 KCl, 10 Tris-HCl, 5 KH$_2$PO$_4$, 1 MgCl$_2$, 2 malate, 2 ATP, 1 dithiothreitol, 0.2 EDTA, 0.3% fatty acid–free BSA, 2 L-carnitine, 0.05 coenzyme A and 0.2 [1-$^{14}$C]-palmitate (0.5 μCi/ml). The reaction was stopped by the addition of 1 M perchloric acid. CO$_2$ produced from the
reaction was collected in 1 M NaOH. Palmitate oxidation rates were determined by counting the $^{14}$C radioactivity of captured CO$_2$ and acid-soluble metabolites.

### 5.2.8 Molecular Docking Simulation

Mtr or metformin was docked to a series of selected targets, including HSP90 (PDB Code: 3T0Z), HSF1 (2LDU), HSP72 (2LDU) and SREBP-1c (1AM9) separately. An X-ray crystal structure of each protein target with relatively high resolution and complete structure was acquired from the Protein Data Bank (http://www.rcsb.org) and used for the docking simulation. Molecular docking simulation was performed using the default setting unless stated otherwise. Briefly, the X-ray crystal structures were firstly prepared with the ‘Protein Preparation Wizard’ in Schrödinger suite. The unwanted water molecules as well as cofactors were removed from the target protein, and hydrogen atoms were added and optimised in order to obtain a better hydrogen bond assignment. The grid box centre of each protein was set according to its co-crystal ligand, and the size of each protein was defined as 20×20×20 Å$^3$. The coordinates of Mtr and metformin were initially built using Epik (version 2.2, Schrödinger LLC, USA) with pH of 7.0 and OPLS_2005 force field. Glide (5.5, Schrödinger LLC, USA) was used to perform the docking simulations in XP Mode and each docking routine returned top 10 ranked docked poses for each ligand. Finally, the most reliable binding pose for each docking routine was selected from the molecular docking results by visual inspection of ligand pose and quantitative scoring. This simulation was performed by Dr Fang Bai in Prof. Huangliang Jiang’s Drug Discovery and Design Center of Shanghai Institute of Materia Medica.

### 5.2.9 Preliminary Study in Humans with Dyslipidaemia

To test the feasibility of the repurposed application of Mtr in clinical settings, a preliminary study was designed in collaboration with Dr Luping Ren in the General Hospital of Hebei (Shijiazhuang, China) based on the results of the animal study.
Five hypertriglyceridemia patients (2 males and 3 females; 52.6 ± 5.2 years) volunteered for this study. Subjects were given 0.3 g of Mtr orally three times per day for one month (a dosage recommended for the treatment of hepatitis). Blood samples were collected in the morning after an overnight fast, centrifuged and stored at -80°C for the measurement of fasting plasma triglyceride, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) as well as creatinine (Cr) before and at the end of the Mtr treatment. The protocol was approved by the General Hospital of Hebei. Written informed consents were obtained from all patients before all procedures.

5.2.10 Statistical Analyses
Data are presented as the mean ± SE. For calculation of statistical differences, GraphPad Prism software was used to perform two-tailed Student's t-test and Pearson’s two-sided correlation test, where applicable. Differences at p < 0.05 were considered to be statistically significant. No statistical method was used to predetermine sample size.

5.3 RESULTS
5.3.1 Effects of Mtr on Glucose Tolerance of HFC-fed mice
A mouse model of obesity and insulin resistance was first induced by HFC feeding. As shown in Table 5.1, HFC feeding induced a ~30% increase in body weight at week 10 compared to CH diet. HFC mice were glucose intolerant compared to CH mice as indicated by a 60% increase of incremental area under the curve (iAUC) during a GTT (data not shown). Neither Mtr nor metformin treatment affects calorie intake in CH or HFC mice. Both Mtr and metformin significantly reduce body weight gain and adiposity in CH or HFC mice (Table 5.1). In the fasting state, Mtr significantly reduced both blood glucose and plasma insulin levels (Figure 5.2A and B). In contrast, metformin, at a dosage of 250 mg/kg/day, was less
effective than Mtr in reducing basal blood glucose and plasma insulin in HFC mice (Figure 5.2A and B). Furthermore, Mtr improved glucose tolerance (~15% reduction in iAUC) (Figure 5.2C and E) and consistently lowered plasma insulin levels throughout the whole GTT (Figure 5.2C and F), while metformin mildly reduced plasma insulin levels during the GTT but had no effect on glucose tolerance compared to HFC mice (Figure 5.2C-F). When expressed as blood glucose iAUC × plasma insulin AUC, both Mtr and metformin significantly ameliorated whole body insulin resistance (Figure 5.2G).

5.3.2 Effects of Mtr on Hepatic Steatosis and Liver Histology

As discussed in Section 1.2.2, ectopic lipid accumulation in insulin-target tissues is believed to be closely linked to insulin resistance [40]. To investigate if Mtr improved glucose tolerance by reducing lipid accumulation in insulin-target tissues, the triglyceride content in liver and muscle was measured. HFC feeding markedly increased triglyceride level by 2-fold in both liver and muscle compared to CH diet. Mtr treatment resulted in an apparent reduction (~25%) of triglyceride level in the liver (but not muscle) of HFC mice. In contrast, metformin had no effect on triglyceride level in the liver or muscle (Figure 5.3A). Plasma triglyceride level was not affected by HFC feeding, Mtr or metformin treatment (Table 5.1). H&E staining of liver sections showed that HFC feeding resulted in a dramatic increase of cells with swollen and rarefied cytoplasm (ballooning), which was significantly reduced after four-week Mtr (but not metformin) treatment (Figure 5.3B; Table 5.1).
Figure 5.2 Effects of matrine on blood glucose, plasma insulin and glucose tolerance. Mice were fed a chow (CH) or a high-fat high-cholesterol (HFC) diet for 14 weeks, and matrine (Mtr) or metformin (Met) was administrated for the last four weeks. After four weeks of drug treatment, blood glucose (A) and plasma insulin (B) were measured following a 5-7 hour fast. A glucose tolerance test (1 g/kg, i.p.) was performed with measurements of blood glucose (C) and plasma insulin (D) after two weeks of drug treatment. Incremental area under the curve (iAUC) of blood glucose (E). Area under the curve (AUC) of plasma insulin (F). Whole body insulin index (G) was expressed as blood glucose AUC × plasma insulin AUC. Data are expressed as means ± SE. * p < 0.05, ** p < 0.01 vs. CH-Veh; † p < 0.05, †† p < 0.01 vs. HFC-Veh, n = 8 per group.
Table 5.1 Metabolic characteristics before and after treatments.

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>CH Mtr</th>
<th>HFC</th>
<th>HFC Mtr</th>
<th>HFC Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW Before Treatment</td>
<td>29.8 ± 0.9</td>
<td>29.3 ± 0.5</td>
<td>38.0 ± 1.8**</td>
<td>37.6 ± 1.1</td>
<td>37.3 ± 1.6</td>
</tr>
<tr>
<td>BW After Treatment</td>
<td>30.2 ± 1.0</td>
<td>28.2 ± 0.5</td>
<td>39.8 ± 2.0**</td>
<td>32.5 ± 0.5††</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>BW Gain during treatment (g)</td>
<td>0.4 ± 0.2</td>
<td>-1.2 ± 0.3**</td>
<td>1.7 ± 0.4*</td>
<td>-5.1 ± 0.8††</td>
<td>-1.8 ± 0.5††</td>
</tr>
<tr>
<td>Calorie Intake (kcal/mouse/day)</td>
<td>12.1 ± 0.5</td>
<td>12.0 ± 0.6</td>
<td>15.0 ± 0.9</td>
<td>14.6 ± 0.2</td>
<td>14.5 ± 0.7</td>
</tr>
<tr>
<td>Liver/BW (%)</td>
<td>4.0 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>EPI/BW (%)</td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.1**</td>
<td>5.0 ± 0.5**</td>
<td>3.2 ± 0.5†</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Plasma Triglyceride (mM)</td>
<td>0.9 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Plasma AST (U/L)</td>
<td>19.5 ± 2.4</td>
<td>22.1 ± 2.2</td>
<td>21.7 ± 1.9</td>
<td>17.6 ± 5.8</td>
<td>18.7 ± 0.8</td>
</tr>
<tr>
<td>Plasma ALT (U/L)</td>
<td>11.0 ± 2.3</td>
<td>12.2 ± 2.4</td>
<td>12.7 ± 2.0</td>
<td>7.5 ± 1.9</td>
<td>5.9 ± 0.7†</td>
</tr>
<tr>
<td>Hepatocyte Ballooning</td>
<td>0.4 ± 0.2</td>
<td>0.0 ± 0.0*</td>
<td>1.6 ± 0.2**</td>
<td>0.7 ± 0.1††</td>
<td>1.4 ± 0.2**</td>
</tr>
</tbody>
</table>

C57BL/6J mice were fed either a chow (CH) or a high-fat high-cholesterol (HFC) diet for 14 weeks. Matrine (Mtr, 100mg/kg/day) or metformin (Met, 250mg/kg/day) was administered as a food additive for the last four weeks of the study. Body weight (BW) was measured before the administration of drugs (week 11) and at the end of the study (week 14). Calorie intake was monitored twice a week. Plasma was collected before the tissue collection at week 14 for the subsequent analysis of triglyceride, AST and ALT. Liver and epididymal fat (EPI) were collected and weighted during the tissue. Data are expressed as means ± SE. * p < 0.05, ** p < 0.01 vs. CH; † p <0.05, †† p <0.01 vs. HFC; # p < 0.05, ## p < 0.01 vs. HFC mice treated with matrine, n = 8 per group.
Figure 5.3 Effects of matrine on ectopic lipid accumulation and liver morphology. After four weeks of drug treatment, mice were euthanized following a 5-7 hour fast. (A) Liver and muscle were collected for the measurement of triglyceride levels. (B) Representative H&E staining (40x) images of liver sections. Scale bar: 50 μm. CH, chow diet; HFC, high-fat high-cholesterol diet; Met, metformin; Mtr, matrine. Data are expressed as means ± SE. * p < 0.05, ** p < 0.01 vs. CH-Veh; † p < 0.05, †† p < 0.01 vs. HFC-Veh, n = 8 per group.
5.3.3 Effects of Mtr on the Lipogenic Pathway in the Liver

To determine whether the Mtr-mediated reduction of hepatic lipid accumulation was a result of reduced lipid synthesis, the expression level of lipogenic proteins in the liver was measured. HFC feeding induced mark increases of mature form of SREBP-1c (nSREBP-1c, 2.5-fold) and SCD-1 (3-fold) compared to CH diet (Figure 5.4A and D). Mtr treatment reduced ACC, FAS and SCD-1 protein levels in CH-fed mice, and normalised the elevated level of nSREBP-1c and SCD-1 in HFC mice (Figure 5.4A-D). Metformin had no effect on nSREBP-1c, ACC or FAS in HFC mice, but normalised SCD-1 to a level similar to the Mtr treatment (Figure 5.4A-D). These data suggested Mtr-induced reduction of hepatic lipid accumulation might be a result of suppressed lipogenic pathways.
Figure 5.4 Effects of matrine on the lipogenic pathway. After four weeks of drug treatment, mice were euthanized following a 5-7 hour fast. (A) Nuclear fraction of the liver was isolated and immunoblotted with specific antibodies for SREBP-1. Liver lysate was immunoblotted with specific antibodies for (B) ACC, (C) FAS and (D) SCD-1. CH, chow diet; HFC, high-fat high-cholesterol diet; Met, metformin; Mtr, matrine. Densitometry and representative blots are presented. Data are expressed as means ± SE. * p < 0.05, ** p < 0.01 vs. CH-Veh; † p < 0.05, †† p < 0.01 vs. HFC-Veh, n = 8 per group.
5.3.4 Effects of Mtr on Fatty Acid Oxidation and Energy Expenditure

Increased fatty acid oxidation and energy expenditure can also contribute to the reduction of hepatic lipid accumulation as well as adiposity. To test this possibility, the protein expression or activity of a number of markers for energy metabolism was first measured. Mtr significantly increased the hepatic protein expression of UCP2 in both CH and HFC mice (Figure 5.5A) and mildly (p = 0.09) increased the activity of citrate synthase in HFC mice (Figure 5.5B), but it had no effect on the activity of β-hydroxyacyl-CoA dehydrogenase (Figure 5.5C). In contrast to Mtr, metformin had no effect on the protein expression or activities of these markers (Figure 5.5A-C). The effect of Mtr on fatty acid oxidation was further examined by incubating liver homogenate prepared from 10-week old CH mice in a respiration buffer containing [1-14C]-palmitate. Mtr dose-dependently increased palmitate oxidation by up to 15% (at 100 μM) (Figure 5.5D).

Finally, the oxygen consumption (VO2) and respiratory exchange ratio (RER) in CH-fed mice administrated orally with Mtr or metformin were measured. Consistent with results on the reduction of body weight gain, hepatic lipid accumulation and adiposity, Mtr significantly increased energy expenditure (10% increase of VO2) and fat oxidation (4% decrease of RER) for at least 7 hours after the oral administration in CH-fed mice (Figure 5.6). In contrast to Mtr, metformin increased VO2 without altering RER compared to CH mice, indicating metformin increased energy expenditure without promoting fatty acid as the predominant fuel for energy production (Figure 5.6).
Figure 5.5 Effects of matrine on fatty acid oxidation in the liver. 10-week old C57BL/6J mice were fed a chow (CH) or a high-fat high-cholesterol (HFC) diet for 14 weeks, and matrine (Mtr. 100 mg/kg/day in diet) or metformin (Met. 250 mg/kg/day) was administrated for the last four weeks. (A) After four weeks of drug treatment, mice were euthanized following a 5-7 hour fast, and the liver was collected immunoblotted with specific antibodies for UCP2. (B) Activities of citrate synthase (CS) and (C) β-hydroxyacyl-CoA dehydrogenase (β-HAD) isolated from the liver were measured (n = 8 per group). Data are expressed as means ± SE. * p < 0.05 vs. CH-Veh; † p < 0.05 vs. HFC-Veh. (D) Palmitate oxidation rate was measured by incubating liver homogenate prepared from a different batch of 10-week old CH-fed mice in an oxidation buffer containing [1-14C]-palmitate at 37°C for 1hr. Palmitate oxidation rates were determined by counting the 14C radioactivity of acid-soluble metabolites and captured CO₂ (n = 2-4 per group from 4 independent experiments). Data are expressed as means ± SE. * p < 0.05 vs. vehicle.
Figure 5.6 Effects of matrine on whole-body energy expenditure and fuel preference. A different batch of 12-week old chow-fed mice were acclimatised in a metabolic cage for 24 hours followed by administration of vehicle (0.5% methylcellulose), matrine (100 mg/kg) or metformin (250mg/kg) by oral gavage. (A) Oxygen consumption and (B) respiratory exchange ratio (RER) were monitored for seven hours after the drug administration (n = 8 per group for vehicle; 5, matrine; 9, metformin). CH, chow diet; HFC, high-fat high-cholesterol diet; Met, metformin; Mtr, matrine. Data are expressed as means ± SE. * p < 0.05 vs. CH-Veh.

5.3.5 Effects of Mtr on the Activation of AMPK or PPARα

AMPK, a sensor of cellular energy status, represents an attractive therapeutic target for the treatment of T2D [182]. Metformin [183] as well as a number of small molecules including berberine [183] and triterpenoids [124], exert their anti-diabetic effects at least partly by activating AMPK through a disturbance of the mitochondrial respiratory chain. To investigate
if the anti-diabetic effects of Mtr involved similar mechanisms, the mitochondria respiration rate in the presence of Mtr as well as the phosphorylation of AMPK and ACC in livers of Mtr-treated mice were assessed. In contrast to berberine, which dose-dependently inhibited respiration in mitochondria isolated from the muscle of mice by suppressing mitochondrial complex I, Mtr did not affect the oxygen consumption rate at a dose up to 30 μM (Figure 5.7). Consistent with the unimpaired mitochondrial respiratory chain, the phosphorylation status of AMPK and its substrate ACC was unaltered in the liver of Mtr-treated mice (Figure 5.8A).

Previous studies including ours [92, 181] demonstrated that activation of peroxisome proliferator-activated receptor α (PPARα) increases fatty acid oxidation, reduces liver triglyceride and improves insulin resistance. Despite similar metabolic effects were observed after the Mtr treatment, the hepatic protein level of ACOX-1, a marker of PPARα, remained unchanged (Figure 5.8B) in the Mtr-treated mice indicating the PPARα pathway was not activated. Collectively, these results suggested the anti-diabetic effects of Mtr might not involve the activation of AMPK or PPARα.

![Figure 5.7 Effects of matrine on mitochondrial respiration. Oxygen consumption rates were measured in isolated muscle mitochondria from chow-fed mice using a Clark-type oxygen electrode at 37°C in substrate combinations targeting respiratory complex I (pyruvate and malate) or complex II (succinate and rotenone). BBR, berberine; mal, malate; Mtr,](image_url)
matrine; pyr, pyruvate; rot, rotenone; suc, succinate. Data are expressed as means ± SE. n = 3 per group. * p < 0.05 vs. vehicle.

Figure 5.8 Effects of matrine on AMPK and PPARα. 10-week old C57BL/6J mice were fed a chow (CH) or a high-fat high-cholesterol (HFC) diet for 14 weeks, and matrine (Mtr, 100 mg/kg/day in diet) or metformin (Met, 250 mg/kg/day) was administrated for the last four weeks. After four weeks of drug treatment, liver lysate was immunoblotted with specific antibodies for (A) phosphorylated (p-)(Thr172) or total (t-) AMPK, (B) p-(Ser79) or t-ACC and (C) ACOX-1. Liver from fenofibrate (Feno)-treated HFC mice was used as a positive
control for the immunoblotting of ACOX1. Densitometry and representative blots are presented. Data are expressed as means ± SE. n = 8 per group. * p < 0.05 vs. CH-Veh.

5.3.6 Effects of Mtr on HSP72 Expression in the Liver

Heat shock protein 72 (HSP72), a highly conserved chaperone protein, has been suggested as a potential therapeutic target for T2D [184]. It was reported recently that attenuated expression of HSP72 is associated with a number of metabolic diseases including obesity [185] and T2D [186]. Importantly, up-regulation of HSP72 has been shown to improve insulin resistance in genetic or HFC diet-induced obese mice [185]. Interestingly, HSP90, a negative regulator of HSP72 [187], is shown to be essential for the progression of viral hepatitis [188, 189], which are treated by Mtr clinically. To assess whether HSP72 or HSP90 might involve in the therapeutic effects of Mtr, molecular docking simulation was used to estimate the binding strength between Mtr and a number of protein targets. Docking simulation revealed the predicted binding affinity of Mtr to HSP90 is much stronger than HSF1, HSP72 or SREBP-1c (Table 5.2; Figure 5.9). Meanwhile, parallel docking simulations on Mtr and ganetespib (a reported HSP90 inhibitor [190]) revealed that these two molecules interact with HSP90 with a similar binding affinity (-6.7 and -7.0 kcal/mol separately). From the predicted binding pose, two hydrogen bonds between Mtr and two residues of HSP90 (T184 and G108), and two water bridged hydrogen bonds between carbonyl group of Mtr and D93 as well as G97, were identified. In addition, Mtr also form hydrophobic contacts with F138, M98 and L107 (Figure 5.9). In contrast to the strong binding affinity between Mtr and HSP90, the energy required for metformin to bind to selected protein targets was predicted to be much greater, rendering the direct interaction less likely to happen between metformin and these proteins targets (Table 5.2).
Figure 5.9 Predicted binding pose of matrine or metformin to HSP90. The lilac cartoon represents HSP90, the violet stick models are important amino acid residues, the orange stick-ball models are matrine (a) or metformin (b), red spheres are conserved water molecules and the dash lines represent the hydrogen bonds.

Table 5.2 Docking results of matrine and metformin to selected protein targets.

<table>
<thead>
<tr>
<th>Target Name</th>
<th>HSP90</th>
<th>HSF1</th>
<th>HSP72</th>
<th>SREBP-1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB Code</td>
<td>3T0Z</td>
<td>2LDU</td>
<td>3ATU</td>
<td>1AM9</td>
</tr>
<tr>
<td>Ligand Scoring (kcal/mol)</td>
<td>Matrine</td>
<td>-6.743</td>
<td>-2.419</td>
<td>-2.878</td>
</tr>
<tr>
<td></td>
<td>Metformin</td>
<td>-4.315</td>
<td>-2.480</td>
<td>-4.943</td>
</tr>
<tr>
<td></td>
<td>Ganetespib</td>
<td>-7.000</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The interactions between matrine or metformin and a series of selected protein targets were simulated by software Schrödinger suite (Schrödinger LLC, USA). X-ray crystal structures of protein targets with relatively high resolution and complete structure were acquired from the Protein Data Bank (http://www.rcsb.org/pdb/). Ganetespib is a reported HSP90 inhibitor. n.d., not determined.

To determine whether the possible interaction between Mtr and HSP90 would have any effect on the expression of HSP72, the hepatic protein expression of HSP72 was measured.
Consistent with previous reports, HSP72 expression was blunted (40% reduction compared to CH, p<0.05) by HFC feeding [185]. Four-week Mtr treatment elevated (~50% increase, p<0.05) the protein expression of HSP72 in both CH and HFC mice, but had no effect on the protein level of HSP90 and HSF1 (transcription regulators of HSP72). In contrast to Mtr treatment, metformin failed to restore the reduced HSP72 level in HFC mice (Figure 5.10A and B). To investigate the relationship of Mtr-induced metabolic effects to changes in HSP72 expression, the liver triglyceride and glucose iAUC were compared with the relative change in hepatic HSP72 expression. As shown in Figure 5.10C, HSP72 expression significantly correlate with liver triglyceride level (r=-0.487, p<0.01) and glucose iAUC (r=-0.427, p<0.05).
Figure 5.10 Effects of matrine on HS72, HSP90 and HSF1. (A) Representative blots and densitometry of (A) HSP72, (B) HSP90 and HSF1 in the liver after four weeks of matrine (Mtr. 100 mg/kg/day in diet) or metformin (Met. 250 mg/kg/day) treatment. CH, chow diet; HFC, high-fat high-cholesterol diet. Data are expressed as means ± SE. * p < 0.05 vs. CH-Veh; † p < 0.05 vs. HFC-Veh, n = 8 per group. (C) Correlation of hepatic HSP72 expression with liver triglyceride or incremental area under the curve of blood glucose. Results from CH-Veh, CH-Mtr, HFC-Veh and HFC-Mtr groups are pooled for the correlation analysis determined by Pearson’s two-sided correlation test.
5.3.7 Effects of Mtr on Hypertriglyceridemia in Humans

Five volunteers with hypertriglyceridemia (plasma triglycerides > 2.49 mM) were recruited to test the effect of matrine on plasma lipids. One month of oral matrine treatment significantly lowered plasma triglyceride by 50% (p<0.02) and increased high-density lipoprotein by 13% (p<0.01), without affecting kidney and liver functions as indicated by the unchanged levels of blood urea nitrogen, creatinine, alanine transaminase and aspartate transaminase. Matrine also mildly reduced plasma cholesterol and low-density lipoprotein (20-30%, p=0.07) in these volunteers (Table 5.3).

Table 5.3 Effects of matrine in patients with hypertriglyceridemia.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Normal Range</th>
<th>Before</th>
<th>After</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TG (mM)</td>
<td>5</td>
<td>0.56 - 1.70</td>
<td>4.66 ± 0.87</td>
<td>2.32 ± 0.50</td>
<td>0.017</td>
</tr>
<tr>
<td>Plasma Chol (mM)</td>
<td>4</td>
<td>2.80 - 5.70</td>
<td>4.57 ± 0.37</td>
<td>3.71 ± 0.23</td>
<td>0.070</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>4</td>
<td>0.78 - 1.55</td>
<td>0.87 ± 0.14</td>
<td>0.98 ± 0.15</td>
<td>0.003</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>4</td>
<td>1.68 - 4.53</td>
<td>2.96 ± 0.45</td>
<td>2.13 ± 0.32</td>
<td>0.071</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>5</td>
<td>1 - 40</td>
<td>31.00 ± 7.42</td>
<td>27.40 ± 2.20</td>
<td>0.619</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>5</td>
<td>2 - 40</td>
<td>37.20 ± 14.1</td>
<td>31.80 ± 8.27</td>
<td>0.438</td>
</tr>
<tr>
<td>BUN (mM)</td>
<td>3</td>
<td>2.50 - 7.10</td>
<td>4.03 ± 0.20</td>
<td>3.65 ± 0.25</td>
<td>0.526</td>
</tr>
<tr>
<td>Cr (μM)</td>
<td>3</td>
<td>53 - 132</td>
<td>78.40 ± 3.73</td>
<td>77.87 ± 1.23</td>
<td>0.226</td>
</tr>
</tbody>
</table>

TG, triglyceride. Chol, cholesterol. HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; BUN, blood urea nitrogen; Cr, creatinine. Data are expressed as means ± SE.
5.4 DISCUSSION

The study in this Chapter demonstrated that chronic administration of Mtr, a drug used clinically for viral hepatitis and hepatic carcinoma, substantially ameliorated visceral adiposity and glucose intolerance as effectively as metformin in HFC-fed mice, with additional effects to reduce hepatic steatosis through inhibiting the lipogenic pathway and promoting fatty acid oxidation. The metabolic effect of Mtr was associated with an up-regulation of HSP72, suggesting Mtr might be a novel drug for the treatment of hepatic steatosis and T2D by a mechanism different from the current anti-diabetic drugs.

The liver is a key tissue for the homeostasis of both blood glucose and lipids in the whole body. Recent studies demonstrate that lipid accumulation (hepatic steatosis) is strongly associated with insulin resistance in the liver [140, 191], and patients with benign hepatic steatosis might progress to more severe steatohepatitis (a state characterised by the presence of hepatocyte injury) [49]. Similar to metformin, of which the main site of action is in the liver [192], Mtr has been reported to have a high tissue distribution in the liver compared to other organs (e.g. muscle and fat) after an oral administration [193]. This study demonstrated that four-week Mtr treatment significantly reduced lipid accumulation in the liver, but not muscle, indicating the liver might be the major target tissue of Mtr. To further investigate the underlying mechanism of the lipid-lowering effect of Mtr in the liver, the hepatic protein expression of a number of lipogenic markers was examined and a significant reduction of mature SREBP-1 and SCD-1 in Mtr-treated HFC-fed mice was observed. In addition to the suppressed lipogenic pathway, the lipid-lowering effect of Mtr in the liver might be attributed to its stimulatory effects on energy expenditure and fatty acid oxidation in the whole body, as indicated by increased oxygen consumption and decreased RER in CH-fed mice received a bolus of Mtr. In agreement with the effect on the in vivo metabolic profile, Mtr also dose-dependently enhanced fatty acid oxidation in liver homogenate in a functional ex vivo assay.
Furthermore, four-week administration of Mtr did not induce liver injuries as indicated by unchanged plasma AST and ALT levels (Table 5.1).

AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy sensing enzyme, which have received considerable attention as a pharmaceutical target for anti-diabetic drugs [182]. It is well documented that activation of AMPK can suppress lipogenesis and stimulate fatty acid oxidation [182]. In the last decade, enormous efforts including ours [124, 183] have been focused on activation of AMPK as a mean for anti-diabetic treatment. While anti-diabetic drug metformin is believed to exert its therapeutic efficacy partly via the activation of AMPK as a result of suppressed mitochondrial electron transport chain complex 1 [192], its efficacy in the long term is insufficient when being used alone, and additional therapeutics (e.g. sulfonylurea etc.) with molecular actions different from metformin are required [71]. Results in this study demonstrated that Mtr improved glucose tolerance and fasting glucose homeostasis in HFC-induced insulin resistance mice as effectively as metformin. Importantly, Mtr exerts these metabolic effects through a distinct pathway from metformin. These results showed that Mtr neither increased the phosphorylation of AMPK or its downstream target ACC in the liver, nor disturbed the mitochondria electron transport chain.

The neutral effect of Mtr on mitochondrial respiration might be an attractive property for T2D therapy, as inhibition of mitochondrial respiration might lead to lactic acidosis, which limits the use of metformin in T2D patients with chronic renal disease [71, 192]. Despite some reports of possible benefits for non-alcoholic fatty liver disease (NAFLD) in laboratories studies [194], metformin it is not regarded as an effective treatment for NAFLD [195], due to its lack of efficacy on the improvement of liver histology such as histological steatosis and fibrosis [196]. In comparison, Mtr appears to display a distinct profile for the correction of key elements (e.g. hepatosteatosis and elevated expression of TNFα) of NAFLD in additional to its metabolic effect on insulin resistance. It has also been reported that Mtr is able to protect
the liver from carbon tetrachloride-induced fibrosis in rats [197]. These findings together raise the possibility that Mtr might offer additional therapeutic effects to manifestations of metabolic syndrome in the liver.

It was also examined if the observed therapeutic effects may be explained by a possible stimulation of PPARα, which is known to eliminate insulin resistance and fatty liver in rodents [181]. The lack of effect on ACOX1 seems to rule out this possibility. Molecular docking simulation in the current study revealed that Mtr interacts with a number of amino acid residues (Figure 5.9) of HSP90 in an N-terminal nucleotide-binding pocket (residues 9-232), which is also the binding domain for geldanamycin (an HSP90 inhibitor) [198], ATP (a substrate for HSP90) [199] and a number of other HSP90 inhibitors [190]. Heat shock proteins are a group of highly conserved chaperones providing cytoprotection and assisting protein folding. In addition to its general biological function, heat shock protein 90 (HSP90) is also reported to implicate in the development of a number of diseases, including hepatic viral infection [200]. HSP90 is essential for the formation of complexes that are critical for the replication of hepatitis B or C virus [188, 189] and inhibitors of HSP90, such as geldanamycin, have been shown to inhibit the progression of hepatitis C viral infection [189]. These observation collectively raises the possibility that Mtr, a clinically used drug for hepatitis treatment, might alter the protein expression of HSP72 through interfering with the interaction of HSP90 and its client proteins HSF1 [201], a transcription factor regulating the expression of HSP72 [187].

Recent studies showed that HSP72 expression is reduced in the skeletal muscle of patients with obesity/insulin resistance [185] or T2D [186]. Heat treatment-induced HSP72 expression in both muscle and liver is blunted by HFC feeding (which can induce insulin resistance) in mice [185]. Consistent with these reports, a similar reduction of HSP72 expression in the liver was observed in the HFC-fed mice. While a significant correlation between HSP72 expression
in the skeletal muscle and insulin resistance was reported by Kurucz et al. [186], a similar correlation between the HSP72 expression and hepatic steatosis/glucose tolerance was observed in this study, suggesting HSP72 might be a key mediator of the metabolic effect of Mtr. In support of this notion, heat treatment was recently reported to improve glucose tolerance and prevent skeletal muscle insulin resistance in high-fat fed rats with associated up-regulation of HSP72 [202].

In summary, the study described in this Chapter has identified the hepatoprotective drug Mtr as a promising novel anti-diabetic drug with liver as an important target organ. Results from this study demonstrated that Mtr reduced obesity and glucose intolerance as effectively as metformin in insulin resistant HFC-fed mice. Importantly, Mtr exerts distinct mechanisms with HSP72 as a possible upstream target (Figure 5.11) and marked effects on key aspects of NAFLD.
Figure 5.11 A proposed mechanism for the therapeutic effects of matrine. Matrine treatment up-regulates the expression of HSP72 in the liver. HSP72 may stimulate energy expenditure and fatty acid oxidation, and suppress lipogenesis. The overall result is a reduction of lipid accumulation in the liver, thus alleviates the glucose intolerance and hepatic steatosis induced by high-fat feeding.
Chapter 6

General Discussion
The aim of this last chapter is to bring together the main findings of the work carried out for this thesis. It finishes with two sections pointing to some future directions of the investigations for OA and Mtr.

6.1 MAJOR FINDINGS

As reviewed in Chapter 1, it is clear that the global prevalence of diabetes is escalating at an unprecedented speed. Meanwhile, diabetes has placed an enormous burden on not only the patient but also the society due to its chronic nature and the severity of its various complications resulted from hyperglycaemia. The dramatically increased cost involved in treating this disease has turned it into a major threat to global development. As T2D is affecting approx. 90% of all people with diabetes, there is still an urgent need for the development of new classes of medications for this disease to address different issues faced by the current therapeutics, e.g. inconvenient side effects or limited efficacy for certain population of patients.

The conventional approach for drug discovery and development (D&D) often involves cell-based high-throughput screenings of large synthetic compound libraries. This approach has been widely used by the pharmaceutical industry in the past three decades, as it was thought to be more cost-effective in developing new drugs. However, a growing body of evidence shows that the pharmaceutical industry is facing a high attrition rate and unimproved new-drug output despite the cost involved in the D&D has increased dramatically.

With a consideration of the screening capacity in an academic institution, the project described in this thesis employed an alternative approach to identify potential compounds of interest for the treatment of T2D (Chapter 3). The source of test compounds was confined to natural products, which are reported to be more structurally diverse and biochemically specific. Structural analysis based on Lipinski’s rule of five was performed for test
compounds with known chemical structure to ensure only compounds with good oral bioavailability were selected for the subsequent animal-based screening. HF feeding and low-dose streptozotocin treatment were used to generate a mouse model of T2D, as HF feeding is known to induce insulin resistance and the use of low-dose streptozotocin compromises β-cell function but does not eliminate insulin from circulation completely. In this type 2 diabetes mouse model, OA and Mtr displayed favourable effects on several key metabolic parameters (including basal blood glucose, plasma and liver triglyceride and adiposity). In addition to these beneficial effects, the chemical structures of OA and Mtr suggest that they have good oral availability and can be modified to generate a series of derivatives. Therefore, OA and Mtr were selected for further detailed investigations.

Following the identification of two investigational compounds as described in Chapter 3, the chronic efficacy of the first compound, OA, on hyperglycaemia was investigated in type 2 diabetic mice (Chapter 4). The most important finding from this study is that the OA-induced anti-hyperglycaemic effects were sustained for the entire post-treatment period of the study (four weeks) despite the reoccurrence of dyslipidaemia. Several possible mechanisms for the anti-hyperglycaemic effect of OA were investigated systematically in the same study. Results from this investigation suggested the effects of OA on β-cell functions, glucose excretion from the urine and food intake were unlikely to be the underlying mechanism responsible for its anti-hyperglycaemic effect. The use of glucose tracers, $[^3\text{H}]$-2DG and D-$[^14\text{C}]$ glucose, further excluded changes in glucose influx into muscle, adipose tissue or liver in this effect. Further examination suggested liver might be a major site of action of OA, and the anti-hyperglycaemic effect of OA was associated with a suppression of glucose production, which might be mediated by the Akt/FoxO1 pathway. A second important finding in this study was the beneficial effect of OA in reversing the damaged kidney morphology in T2D mice. The findings in this study provided a proof of concept for exploring triterpenoids as a promising
source to identify new drugs for the long-term control of hyperglycaemia and treatment of diabetic kidney complications.

In the third study (Chapter 5), the therapeutic effect of Mtr on insulin resistance and hepatic steatosis was investigated in HFC mice. The first important finding of this study is that *Mtr, a hepatoprotective drug used in clinic for the treatment of hepatitis B, was also effective to improve physiological parameters related to the treatment of T2D* (including glucose intolerance, dyslipidaemia, hepatic steatosis and adiposity) without affecting caloric intake in HFC mice. The Mtr-induced improvement in the metabolic profile of HFC mice was accompanied with a suppressed lipogenic pathway and increased fatty acid oxidation. The second important finding is that *Mtr exerted its metabolic effects through a distinct mechanism compared to the most commonly used anti-diabetic drug metformin*. Mtr treatment did not suppress mitochondrial respiration chain or activate AMPK as metformin does, and Mtr did not activate PPARγ either. Instead, the treatment of Mtr was associated with an up-regulation of hepatic protein expression of HSP72, as expected based on an *in silico* molecular docking simulation. Finally, the preliminary study in humans suggests the feasibility to test this potential new application in humans by demonstrating an efficacy of Mtr in reducing hypertriglyceridemia without adverse effects in the kidney and liver. Given that Mtr is well tolerated in humans during chronic use, these results suggest that Mtr is a promising compound to be used in humans for the metabolic syndrome with favourable effects on the associated NAFLD by a mechanism different from the current treatments.

Overall, these studies support the notion that phenotypic screenings of natural products in combination with data mining of biological and chemical data are a viable approach for the discovery of new promising compounds for drug development. While results presented in Chapter 4 and Chapter 5 suggest OA, Mtr and their derivatives can be explored for the treatment of T2D and related metabolic diseases, there are several questions needed to be
addressed, and they will be discussed in the following two sections along with further comments on each individual study.

6.2 FUTURE DIRECTIONS FOR OLEANOLIC ACID

The study presented in Chapter 4 demonstrated a promising anti-hyperglycaemic effect of OA which was sustained beyond the treatment period. While this finding is exciting and might provide novel treatment for glycaemic management in T2D, several questions remain to be answered.

The first question is: Does OA indeed reduce hyperglycaemia by suppressing gluconeogenesis four weeks after the treatment?

Firstly, results from Chapter 4 systematically rule out a number of possible mechanisms that can be responsible for the anti-hyperglycaemic effect of OA, leaving the suppression of glucose production from the liver as a most likely mechanism (another possible mechanism, which will be discussed later, is the inhibition of glucose digestion/absorption from the intestine, e.g α-glucosidase inhibitors.) especially the gluconeogenic gene expression was shown to be reduced in OA-treated T2D mice. Consistent to this result, a study published during the course of this PhD project demonstrated a similar reduction of G6Pase protein expression in the liver of \( db/db \) mice with two-week OA treatment (20 mg/kg/day, i.p.) [203]. However, both studies rely on markers of gluconeogenesis, and neither of them demonstrated functionally the OA-induced suppression of hepatic glucose. A hyperinsulinaemic-euglycaemic clamp should be performed to assess the effect of OA on whole body or tissue-specific insulin sensitivity as well as hepatic glucose output [204]. Alternatively, a pyruvate tolerance test should be performed to confirm that the suppression of glucose production did play a role in the anti-hyperglycaemic effect of OA four weeks after the treatment. Alternatively, the OA-induced suppression of hepatic glucose production at least should be
demonstrated in vitro, e.g. a glucose production assay in Fao cells. Fao cells are rat hepatoma cells, which have been shown to be capable for gluconeogenesis and respond well to insulin’s suppression of glucose production [205].

Secondly, OA was showed to inhibit α-glucosidase and α-amylase activities. OA inhibits the activity of α-glucosidase in a cell-free spectrophotometrical assay based on the degradation of p-nitrophenyl α-d-glucopyranoside [206, 207]. A methanol extract of Tournefortia hartwegiana containing OA was shown to inhibit α-glucosidase activity in the mucosa isolated from the small intestine of Wistar rats in a dose-dependent manner [208]. Furthermore, OA was reported to inhibit α-amylase activity and a single oral administration of OA reduced postprandial glucose level in diabetic GK/Jcl rats fed starch [209]. Given that the inhibition of α-glucosidase can slow down carbohydrate digestion/absorption and is the major mechanism of several anti-diabetic drugs, the effect of OA-induced inhibition of α-glucosidase/α-amylase might play a role in the anti-hyperglycaemic effect of OA during treatment. However, further studies are required to determine whether this inhibitory effect was sustained four weeks after the removal of treatment and continue to contribute to the glucose-lowering effects of OA.

If the functional study provides further evidence to support the role of suppressed gluconeogenesis in the anti-hyperglycaemic effect of OA, the second question is related to the regulation of gluconeogenesis - Is FoxO1 the sole responsible regulator for the OA-induced suppression of gluconeogenesis?

Gluconeogenesis is an energy-consuming process to generate glucose from non-carbohydrate carbon substrates and plays an important role in regulating blood glucose levels. Two signalling pathways, the insulin-regulated PI3K pathway and AMPK pathway, are involved in the suppression of gluconeogenesis [210]. In addition to the insulin signalling pathway (which
was described in Chapter 4), the AMPK pathway was also examined four weeks after the removal of OA. However, the activation of AMPK and its downstream target ACC was not detectable in either liver or muscle of OA-treated mice (data not shown), suggesting the AMPK pathway might not play a role in regulating the OA-induced suppression of gluconeogenesis after the cessation of treatment.

In addition to FoxO1, a number of insulin-regulatable transcription factors (e.g. SREBP-1c [211, 212], LXR [213, 214] and FXR [215]) and coactivator proteins (e.g. PGC-1α [216, 217]) are known to regulate the process of gluconeogenesis. Overexpression or activation of SREBP-1c, LXR and FXR is reported to suppress gluconeogenesis, while PGC-1α is an important coactivator involved in the gluconeogenic gene expression. It is reported that OA does not activate LXR in 3T3L1 adipocytes [218], and OA has no effect on the activation of FXR [219] or even dose-dependently blocks the CDCA-induced FXR activation [220]. Given that OA suppressed plasma and liver triglyceride levels during treatment and had no effect on these post-treatment, it is unlikely that OA activated SREBP-1c as activation of SREBP-1c will promote lipid synthesis (as discussed in Section 1.3.1.1). PGC-1α is well-known for its regulation on mitochondrial biogenesis in addition to gluconeogenesis [70]. A recent study reports that OA inhibits the decrease of PGC-1α mRNA expression in db/db mice [203]. However, the effects of PGC-1α on mitochondrial biogenesis and gluconeogenesis are differentially regulated by phosphorylation [221]. Given that the gluconeogenesis is inhibited in the db/db mice treated with OA despite an increased PGC-1α expression [203] and FoxO1 knock-out render PGC-1α incapable to induce gluconeogenic gene expression [222], it is likely that PGC-1α might act as a downstream mediator of FoxO1 and its promoter activity is also suppressed (despite there might be an up-regulation of its protein level) in this study, due to an OA-induced reduction of FoxO1 level.
The third and most intriguing question remains to be answered in this study is: *how is the anti-hyperglycaemic effects of OA sustained beyond the treatment?*

Results from Chapter 4 demonstrated a suppressed G6Pase gene expression associated with a marked reduction of FoxO1 protein level. While this reduction of total FoxO1 protein level can be attributed to an increased inhibitory phosphorylation on Ser256 (which leads to the nuclear exclusion and degradation), a decrease in FoxO1 expression can also contribute to this reduction of total protein level. The expression level of FoxO1 deserves further investigations, as it might shed light on the underlying mechanism by which OA exert sustained anti-hyperglycaemic effects in type 2 diabetic mice.

Sustained end-organ effects after exposure to hyperglycaemia in both type 1 and 2 diabetes have been demonstrated in clinic [223, 224] and animal models [225]. A growing body of evidence suggests that epigenetic changes (e.g. DNA methylation and histone modification) might play a role in the long-term metabolic memories [226-230]. It has been demonstrated that inhibition of histone demethylase LSD1 or increased acetylation at Lys 9 on histone H3 over gluconeogenic genes contribute to their transcription activations and increased hepatic glucose production [231, 232]. Similarly, FoxO1 gene is reported to be hypermethylated in rheumatoid arthritis, whereas hypermethylation is associated with decreased gene expression [233]. It is exciting to hypothesize that OA-treatment might induce similar epigenetic events to alter the FoxO1 gene methylation and/or modify the acetylation/methylation status of related histone proteins, resulting in a sustained reduction of FoxO1 expression. Evidence supporting this hypothesis comes from a study demonstrating that a derivative of OA (ursolic acid) profoundly inhibits the activity of histone deacetylases 1, 3, 4, 5 and 6, and increases histone H3 acetylation [234]. It would be important to test this hypothesis in the future study.
In addition to the potential epigenetic regulation of FoxO1 expression, enzymes involved in histone modifications have been shown to regulate FoxO1 activity through post-translational modification, e.g. acetylation or deacetylation [235, 236].

A hypothesized regulation of OA on FoxO1 level and gluconeogenesis is summarised in Figure 6.1.

**Figure 6.1 A hypothesized regulation of oleanolic acid on gluconeogenesis** Oleanolic acid (OA) might regulate the total protein level of FoxO1 and gluconeogenesis through both post-translational (e.g. phosphorylation and acetylation) and/or epigenetic modifications. ac-FoxO1, acetylation of FoxO1; p-FoxO1, phosphorylation of FoxO1. Solid lines indicate known pathways/reported observations; dotted lines indicate hypothesized regulations. Pathways marked in grey are for future studies.
6.3 FUTURE DIRECTIONS FOR MATRINE

The study presented in Chapter 5 demonstrated the potential of Mtr for the treatment of a medical condition (T2D) other than originally intended (hepatitis B). Results from this study showed that Mtr significantly improves glucose tolerance and reduces hepatosteatosis in HFC-fed mice. The study in humans also provides an initial proof-of-concept for its efficacy for dyslipidaemia without significant noticeable adverse effects. Collectively, results presented in this study suggest that Mtr may be a promising novel therapeutic for T2D with favourable effects on the associated fatty liver disease.

The approach of investigating and developing drugs with established safety in humans for efficacy in a disease other than the one originally intended is called drug repurposing (also known as drug redirecting, repositioning or reprofiling). Drug repurposing is gaining increasing interest in recent years as it can potentially avoid many common issues (e.g. poor bioavailability, side effects) encountered by new identified leads in conventional D&D [167]. Compared to the conventional D&D, drug repurposing might substantially reduce the development duration, risk and cost involved in the drug development due to the well-known safety and pharmacokinetic profiles of the compound of interest [237]. Successful examples resulted from this approach include duloxetine (it was initially developed for depression, and is now widely used for the treatment of stress urinary incontinence) [238], sildenafil (it was intended to relax coronary arteries, however the desired cardiovascular effects were not observed. It later turned out to be a blockbuster drug, with a peak annual sale > US$1 billion, for erectile dysfunction) [239] and zidovudine (it failed to be developed into an anti-cancer drug, but it was found to inhibit the replication of human immunodeficiency virus and approved as the first anti-HIV therapy) [240].
While drug repositioning offers a number of advantages over conventional D&D and successful repositioning cases are increasing in number, this approach also faces several challenges. One of these challenges is the possible lack of a clinically validated mechanism of action for the treatment of the new disease [237].

In this study, the therapeutic effect of Mtr was associated with an up-regulation of HSP72 in the liver. A number of potential anti-diabetic compounds, including resveratrol [241] and BGP-15 [185], were shown to induce HSP72 expression in vitro and/or in vivo. Transgenic overexpression of HSP72 was reported to prevent high-fat diet-induced increase of body weight, improves glucose tolerance and insulin sensitivity [185]. In line with this report, a randomised, double-blind, placebo-controlled clinical trial involved 47 patients with impaired glucose tolerance has shown that BGP-15 significantly improved whole-body and muscle insulin sensitivity [242]. While results in this study demonstrated a significant inversed correlation between the hepatic HSP72 protein levels with both hepatic steatosis and glucose intolerance, the precise role of HSP72 up-regulation in the therapeutic effects of Mtr remains unknown. To address this question, additional studies are required to assess the metabolic effects of Mtr in HSP72-knockout mice [185].

In addition to its clinical use for hepatic viral infection and the therapeutic effects reported in this thesis, Mtr has been shown to possess several other pharmacological properties. It protects rats from liver fibrosis induced by CCl$_4$ [197, 243] and lung injury induced by LPS [244]. It can also suppress tumour growth [245] and alleviate cachexia-related symptoms induced by colon26 adenocarcinoma in mice [246]. These anti-fibrosis, -inflammation and –tumour properties were attributed to its effects to reduce the expression of inflammatory mediators (e.g. TNF$\alpha$, NF$\kappa$B and TGF-$\beta$) [197, 244], and induce apoptosis in carcinoma cells or tissues (by up-regulation of Bax, Fas/FasL and/or caspase-3, and/or down-regulation of Bcl2) [245, 247-249]. Intriguingly, modulations of the HSP90/HSF1/HSP72 pathway have
been shown to suppress canonical TGF-β and NFκB signalling (by inhibiting HSP90 [250-252]), and induce apoptosis (by overexpressing HSP72 [253] or inhibiting HSP90 [254]). These observations raise an exciting possibility that a unifying mechanism underlying these seemingly disparate effects of Mtr might exist and involve the modulation of Mtr on the HSP pathway (Figure 6.2).

In light of these lipid-lowering, anti-inflammation and –fibrosis properties of Mtr, it would be worthwhile investigating the possibility of repurposing Mtr for NAFLD in the future study. NAFLD is an umbrella term used to describe a range of related disorders in the liver, including hepatic steatosis, non-alcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma [49]. While hepatic steatosis (characterised by the increased intra-cellular deposition of triglyceride in hepatocytes) is benign, it can progress to NASH, which is distinguished from simple steatosis by the presence of hepatocyte injury (e.g. hepatocyte ballooning), inflammation and/or collagen deposition (fibrosis). 10–29% of individuals with NASH develop cirrhosis within 10 years. As the exact mechanism underlying the development of NASH is still unclear and currently there is no specific therapeutic for the treatment of NAFLD [195], the drug discovery and development for NAFLD presents a great challenge as well as an invaluable opportunity. Prior knowledge of Mtr on its pharmacokinetics, drug safety profiles and therapeutic properties, will hopefully serve the development of new treatments for NAFLD.
Figure 6.2 Potential applications of matrine and the possible role of HSP90 and HSP72.
Matrine (Mtr) is a drug used in clinic for the treatment of hepatic viral infection. In addition to its effects to improve hepatic steatosis and glucose intolerance as demonstrated by this thesis, anti-inflammation, -fibrosis and -tumour properties of Mtr have also been reported. Inhibition of HSP90 and/or up-regulation of HSP72 can lead to similar effects. These observations suggest Mtr might be repurposed for other medical conditions including type 2 diabetes, non-alcoholic fatty liver disease and certain cancers.
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Publications
Oleanolic Acid Reduces Hyperglycemia beyond Treatment Period with Akt/FoxO1-Induced Suppression of Hepatic Gluconeogenesis in Type-2 Diabetic Mice

Xiao-Yi Zeng, Yi-Ping Wang, James Cantley, Tristan J. Iseli, Juan Carlos Molero, Bronwyn D. Hegarty, Edward W. Kraegen, Yang Ye, Ji-Ming Ye

Abstract

The present study investigated the chronic efficacy of oleanolic acid (OA), a triterpenoid selected from our recent screening, on hyperglycemia in type-2 diabetic mice. C57BL/6J mice were fed a high-fat diet followed by low doses of streptozotocin to generate a type-2 diabetic model. OA (100 mg/kg/day) was administered orally for 2 weeks with its effects monitored for 6 weeks. High-fat feeding and streptozotocin generated a steady hyperglycemia (21.2±1.1 mM) but OA administration reversed the hyperglycemia by ~60%. Interestingly, after the cessation of OA administration, the reversed hyperglycemia was sustained for the entire post-treatment period of the study (4 weeks) despite the reoccurrence of dyslipidemia. Examination of insulin secretion and pancreas morphology did not indicate improved β-cell function as a likely mechanism. Urine glucose loss was decreased with substantial improvement of diabetic nephropathy after the OA treatment. Pair-feeding the OA-treated mice to an untreated group ruled out food intake as a main factor attributable for this sustained reduction in hyperglycemia. Studies with the use of glucose tracers revealed no increase in glucose influx into muscle, adipose tissue or liver in the OA-treated mice. Finally, we analyzed key regulators of gluconeogenesis in the liver and found significant increases in the phosphorylation of both Akt and FoxO1 after treatment with OA. Importantly, these increases were significantly correlated with a down-regulation of glucose-6-phosphatase expression. Our findings suggest triterpenoids are a potential source of new efficacious drugs for sustained control of hyperglycemia. The liver appears to be a major site of action, possibly by the suppression of hepatic glucose production via the Akt/FoxO1 axis.

Introduction

The incidence of diabetes is estimated at 220 million worldwide [1] and prolonged hyperglycemia is a major cause of various diabetic complications including nephropathy [2]. Effective control of blood glucose is, therefore, crucial to the treatment of diabetes and the prevention/delay of diabetic complications. Type-2 diabetes accounts for ~90% of all diabetes cases [1] and it results from the metabolic disorders of insulin resistance (diminished sensitivity of the target tissues to insulin action) and β-cell failure (reduced ability of the pancreatic β-cells to produce sufficient insulin). Therefore, improvements of insulin action and β-cell function are important mechanisms for the pharmacological treatment of type-2 diabetes.

Sustained control of hyperglycemia is of great importance to the treatment of type-2 diabetes and it remains a significant challenge. Until recently, the mainstay oral medications to improve insulin action in type-2 diabetes have been biguanides (e.g., metformin) and thiazolidinediones (TZDs) [3]. However, biguanides are not adequate therapies on their own in the long-term [3,4] as they have limited effects in improving insulin action in muscle [5,6]. While TZDs are effective in lowering hyperglycemia, largely by an insulin sensitizing action [7,8], concerns over the adverse effects of TZDs on an increased risk of heart failure [9] and bladder cancer [10] have restricted their long-term use. Other new drugs such as GLP analogues and inhibitors of the sodium glucose co-transporter (SGLT) appear promising [11,12,13], however their long term effectiveness is not clear. Thus there remains an urgent need for the development of new anti-diabetic drugs with sustained efficacy.

We recently found that triterpenoid compounds isolated from bitter melon have potent efficacy in stimulating GLUT4 translocation in L6 myotubes and 3T3L1 adipocytes, along with activation of the AMPK pathway [14]. Our acute studies in mice showed that triterpenoids are able to reduce glucose intolerance in insulin resistant high-fat (HF)-fed mice after a single injection [14]. These findings are encouraging because triterpenoids are a rich natural source for drug discovery, with more than 20,000 of them known to exist in plants [15]. The present study investigated...
whether the triterpenoid, oleanolic acid (OA), is an effective treatment for hyperglycemia in a murine model of type-2 diabetes. The study focused on the OA compound based on our recent screens [14].

OA itself has been used in humans for its potential therapeutic application for cancer [15] and an OA analogue has been shown to alleviate diabetic nephropathy in type-2 diabetic patients [16]. OA and its analogues have been shown to lower hyperglycemia in STZ-treated rodents [17], HF-fed or db/db mice [18], to protect against diabetic nephropathy [17] and to enhance the survival of pancreatic islets [19]. However, all of these studies were relatively short-term (mostly less than 2 weeks) and the sustainability of these therapeutic effects is not known. Here we investigated the therapeutic efficacy of OA in reducing hyperglycemia in a murine diabetic model produced by chronic HF feeding with low doses of STZ [20,21]. We demonstrate a potent glucose-lowering effect that was sustained well beyond the treatment period and was accompanied by a significant improvement in renal structure.

Furthermore, we have identified Akt/FoxO1 mediated suppression of glucose-6-phosphatase (G6Pase), a key regulator of hepatic glucose production, as a likely mechanism underlying the improved glucose homeostasis.

Materials and Methods

Animal model

Male C57BL/6J mice (10 weeks old) were purchased from the Animal Resources Centre (Perth, Australia). The animals were kept in a temperature-controlled room (22±1°C) on a 12-h light/dark cycle with free access to food and water. After 1 week of acclimatization, mice were fed ad libitum for 10 weeks with a standard lab chow diet (CH; 8% calories from fat, 21% calories from protein, and 71% calories from carbohydrate) or a high-fat diet (HF; 45% calories from fat, 20% calories from protein, and 71% calories from carbohydrate) in order to induce insulin resistance [7,8,14,22]. Mice were then injected with either vehicle (saline) or a low dose of streptozotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce diabetes (fasting blood glucose >12 mM) [20,21]. CH-fed mice treated with STZ comprised a model of type-1 diabetes (T1D mice). HF-fed mice treated with STZ comprised a model of type-2 diabetes (T2D mice). One week after the last STZ injection (‘baseline’), a subset of T1D and T2D mice received OA as a food additive at 100 mg·kg⁻¹·day⁻¹ for two weeks (T1D-OA and T2D-OA respectively). This dose of OA was selected on the basis of our previous study [14]. The remaining T1D and T2D mice received their normal CH or HF diet (T1D-Veh and T2D-Veh respectively). Body weight, food intake and fasting blood glucose levels were monitored on a weekly basis until 4 weeks after the cessation of OA administration. All experiments were approved by the Animal Ethics Committees of the Garvan Institute (#0847) and RMIT University (#1012) in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Assessment of effects on hyperglycemia, blood insulin level and glucose tolerance

Blood glucose levels were measured once a week after 5–7 hours of fasting. Blood samples were collected from the tail tip and were analyzed using a glucometer (AccuCheck II; Roche, New South Wales, Castle Hill, Australia). Two weeks after the cessation of OA treatment, i.p. glucose tolerance tests (ipGTT; glucose load 1 g/kg BW) were performed in the 5–7 hours fasted state, as previously described [22]. Briefly, blood samples were obtained from the tail tip at 0, 15, 30 and 60 min for the measurement of blood glucose levels and at 0, 5, 30 and 60 min for the measurement of insulinemia (determined by radioimmunoassay; Linco/Millipore, Billerica, MA).

Insulin secretion assays, pancreatic histology and insulin content measurements

Islet isolation and ex vivo insulin secretion assays were performed as previously described [23]. Mice were killed by cervical dislocation and the pancreas was perfused with 2 ml of Liberase (Roche, Basel, Switzerland) solution (0.25 mg/ml in Krebs-Ringer buffer) via injection into the common bile duct. After pancreatic digestion at 37°C, islets were purified using a Ficoll-paque (GE Healthcare, Chalfont St. Giles, U.K.) gradient. Islets were washed and immediately pre-incubated for 1 hr in Krebs-Ringer buffer containing HEPES (KRHB), 0.1% BSA and 2 mM glucose. Batches of five islets were incubated at 37°C for 1 hr in 130 μl KRHB containing 0.1% BSA and 2, 5.5, 11 or 20 mM glucose. For the measurement of pancreatic insulin content, the pancreas was weighed and then homogenized in ice-cold acid ethanol.

Table 1. Metabolic characteristics of the type-1 and type-2 diabetes mouse models.

<table>
<thead>
<tr>
<th></th>
<th>CH (Normal)</th>
<th>HF (IR)</th>
<th>CH-STZ (T1D)</th>
<th>HF-STZ (T2D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.5±0.4</td>
<td>31.7±0.81</td>
<td>28.8±0.41</td>
<td>27.8±0.21**</td>
</tr>
<tr>
<td>Epi fat (% BW)</td>
<td>1.4±0.1</td>
<td>3.2±0.31†</td>
<td>1.2±0.1†</td>
<td>1.6±0.1**†</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>9.2±0.2</td>
<td>8.3±0.4</td>
<td>13.6±0.6†</td>
<td>17.5±1.2†###</td>
</tr>
<tr>
<td>Blood insulin (pg/ml)</td>
<td>367±29</td>
<td>503±130†</td>
<td>300±26</td>
<td>301±63†</td>
</tr>
<tr>
<td>Plasma triglyceride (mM)</td>
<td>1.2±0.1</td>
<td>1.5±0.1</td>
<td>1.9±0.1†</td>
<td>2.2±0.2†</td>
</tr>
<tr>
<td>Liver triglyceride (μmol/g)</td>
<td>7.8±0.8</td>
<td>18.6±2.0†</td>
<td>9.4±0.9</td>
<td>19.6±1.1†###</td>
</tr>
</tbody>
</table>

C57BL/6J mice were fed with chow diet and injected with saline (CH) or STZ (T1D), or fed with HF diet and injected with saline (HF) or STZ (T2D). IR, insulin resistant. Blood samples were collected from mice in the 5–6 hour fasted state. Blood glucose (n=8–10) and insulin (n=5), and plasma triglyceride (n=7–8) were measured as described in the methods. Body weight, epididymal weight and liver triglycerides were measured at the end of the study (n=10). Data are expressed as means ± SE.

*p<0.05,  
*p<0.01 vs. CH;  
*p<0.05,  
*p<0.01 vs. HF;  
*p<0.05,  
*p<0.01 vs. T1D.

doi:10.1371/journal.pone.0042115.t001
Figure 1. Effects of OA on blood glucose, food intake and body weight in T2D and T1D mice over time. HF-fed mice with STZ injections were treated with (T2D-OA) or without (T2D-Veh) OA in the diet for two weeks, at the end of which OA was removed from the diet. Blood glucose, food intake and body weight were monitored between 14:00 and 16:00 once a week (A, B and C). CH-fed mice with STZ injections were treated with (T1D-OA) or without (T1D-Veh) OA in the diet for two weeks, at the end of which OA was removed from the diet. Effects of OA on hyperglycemia in T1D mice (D). CH, normal chow fed mice. Data are expressed as means ± SE. † p<0.05, ‡ p<0.01 vs. T2D-Veh group, n = 11–16 per group. doi:10.1371/journal.pone.0042115.g001

Figure 2. Effects of OA on glucose tolerance and blood insulin. Studies were performed in mice two weeks after the removal of OA. ipGTT was performed with an injection of glucose (1 g/kg, ip) after 5–7 hours of fasting. Blood glucose was monitored at 0, 15, 30, and 60 min following the glucose injection (A). ipGTT results were quantified by calculating the area under the blood glucose curve (AUC) and the incremental AUC (iAUC) (B). Insulin levels throughout the ipGTT (C). The average value of blood insulin levels from 5 to 60 mins during the ipGTT (D). CH, normal chow fed mice; T2D-Veh, HF-fed mice with STZ injections; T2D-OA, HF-fed mice with STZ injections and OA treatment. ** p<0.01 vs. CH; †† p<0.01 vs. T2D-Veh, n = 5–8 per group. doi:10.1371/journal.pone.0042115.g002
overnight at 4°C with 2% BSA and 5% chick serum) before incubation for 30 min at room temperature with blocking solution (PBS-EDTA). Ted light images were captured (magnification ×200) by ImageJ. A total of 50 randomly-selected cortical tubules in 10 non-overlapping fields (magnification ×200) were measured, and the mean cross-sectional tubule cell height was determined for each section. The degree of glomerular hypertrophy was measured quantitatively. The outline of the glomeruli and glomerular capillary tuft was traced, and the computed area was used as a measure of total glomerular area and tuft area. The mean value of 20 randomly selected glomeruli was determined for each section. The cortical interstitial volume included the tubular basement membrane and peritubular capillaries. To quantitate this area, cortical fields (magnification ×200) were viewed on a video screen, and the area of interstitial space was determined with image analysis software and expressed as a percentage of the total area of the field. The mean percentage area of five non-overlapping cortical fields was calculated for each section.

Figure 3. Effects of OA on insulin secretion and insulin in pancreatic β-cells. Four weeks after the cessation of OA treatment, fresh islets were isolated from T2D mice treated with or without OA, and insulin secretion in response to different glucose concentrations was measured (A). Pancreatic insulin content (B). β-cell area (expressed as a percentage of pancreatic area) (C). Representative images of immunohistochemical staining of β-cells (D). T2D, HF-fed mice with STZ injections; T2D-OA, HF-fed mice with STZ injections and OA treatment. n = 4–6 per group.

Measurement of plasma and liver triglyceride

Plasma was extracted from blood samples collected from the tail tip and stored at ~80°C. Mice were killed by cervical dislocation in the fasted state and liver samples were immediately freeze-clamped. Liver triglycerides were extracted by the method of Folch. The triglyceride level in plasma and liver extract was determined by a Peridochrom triglyceride GPO-PAP kit (Roche Diagnostics) as previously described [7,8,12,22].

Measurement of glucose flux in key tissues for glucose homeostasis

In one subset of mice, [3H] labeled 2-deoxy-D-glucose (2DG; PerkinElmer, USA) and D-[14C] glucose (PerkinElmer, USA) were used to measure glucose metabolism in skeletal muscle, fat and liver, as described in our previous work [8]. Briefly, 4 weeks after the cessation of OA treatment, an ipGTT was performed after 5–7 hours of fasting. Glucose (1 g/kg BW) containing [3H]-2DG (65 µCi/kg BW) and D-[14C] glucose (32 µCi/kg BW) was injected i.p. Plasma samples were obtained from the tail tip at 10, 20, 30 and 40 min after glucose administration for estimation of plasma tracer concentration. At the completion of the ipGTT, mice were culled and tissue samples were immediately freeze-clamped for subsequent analysis.

Analysis of gene expression

Total RNA was extracted from liver tissue using TRIZOL® (Invitrogen, USA) according to the manufacturer’s instructions. Reverse transcription was carried out with 0.2 mg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real time PCR was carried out using the IQ SYBR Green Supermix (2×) (Bio-Rad Laboratories Inc, USA) for G6Pase and PEPCK (Genework, Australia). The gene expression

Assessment of the effects on urine glucose secretion and kidney morphology

Urine samples were collected in the morning three weeks after the completion of OA treatment. The urinary glucose level was measured by the glucose oxidase assay using an automated glucose analyser (YSI 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, Ohio, USA). For the kidney morphology study, kidneys were rapidly removed after cervical dislocation. Coronal sections of renal tissue were immersion-fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 5 µm thick were stained with periodic acid-Schiff and evaluated using methods described previously [25]. To quantitate tubular atrophy, the tubule cell height of an individual cortical tubule was measured using line morphometry (magnification ×200) by ImageJ. The degree of glomerular hypertrophy was measured quantitatively. The outline of the glomeruli and glomerular capillary tuft was traced, and the computed area was used as a measure of total glomerular area and tuft area. The mean value of 20 randomly selected glomeruli was determined for each section. The cortical interstitial volume included the tubular basement membrane and peritubular capillaries. To quantitate this area, cortical fields (magnification ×200) were viewed on a video screen, and the area of interstitial space was determined with image analysis software and expressed as a percentage of the total area of the field. The mean percentage area of five non-overlapping cortical fields was calculated for each section.

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(0.15 M HCl in 75% ethanol) immediately after collection. Insulin concentrations in the incubation medium and pancreatic extracts were determined by a commercial insulin radioimmunoassay kit (Linco/Millipore, Billerica, MA).

Quantification of β-cell area was performed based on previous methods [24]. Each pancreas was removed, cleared of fat and lymph nodes, fixed in 10% neutral buffered formalin and embedded in paraffin wax. 5 µm sections were cut and incubated for 30 min at room temperature with blocking solution (PBS buffer with 2% BSA and 5% chick serum) before incubation overnight at 4°C in blocking solution containing mouse anti-insulin antibody (L2018, Sigma-Aldrich). Sections were then incubated with chicken anti-mouse IgG-Alexa Fluor 594 conjugate (Invitrogen) for 1 hr at room temperature in darkness. Transmitted light images were captured (magnification ×20). To quantitate β-cell area, the outline of the pancreas section and all insulin-positive cells were traced and scored using ImageJ image analysis software [ImageJ, NIH, Bethesda, MD]. Results are expressed as the percentage of the total pancreatic area stained positive for insulin.
from each sample was analyzed in duplicate and normalized against the housekeeper, 18S. The primer sequence (5’ to 3’) of 18S was: CGCCGCTAGAGGTGAAATTCT (sense) and CGAACCTCCGACTTTCGTTCT (antisense); PEPCK: CCA-CAGCTGCTGCAGAACA (sense) and GAAGGGTCG-CATGGCAAA (antisense); G6Pase: AACGCCTTC-TATGTCCT CTTTC (sense) and GTTGCTGTAGTA-GTGGGTGTC (antisense). All reactions were performed on the iQ™ 5 Real Time PCR Detection System (Bio-Rad Laboratories Inc, USA). The results are expressed as relative gene expression using the \( \Delta \Delta Ct \) method.

Western blotting

Western blotting in liver samples was performed as described previously [26] with minor modifications. Briefly, the freeze-clamped liver tissues were homogenized in ice-cold lysis buffer at pH 7.5 containing (in mM): 50 Tris, 150 NaCl, 1% Triton X-100, 10 NaP, 100 NaF, 2 Na3VO4, 1 EDTA, 1 EGTA and 10% glycerol supplemented with protease inhibitor cocktail tablets (Roche Diagnostics Pty Ltd, Australia) and DL-dithiothreitol. Total protein concentrations were assessed using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) before protein samples were denatured in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue). Regulation of glucoenogenesis was assessed by total- and phospho(Ser473)-Akt, as well as total- and phospho(Ser256)-Forkhead box protein O1 (FoxO1, Cell Signaling, USA). Densitometry analysis was performed using Image Lab software (Bio-Rad Laboratories, USA) and representative blots are shown.

Statistical Analyses

Data are presented as means ± SE. One-way analysis of variance was used for comparison of relevant groups. When significant variations were found, the Tukey-Kramer multiple comparisons test was applied. Pearson’s two-sided correlation was used for the correlation analysis. Differences at \( p<0.05 \) were considered to be statistically significant.

Results

Induction of type-1 and type-2 diabetes

We first induced a type 2 diabetes model of hyperglycemia by chronic HF-feeding and low-dose STZ based on previous reports [20,21]. As shown in Table 1, HF feeding caused a small increase in body weight and a 2.3 fold increase in epididymal fat compared to CH mice. HF mice also exhibited increased blood insulin (40%) and liver triglyceride (2.4 fold) compared to CH mice, but the circulating levels of glucose and triglyceride were similar between HF and CH mice. CH-fed mice treated with STZ (T1D thereafter), demonstrated moderate increases in blood glucose and plasma triglyceride compared to CH mice (50–60%, \( p<0.05 \) vs. CH-fed mice), but liver triglyceride content was unchanged. As expected, HF-STZ (T2D thereafter) mice recapitulated several
major characteristic metabolic disorders in type-2 diabetes, namely hyperglycemia (~2 fold), hypoinsulinemia (by 20%), dyslipidemia (80%) and liver steatosis (2.5 fold) compared to CH mice. Consistent with previous reports [21], T2D mice also displayed a lack of glucose response to insulin action during an insulin tolerance test - a typical feature of insulin resistance (data not shown). Compared with HF feeding alone, T2D mice displayed hyperglycemia (>2 fold, p<0.01), hypertriglyceridemia (50%, p<0.05) and reduced plasma insulin level (50%, p<0.01) while retaining a similar level of liver steatosis. Compared with T1D, T2D mice showed more epididymal fat (30%), greater hyperglycemia (~30%) and significantly increased triglyceride content in the liver (~2 fold).

Effects of OA on hyperglycemia and glucose tolerance

Oral administration of OA, at a dose of 100 mg/kg/day, dramatically reversed the hyperglycemia of T2D mice by more than 60% (approaching the level of CH-fed mice) by the end of the two-week treatment (22.5 ± 0.6 vs. 13.2 ± 1.9 mM, p<0.001). Intriguingly, following the removal of OA from the HF diet, the reversed hyperglycemia evident in the T2D-OA group persisted for the rest of the study. Meanwhile the hyperglycemia in the untreated T2D-Veh group remained high (Fig. 1A). Under a similar treatment regime, OA showed no significant effect in lowering the hyperglycemia evident in the T1D Fgnouse model (Fig. 1D). Along with a reduced food intake at the end of the two-week treatment (Fig. 1B), T2D-OA group had a lower body weight than T2D-Veh group at week 3 and 4 (Fig. 1C). However, food intake and body weight of T2D-OA mice was completely recovered by the end of the study (Fig. 1, B and C).

As the anti-hyperglycemic effect of OA was only observed in T2D mice, our subsequent studies were performed only in the T2D mouse model. OA significantly improved the glucose tolerance of T2D mice as evidenced by decreased blood glucose level in T2D-OA compared to T2D-Veh at all time points (p<0.01) during the ipGTT as well as by a reduced AUC under the curve (Fig. 2, A and B). However, when expressed as the incremental area under the curve, we did not find a significant difference between the T2D-Veh and T2D-OA groups. During the ipGTT, blood insulin levels were significantly higher in T2D-OA compared to T2D-Veh mice at 30 min, with a similar trend at 5 min (p = 0.082) (Fig. 2C). However, when expressed as the average value from 5 to 60 min, there was no statistical difference between the T2D-Veh and T2D-OA groups (p = 0.053, Fig. 2D).

Insulin secretion, β-cell numbers and pancreatic insulin content post-OA treatment

Fig. 2C and D appeared to indicate a seeming improvement in β-cell function after treatment of T2D mice with OA, which could provide a mechanism for the improved blood glucose levels in these mice. We therefore further assessed glucose-stimulated insulin secretion in isolated islets. As shown in Fig. 3A, islets isolated from T2D-OA mice showed an insulin secretion response to increasing glucose stimulation that was indistinguishable from T2D-Veh mice. We next examined total pancreatic insulin level and found that the administration of OA had no effect on the total pancreatic insulin content in T2D mice (29.1 ± 10.6 vs. 26.6 ± 6.0 μg/g pancreas in the T2D-Veh group, p>0.05) (Fig. 3B). Finally, immunohistochemical staining of pancreatic sections showed that OA did not affect the total number of β-cells per pancreas in T2D mice (0.29±0.11% vs. 0.27±0.11%, p = 0.994) (Fig. 3C and D).

Alleviation of glucose loss in urine and nephropathy post-OA treatment

In order to investigate if the reduced blood glucose level in T2D-OA mice was due to an increase in the urine excretion of glucose, we measured the glucose level in the urine of T2D mice before and after the treatment with OA. T2D mice excreted more glucose in the urine compared to CH-fed mice at baseline (Fig. 4A). Whilst glucose excretion remained high in T2D mice when measured 3 weeks after the cessation of OA treatment, T2D-OA mice demonstrated a significant drop in urine glucose levels (11.8 ± 2.1 vs. 5.1 ± 0.8 mM before vs. after OA treatment, p<0.01) (Fig. 4B). Morphology studies revealed that the OA treatment dramatically improved kidney structure of T2D mice, as indicated by significant reductions in interstitial volume (13.2 ± 0.7 vs. 24.9 ± 0.8% in untreated T2D, p<0.01) and glomerular tubulur area in T2D-OA mice (3251 ± 41 vs. 4248 ± 33 μm² in untreated T2D, p<0.01) to levels similar to CH-fed mice (Fig. 4B,C). Associated with the normalization of interstitial volume and glomerular tubulur area, the reduced tubular cell height evident in T2D mice was significantly ameliorated in T2D-OA mice (13.6 ± 0.1 vs. 12.4 ± 0.1 μm in untreated T2D, p<0.01) (Fig. 4B and C).

Triglyceride levels in plasma and liver during and after OA treatment

T2D-Veh mice had significantly higher levels of plasma and liver triglyceride compared to CH-fed mice at the end of the two-week treatment period (Fig. 5A) and at 4-weeks post-treatment (Fig. 5B). As expected, two weeks of OA treatment substantially reduced the triglyceride levels in plasma (Fig. 5A) and liver (Fig. 5B) of T2D mice. However, 4-weeks after the termination of OA administration, both plasma and liver triglycerides had returned to the levels of the T2D-Veh group (Fig. 5A, B).

Figure 5. Effects of OA on triglyceride levels in plasma and liver during and after OA treatment. Lipid accumulation in the liver and plasma was assessed both during OA treatment (at week 2) and 4 weeks after the cessation of OA treatment. Mice were euthanized following a 5–7 hour fast and samples of plasma (A) and liver (B) were collected for the measurement of triglyceride levels. T2D-Veh, HF-fed mice with STZ injections; T2D-OA, HF-fed mice with STZ injections and OA treatment. ** p<0.01 vs. CH; †† p<0.01 vs. T2D-Veh, n = 5–8 per group.

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Influence of pair-feeding on hyperglycemia and glucose tolerance

Since a fluctuation in food intake was observed in the T2D-OA group during the initial experiment, we carried out an additional pair-feeding study to determine if the sustained reversal of hyperglycemia during the period of post-OA treatment was due to the difference in food intake. The food intake in pair-fed T2D-Veh and T2D-OA groups (2.7 ± 0.1 vs. 2.5 ± 0.1 g/mouse/day, \( p < 0.05 \)) was well matched (Fig. 6A). T2D-Veh and T2D-OA groups were not different in body weight at the baseline nor at the end of the study (Fig. 6B). The basal blood glucose of the T2D-OA group showed a significant decrease compared to the T2D-Veh group after two weeks of OA treatment despite matching of food intake, and this was maintained for 3-weeks post-treatment (Fig. 6C). Similar to the previous observation, T2D-OA mice were more glucose tolerant than T2D-Veh mice in the ipGTT performed two weeks after the removal of OA (Fig. 6, D and E).

Glucose flux in muscle, fat and liver after OA treatment

To further investigate the mechanism underlying the sustained improvement in glycemia of OA treated T2D mice, we employed \(^{3}H\)-2DG and \(^{14}C\)-glucose tracers to measure the glucose uptake in muscle and adipose tissue during an ipGTT conducted 4 weeks after the cessation of OA treatment. As expected, T2D mice demonstrated reduced glucose uptake in muscle compared to CH-fed mice (Fig. 7A) (14.4 ± 2.9 vs. 7.0 ± 0.5 \( \mu \)mol/100 g/min, \( p < 0.05 \)). A similar tendency was evident in fat tissue (7.2 ± 2.3 vs. 3.3 ± 0.8 \( \mu \)mol/100 g/min, \( p > 0.05 \); Fig. 7B). However, OA treatment of T2D mice did not cause any improvement of glucose uptake in these two tissues (Fig. 7A, B). Furthermore, measurement of glucose incorporation into glycogen and lipid in the liver (as a proxy of glucose influx) did not show any improvement in T2D-OA as compared to the T2D-Veh group (Fig. 7, C and D).

Changes in key regulators of hepatic glucose production after OA treatment

As hepatic glucose production is a major factor affecting glucose homeostasis \([27]\), we investigated the key molecules (Akt and FoxO1 \([28]\)) in the insulin signaling pathway that regulate hepatic gluconeogenesis and glucose production. As shown in Fig. 8A–D, compared to CH-fed mice, T2D mice displayed a 60% reduction in p-/t-Akt. The p-/t-Akt ratio was similar in T2D-OA and T2D-Veh mice. However, the absolute p-Akt level was lower in T2D-Veh mice compared to CH, but restored to CH levels in T2D-OA.
In the case of FoxO1, T2D-OA mice demonstrated substantially increased p-/t-FoxO1 (200% increase) (p, 0.01) and a 50% reduction in total FoxO1 protein compared to both T2D-Veh and CH mice (p, 0.05) (Fig. 8, A,E–G).

We next examined the transcriptional expression levels of the key enzymes controlling gluconeogenesis (PEPCK) and glucose production (G6Pase). As shown in Figure 9A and B, compared to CH-fed mice, T2D mice demonstrated an elevated expression of PEPCK (~80% increase, p<0.05) and G6Pase (~50% increase, p>0.05) at the transcriptional level. Interestingly, treatment of T2D mice with OA tended to reduce G6Pase RNA (~30%, p = 0.09, Fig. 9B), while the level of PEPCK expression remained unchanged. Further analysis showed that there was a significant reversed correlation of both phosphorylated Akt(p<0.05) and phosphorylated FoxO1 (p<0.05) to the transcriptional expression level of G6Pase (Fig. 9C).

**Discussion**

The present study investigated the chronic effect of OA, a triterpenoid selected from our recent screens [14], on hyperglycemia in diabetic mice and the contributing mechanisms involved. Our results show that OA did not affect hyperglycemia in T1D mice, but was able to reverse hyperglycemia in T2D mice, as predicted. Intriguingly, this reversed hyperglycemia was sustained well beyond the treatment period - for at least 4 weeks after cessation of OA treatment. Along with the correction of hyperglycemia, elevated urine glucose loss in T2D mice was also completely reversed and damage to renal structures was markedly attenuated. Although OA has been reported to lower hyperglycemia by counteracting insulin resistance within the period of its administration [29,30,31], to the best of our knowledge the sustained reversal of hyperglycemia for such a long duration after terminating the treatment has not been described before.

Since previous studies reported a hypoglycemic effect in insulin resistant HF-fed [29,30,31] and db/db mice [18], we first investigated the effects of OA on hyperglycemia in a T2D model generated by chronic HF feeding in combination with low doses of STZ. Chronic HF feeding in rodents is a widely used model of insulin resistance, associated with lipid accumulation in muscle and liver [7,32]. However, HF feeding alone is insufficient to cause diabetes due to the capacity of pancreatic β-cells to increase insulin secretion in order to compensate for the insulin resistance [33]. Therefore, we administered multiple low doses of STZ to restrict the ability of pancreatic β-cells to increase insulin secretion, thereby generating hyperglycemia as previously reported [20,21]. As expected, the combination of HF feeding and low doses of STZ resulted in marked hyperglycemia with the characteristics of overt type-2 diabetes, namely, failed compensatory increase in blood insulin levels with concurrent hypertriglyceridemia and fatty liver. Compared with CH-fed mice injected with STZ (T1D), HF-fed mice injected with STZ (T2D) showed significantly more severe hyperglycemia liver steatosis and visceral adiposity (Table 1).

Oral administration of OA to the T2D mouse model progressively reduced hyperglycemia to a level similar to that of normal mice within two weeks. Interestingly, after the termination of OA treatment the reversed hyperglycemia was retained for the remaining 4-week period of the study (Fig. 1A). Along with the reduced blood glucose level, T2D-OA mice also displayed an improved glucose tolerance at the end of the study when measured with the total area of glucose under the curve (Fig. 1A, B). While the hypoglycemic effect of an anti-diabetic compound is expected during the period of treatment, we are not aware of any other report showing similarly sustained correction of hyperglycemia after cessation of drug treatment. This dramatic improvement of
glucose homeostasis for such a long time post-OA treatment is particularly interesting because sustained control of glycemia is an important target in the treatment of type-2 diabetes.

Possible mechanisms of the sustained reversal of hyperglycemia following the treatment of OA were investigated from several different perspectives. The half-life of OA in the circulation is reported to be less than 30 min in mice [34], suggesting that the reversal of hyperglycemia post-OA in the present study is unlikely to result from a prolonged presence of OA in the circulation. We therefore first investigated whether OA may induce sustained improvement of pancreatic β-cell function to increase insulin secretion, given that acute treatment of OA was previously reported to enhance insulin secretion in isolated rat β-cells [35]. Such a possibility has been implicated in several previous studies using a T1D model induced by STZ in CH-fed rodents [17,19]. Conceivably, the sustained reversal of hyperglycemia may be achieved with a compensatory increase in insulin secretion as observed in the HF-fed mice in the present study (Table 1) or improved β-cell function as suggested for incretins [13]. However, our assessment showed that 2-week treatment of T2D mice with OA did not increase the number or enhance the function of β-cells (pancreatic insulin content or insulin secretion of β-cells in response to glucose stimulation). Furthermore, the blood insulin profiles during the ipGTT were not different between T2D mice with/without OA treatment. These results are also consistent with the inability of OA to lower hyperglycemia in STZ-injected CH-fed mice in the present study. The reason for the discrepancy of our results from the previous reports in this T1D rodent model [17,19] is not clear. However, our findings in T2D and T1D models are internally consistent and collectively indicate that it is unlikely that OA exerts its beneficial effects on glycemia through an improvement in β-cell function.

It has been proposed that the inhibition of SGLT2 (a transporter for glucose re-absorption in the kidney) can increase glucose excretion in the urine and thereby reduce hyperglycemia for the treatment of type-2 diabetes [12,13]. We therefore examined whether the sustained anti-hyperglycemic effect of OA was achieved by increasing urine glucose excretion. However, in contrast to an increase in the glucose excretion through the kidney, OA treatment significantly reduced the urine glucose level in T2D mice to the level of CH-fed mice. Furthermore, the diabetic nephropathy evident in T2D-Veh mice was abolished in T2D-OA treated mice.

Figure 8. Changes in Akt and FoxO1 in the liver 4 weeks after the removal of OA. Four weeks after the cessation of OA treatment, mice were sacrificed following a 5–7 hour fast. Liver samples were freeze-clamped and stored at −80°C for subsequent Western blotting analysis. Representative Western blot images of phosphorylated and total Akt and FoxO1(A). Quantification of p-Akt/GAPDH (B), t-Akt/GAPDH (C), p-/t-Akt (D), p-FoxO1/GAPDH (E), t-FoxO1/GAPDH (F) and p-/t-FoxO1 (G). * p<0.05 vs. CH; † p<0.05, †† p<0.01 vs. T2D-Veh, n=6–8 per group. doi:10.1371/journal.pone.0042115.g008
mice treated with OA, as indicated by increased tubular cell height, and decreased interstitial volume and glomerular turf area. These results rule out increased urine excretion of glucose as a mechanism for the reduced glucose level following OA treatment. These findings are consistent with a recent clinical trial which demonstrated the beneficial effects of an OA derivative for nephropathy in type-2 diabetes patients [9]. As OA can improve renal functions in diabetic mice by inhibiting the formation of advanced glycation endproducts [17], it is possible that the reversal of hyperglycemia following the treatment of OA contributed to the alleviated nephropathy.

The reduced food intake evident in T2D-OA mice during the period of OA treatment presented another potential mechanism underlying the sustained reduction in glycemia. Reduced food intake has been shown to have effects on fasting blood glucose levels, glucose tolerance and insulin sensitivity in mice and rats [36,37]. However, it is unknown whether the on glycemia, can be maintained for days after food intake has normalised. To investigate possible influences of the different food intake pattern of T2D-Veh and T2D-OA groups, we conducted a pair-feeding study with an additional two groups of mice. Despite food intake of the pair-fed T2D-Veh and T2D-OA groups being matched throughout the study, T2D-Veh mice maintained significantly higher glycemia than T2D-OA mice from the second week of OA treatment and through to the end of the post-treatment period (Fig. 6A–C). Furthermore, pair-fed T2D-Veh mice were not as glucose tolerant as T2D-OA mice as evidenced by an ipGTT performed at the end of the study (Fig. 6D, E). These data indicate that the effect of OA to improve glucose homeostasis in diabetic mice does not rely on its effect to reduce food intake. In support of this interpretation, a recent study has demonstrated that an OA analogue can lower blood glucose in both HF-fed and db/db mice without affecting food intake [18].

Several studies have shown that triterpenoids, including OA, can reverse insulin resistance and glucose intolerance during the treatment period [18,29,30,38]. The present study similarly observed that T2D-OA mice displayed significantly lower plasma glucose levels during ipGTT. Since OA treatment attenuates the glucose intolerance with similar blood insulin levels as T2D-Veh mice, the improved glucose tolerance and glycemia in T2D-OA mice is probably due to improved insulin sensitivity of the peripheral tissues.

Insulin resistance is closely related with increased accumulation of lipids in peripheral tissues [39] and a reduction in lipid content in these tissues is an effective means to improve insulin sensitivity [7]. The reversal of insulin resistance and glucose intolerance during OA treatment has been associated with a reduction of lipid accumulation in muscle and liver [18,29,30,38]. As triterpenoids can acutely activate AMPK and promote fat oxidation [14], one plausible mechanism of the sustained efficacy to reduce hyperglycemia in the present study could conceivably be due to improved insulin action as a result of reduced lipid accumulation in muscle and liver. Such a mechanism has been demonstrated with berberine [40], and Abbott compound A [41] during the period of treatment. To investigate if OA improves insulin sensitivity through a similar mechanism, we measured the triglyceride levels
in plasma and liver because a reduction in hepatic steatosis is able to normalize glyceria in type-2 diabetes [42]. Indeed, associated with the corrected glyceria, the triglyceride levels in plasma and liver were reduced to almost the normal levels of CH-mice during the period of OA administration. These results are similar to those recently reported in db/db mice within the treatment duration of an OA analogue, along with increased AMPK activity [18]. However, as the efficacy of OA on triglyceride levels in plasma and liver did not persist after cessation of OA treatment, the sustained reduction in glyceria is likely to involve alternative/additional mechanisms rather than the improvement in dyslipidemia and hepatic steatosis alone.

Glucose uptake into skeletal muscle and hepatic production of glucose are two major metabolic pathways responsible for glucose homeostasis. With the use of [3H]-2DG and D-[14C] glucose tracers, we demonstrated a reduction of glucose uptake into muscle and adipose tissue in T2D-Veh mice and there was no improvement of glucose uptake in these two tissues in T2D-OA mice. The lack of an effect of OA upon glucose uptake into muscle and liver indicated that the liver may be the major site for the sustained improvement of glyceria after the treatment with OA. To investigate the possible role of the liver, we first assessed glucose influx into this organ, but found no improvement in glucose incorporation into either glycogen or triglyceride (note that measurement of glucose uptake with 2DG in the liver is not valid). We next examined indicators of hepatic glucose production (efflux). Interestingly, we found that the insulin signaling transduction (indicated by the phosphorylation of Akt and FoxO1) regulating gluconeogenesis was significantly improved in OA treated mice. FoxO1 is a key transcription factor regulating hepatic gluconeogenesis. The phosphorylation of FoxO1 by Akt leads to its expulsion from the nucleus for degradation [28,43]. A recent study has indicated that the Akt-FoxO1 signaling plays a key role in controlling the expression of G6Pase [43]. Indeed, we found a trend of reduced expression of G6Pase (p = 0.09). Importantly the suppressed expression of G6Pase was significantly correlated with increased phosphorylation of Akt and FoxO1 (both p<0.05). As G6Pase is the gate-keeping enzyme for hepatic glucose production, our data indicate that its down-regulation induced by the Akt/FoxO1 signaling pathway is a likely mechanism for the sustained improvement of glyceria after the treatment with OA (Fig. 9E).

In summary, the present study demonstrated a sustained effect of OA to reverse hyperglyceria in T2D mice induced by HF-feeding and STZ. Our data indicate that OA, a triterpenoid abundantly present in natural products, may be a potential drug for the sustained control of hyperglyceria in type-2 diabetes and related kidney complications independent of lipid metabolism, insulin secretion and glucose disappearance into muscle and fat tissue. Furthermore, our data suggest that the sustained improvement of glucose homeostasis is due, at least in part, to a suppression of gluconeogenesis in the liver mediated by the Akt/FoxO1 axis. The findings in our study also provide a proof of concept for the potential of triterpenoids as a promising source to explore new drugs for the long-term control or cure of hyperglyceria and diabetic kidney complications.

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Author Contributions

Conceived and designed the experiments: JMY YY XYZ. Performed the experiments: XYZ JC YPW TI JMY. Analyzed the data: XYZ YPW JC JCM JMY. Contributed reagents/materials/analysis tools: JMY YY XYZ. Experiments: XYZ JC YPW TI JMY. Conceived and designed the experiments: JMY YY XYZ. Performed the experiments: JMY YY XYZ. Analyzed the data: JMY YY XYZ. Contributed reagents/materials/analysis tools: JMY YY XYZ. Wrote the paper: XYZ JMY JC.

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