MECHANISM-BASED DISCOVERY OF NEW ANTI-DIABETIC DRUGS FROM NATURAL PRODUCTS IN TRADITIONAL CHINESE MEDICINE

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(BSc)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

March 2014

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DECLARATION OF ORIGINALITY

I hereby declare that this thesis and the research represent my own studies while I was a student in the Molecular Pharmacology for Diabetes Group, Discipline of Complementary Medicine, School of Health Sciences at RMIT University, and also to the best of my knowledge it contains neither materials previously published or written by any other person, nor materials which to a substantial extent has been accepted for the award of any other degree, diploma or other qualifications at RMIT University or any other educational institution, except where due acknowledge is made in this thesis. Any contribution made to the research by others, with whom I have studied at RMIT University or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project’s design and conception or in style, presentation and linguistic expression is acknowledged.

(Signed).......................... .................

Xiu Zhou
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Xiu Zhou, March 2014
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</tr>
<tr>
<td>ACC</td>
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<tr>
<td>ADP</td>
<td>adenosine 5′-diphosphate sodium salt</td>
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<td>AF</td>
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<td>AGPAT</td>
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<tr>
<td>iAUC</td>
<td>incremental area under the curve</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
</tr>
<tr>
<td>ipGTT</td>
<td>intraperitoneal glucose tolerance tests</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>potassium phosphate monobasic</td>
</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LCACoA</td>
<td>long-chain fatty acyl CoA</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LKB1</td>
<td>liver kinase B1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacylglycerol</td>
</tr>
<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>MGAT</td>
<td>MAG acyltransferase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>sodium phosphate dibasic dihydrate</td>
</tr>
<tr>
<td>Na$_3$VO$_4$</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NDSS</td>
<td>National Diabetes Service Scheme</td>
</tr>
<tr>
<td>NEC</td>
<td>new chemical entities</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonide-P 40 Substitute</td>
</tr>
<tr>
<td>OA</td>
<td>oleanolic acid</td>
</tr>
<tr>
<td>OGT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>ORO</td>
<td>oil red O</td>
</tr>
<tr>
<td>PAP</td>
<td>phosphatidic-acid phosphohydrolase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PDK</td>
<td>phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4, 5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphoinositide-3, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKCs</td>
<td>protein kinase Cs</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PSG</td>
<td>penicillin streptomycin L-glutamate</td>
</tr>
<tr>
<td>PTT</td>
<td>pyruvate tolerance test</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RSG</td>
<td>rosiglitazone</td>
</tr>
<tr>
<td>SCD</td>
<td>stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol regulatory element-binding protein-1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrilamide gel electrophoresis</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylene diamine</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TOFA</td>
<td>5-(tetradecyloxy)-2-furancarboxylic acid</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</tbody>
</table>
PUBLICATIONS ORIGINATING FROM THIS THESIS

1. XY Zeng, X Zhou (co-first author), J Xu, SM Chan, CL Xue, JC Molero and JM Ye.

Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds, *Biochemical Pharmacology*. 84: 830-837, 2012


3. X Zhou, XY Zeng, MH Chan, H Wang, S Li, E Jo, CL Xue, M Tan, JC Molero and JM Ye. The sustained suppression of hepatic FoxO1 and G6Pase induced by oleanolic acid in diabetic mice is associated with acetylation modifications. Submitted to *Biochemical Pharmacology*, 2014


CONTRIBUTION TO OTHER PUBLICATIONS

1. Q Gu, S Wang, Q Ding, X Zhou, J Fang, JM Ye and J Xu. Anti-Diabetes Agents from Synthetic and Natural Products, Their Targets, and Agent-target Networks. Review, Submitted to Nature Medicine, 2014

2. XY Zeng, H Wang, F Bai, X Zhou, S Li, LP Ren, CL Xue, HL Jiang, LH Hu and JM Ye. Matrine is a promising novel drug for hepatic steatosis and glucose intolerance with HSP72 as a target: comparison with metformin. Submitted to Science Translational Medicine, 2014

3. H Wang, RQ Sun, XY Zeng, X Zhou, SM Chan, S Li, E Jo, JC Molero and JM Ye. Suppression of autophagy contributes to fructose diet-induced hepatic ER stress and insulin resistance. Submitted to Endocrinology, 2014
ORAL PRESENTATIONS AT SCIENTIFIC MEETINGS
(National and International)


SUMMARY

Insulin resistance is a fundamental metabolic defect of type 2 diabetes. Excessive lipid accumulation in the muscle and liver is the most common cause of insulin resistance. Hence, reducing lipid levels in these tissues can potentially reverse insulin resistance as an effective approach for the treatment of type 2 diabetes.

Although there are a number of anti-diabetic drugs in clinical use, the long-term effects of the current medications are still unsatisfactory. Some of them have various degrees of adverse effects which limit their usage. Natural products have been recently rejuvenated as an important source for the discovery of anti-diabetic drugs due to their rich source and chemical diversity. Traditional Chinese Medicine have been used for centuries to treat diseases including diabetes, hence there are already vast literatures available for data mining, virtual screening and computer modelling for drug discovery and development process.

The hypothesis in this thesis was that compounds derived from natural products in Traditional Chinese Medicine with lipid-lowering efficacy have therapeutic potential for the treatment of type 2 diabetes by reversing lipid-induced insulin resistance.

In order to identify the compounds with potential anti-diabetic properties by reducing lipid accumulation, a novel biochemical screening assay was developed using 3T3-L1 adipocytes and triglyceride content was assessed as readout of cellular lipids level. Potassium hydroxide (KOH) was used in this newly-developed assay to overcome the obstacles of conventional
chloroform/methanol (C/M) method in triglyceride extraction. This greatly simplified the procedures and enhanced the efficiency of the assay as a relatively high-throughput screening tool.

By using this assay, I have screened more than 200 candidates selected from Traditional Chinese Medicine after careful evaluation of the relevant indications and computer-based modelling. Out of these compounds, I have identified 76 hits from more than 10 different classes in terms of the reduction of triglyceride content by either >50% or >75% at tested concentrations. These results indicated that the approach I have used is highly effective in identifying the potential candidates with 38% successful rate.

Based on the screening results and other selection criteria (abundance, chemical diversity, quantity, novelty and Lipinski’s rule of five) established in our research group, I selected albiflorin and oleanolic acid to test their in vivo efficacy and investigated the mechanisms involved.

In the first animal study, the efficacy of albiflorin was tested in insulin resistant mice induced by high-fat cholesterol diet, a commonly used animal model which resembles lipid-induced insulin resistance in humans. Administration of albiflorin significantly reduced triglyceride and cholesterol levels in the liver of high-fat cholesterol fed mice. Interestingly, further examinations of the liver samples showed significant reduction in the mRNA expression of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) by the treatment of albiflorin. These results suggest that albiflorin may have potential therapeutics for hepatic
steatosis and associated liver metabolic conditions, such as non-alcoholic steatohepatitis (NASH). To further test the effects of albiflorin on insulin resistance, I examined its efficacy in a more established insulin resistant mice model induced by high-fat diet. Albiflorin-treated mice significantly improved glucose intolerance in insulin resistant mice. However, further studies are required with more sensitive and reliable technique, such as hyperinsulinaemic-euglycaemic clamp with glucose tracers, to confirm its therapeutic potential for insulin resistance.

In the second animal study, I investigated oleanolic acid (OA), a triterpenoid which was previous reported to have beneficial effects on diabetes via activating AMP-activated protein kinase (AMPK). In this study, I used a model of type 2 diabetes and focused on its effects on hyperglycaemia. The type 2 diabetes was generated by a combination of high-fat diet (to induce insulin resistance) and multiple low doses of streptozotocin injections (to inhibit insulin levels by 50%). OA-treated type 2 diabetic mice effectively reduced hyperlipidaemia and reversed hyperglycaemia with liver being the major target tissue of its action. Moreover, the anti-hyperglycaemia effect of OA to type 2 diabetic mice was sustained far beyond the treatment period. As such sustained response has not been described for other anti-diabetic treatments, my subsequent studies focused on the mechanisms for the persistent effective maintenance of the corrected hyperglycaemia. By comparing the changes of key regulators in the liver during OA treatment compared with post-OA administration, I found that the OA-induced changes in forkhead box protein O 1 (FoxO1), glucose-6-phosphatase (G6Pase),
histone acetyl-transferase 1 (HAT1) and histone deacetylases (HDACs) persisted during the post-OA treatment period where the increased phosphorylation of AMPK, sirtuin 1 (SIRT1) content and the reduced liver triglyceride had subsided. Further studies indicated that the anti-hyperglycaemic effects observed after cessation of OA treatment may result from persistent acetylation of FoxO1 to suppress the hepatic gluconeogenic pathway.

In summary, the results of my PhD project indicate that using targeted screening approach for the intracellular lipid-lowering efficacy is an effective phenotypic screening tool, which is capable of identifying potential new anti-obese and anti-diabetic compounds via different metabolisms. The present study highlights that mechanism-based discovery of compounds derived from our unique natural products library in Traditional Chinese Medicine have potential for the treatment of type 2 diabetes by reversing lipid-induced insulin resistance.
CHAPTER 1
INTRODUCTION AND
LITERATURE REVIEW
1.1 DIABETES MELLITUS

Diabetes mellitus, simply referred as diabetes, is a chronic metabolic disease characterised by elevated blood glucose levels (hyperglycaemia) with fasting blood glucose level $\geq 7.0$ mM [1].

Diabetes results from either the pancreas is not able to produce insulin (type 1 diabetes) or the insulin is not sufficient and the insulin target tissues cannot make response to insulin effectively and efficiently (type 2 diabetes) [1]. In this thesis, I investigated possible novel treatments for type 2 diabetes by targeting the improvement of the responsiveness of tissues to insulin action.

1.1.1 Prevalence of Diabetes in the Global and Australia

The epidemics of diabetes are rapidly increasing and diabetes has become a worldwide health threat in the past decades [2]. According to a statistical report, it is estimated that 382 million people worldwide, or 8.3% adults, have diabetes in 2013 (Figure 1), and if these trends continue, this number will rise to 592 million by 2035 [3].

In Australia, it is estimated that more than 100,000 people develop diabetes each year. A total of 1.7 million Australians have diabetes in 2013 including 1.1 million Australians diagnosed with diabetes. Diabetes has become the 6th leading cause of death in Australia and it is estimated that about 3.3 million Australians will have type 2 diabetes by 2031 [4].
Figure 1. Number of people with diabetes (20-79 years), 2013. This map presents the number of people with diabetes in the top ten countries worldwide. Data are based on the statistical report from International Diabetes Federation (IDF), Diabetes Atlas, 6th edition, 2013 [3].
1.1.2 Global Burden of Diabetes

Diabetes primarily afflicts people between 40 and 59 years of age. 80% of people with diabetes live in low- and middle- income countries [3]. Diabetes causes serious economic burden on individuals and families, national health systems, and countries. The cost of diabetes accounts for 10.8% (approximately 548 billion US dollars) of the total health expenditure worldwide in 2013 [3]. Compared with high-income countries, there is a huge burden on healthcare systems and governments in low- and middle-income countries due to the lack of access to health insurance and medical services [3].

1.1.3 Diabetes-associated Complications

The mortality rate of diabetes varies sharply with the prosperity of the country. Overall, low- and middle- income countries have more than double the mortality rate compared to high-income countries. In 2013, about 5.1 million people aged 20–79 years died from diabetes, accounting for 8.4% of all mortality globally among people in the same age range [3]. This number of deaths caused by diabetes showed a 27% increase in 2011 [5]. The increasing mortality rate of diabetes is not only caused by diabetes itself, but also largely due to its complications.

People with diabetes are at a high risk of developing a number of life-threatening health problems [3]. Untreated diabetes can lead to cardiovascular disease [6], retinopathy[7], nephropathy [8], neuropathy [9], food disorders [10], and pregnant complications [11]. Diabetes also can interfere with wound healing [12] (Figure 2).
Figure 2. The major diabetes-associated complications. This figure presents the major complications caused by diabetes in different organs, including brain, eyes, heart and kidney etc. Adapted from the International Diabetes Federation (IDF), Diabetes Atlas, 6th edition, 2013 [3].

1.1.4 Main Types of Diabetes

*Type 1 diabetes* is also referred as insulin-dependent diabetes mellitus or juvenile-onset diabetes. It accounts for 5%~10% of all cases of diabetes. Historically, type 1 diabetes was
largely diagnosed in children, teenagers and young adults. However, this view has changed over the past decade, type 1 diabetes can develop at any age [13]. Type 1 diabetes is commonly caused by an auto-immune response of the body. In this condition, body fails to recognise the β-cells in the pancreas and then destroys them with antibodies and white blood cells, resulting in pancreatic defects in insulin secretion [14-17]. Therefore, the pancreas of type 1 diabetic people could not produce insulin. People with type 1 diabetes must rely on insulin injections or a continuous infusion of insulin via an insulin pump. Recently, there has been a rapid revolution of the care and treatment for people with type 1 diabetes, such as genetically engineered insulin and glucose monitoring devices, in order to control the blood glucose levels and to prevent or delay the diabetes-related complications [18]. In addition, pancreas transplantation procedure has been applied to type 1 diabetic people. However, this approach still holds many limitations and needs further investigations [19].

**Type 2 diabetes** is formerly known as non-insulin dependent diabetes mellitus or adult-onset diabetes. It accounts for 85%~90% of all cases of diabetes. Type 2 diabetes is commonly diagnosed in the aged population (over the age of 45 years) [20] and now it is increasingly developing in younger people, even children under 14 years of age. Type 2 diabetes is caused by metabolic disorders of insulin resistance which leads to the progression to high blood glucose levels [1]. In most people diagnosed with type 2 diabetes, their insulin levels in the blood are normal or elevated initially and at this early stage people usually can manage their conditions to delay and in some cases even prevent the disease by adopting a healthy lifestyle,
including regular physical activities, healthy food intake and losing excess body weight. However, at the later stage of the process, many people with type 2 diabetes become insulin deficient and may require oral medications and/or eventually need insulin injections when other medications fail to control blood glucose levels adequately. Over 40% of people with type 2 diabetes require insulin as part of their diabetes management plan. Type 2 diabetes may be controlled by lifestyle and medications [21]. The current pharmacological treatments of type 2 diabetes are described in Section 1.1.5.

**Gestational diabetes** is another form of diabetes. It is characterised by high blood glucose levels during the late stage of pregnancy (usually between the 24th and 28th week) [22]. Gestational diabetes affects about 4% of pregnancies worldwide and is associated with complications to both mother and baby. Gestational diabetes usually resolves after the delivery of foetus but women with gestational diabetes and their children are at a higher risk of developing type 2 diabetes [23]. Approximately half of women with a history of gestational diabetes have developed type 2 diabetes within five to ten years after delivery [22, 24]. Because gestational diabetes can affect both mother and baby, it is essential to treat it as early as possible. Treatments for gestational diabetes include special dietary plans and regular physical activities to keep normal blood glucose levels, which are same to those pregnant women who do not have gestational diabetes [24]. Daily blood glucose testing and insulin injections may be also included [22].
1.1.5 Most Common Diabetes - Type 2 Diabetes

Type 2 diabetes is the most common type of diabetes and is a global public health crisis that threatens the economies of all countries worldwide. Type 2 diabetes accounts for 90% of all diabetic people which results from insulin resistance and subsequent pancreatic β-cells failure as described in Section 1.1.4. According to the National Diabetes Service Scheme (NDSS)’s report, in 2013, about 940,000 people have type 2 diabetes in Australia [25] (Figure 3).

Figure 3. The prevalence (%) of type 2 diabetes in Australia. The map indicates the percentage of people with type 2 diabetes in each state of Australia. Data are based on the report from National Diabetes Service Scheme (NDSS), the Australian Diabetes Map. Last updated 30th September 2013 [25].
Chapter One - Introduction

The current diagnostic criteria for type 2 diabetes is fasting plasma glucose $\geq 7.0$ mM (126 mg/dl) and/or plasma glucose $\geq 11.1$ mM (200 mg/dl) 2 hours after an oral glucose tolerance test (OGTT; 1.75 g glucose/kg of body weight, maximum 75 g glucose) [26]. World Health Organization (WHO) has reduced the threshold of glucose level from $\geq 8.0$ mM (1980) to $\geq 7.8$ (1985) and to $\geq 7.0$ mM (1999 and 2006), which is mainly due to the evolution of environmental risk factors [27], including modern lifestyles such as the abundance of caloric intake, reduced physical activities [28], increased smoking and heavy alcohol consumption [29, 30], and internal environmental biomarkers as inflammatory factors [31]. Moreover, adipocytokines and hepatocyte factors [32] may also play important roles in type 2 diabetes epidemics.

1.2 INSULIN ACTION

1.2.1 The Metabolic Role of Insulin

Insulin is the principal hormone controlling blood glucose levels and is synthesised by the $\beta$-cells in the pancreas. The level of insulin is a central metabolic control mechanism. The immediate effect of insulin is to promote storage of dietary calories and thus lower the circulating levels of glucose coming from carbohydrate throughout the whole body. For example, after ingestion of a meal, dietary carbohydrates increase plasma glucose level, which is sensed by pancreatic $\beta$-cells, promoting insulin secretion into the bloodstream. This suppresses the glucose production in the liver and stimulates the uptake of glucose by peripheral tissues (skeletal muscle and adipose tissue) [33] (Figure 4).
Figure 4. Insulin production and action. Adapted from the International Diabetes Federation (IDF), Diabetes Atlas, 6th edition, 2013 [3].

Therefore, liver, skeletal muscle and adipose tissue are the main target tissues of insulin. In the liver, insulin increases the activity and/or expression of enzymes that enhances glycogen, lipid and protein synthesis. At the same time, insulin suppresses the activity and/or expression of those enzymes that catalyse gluconeogenesis and glycogenolysis, which breaks down the glycogen stored in the liver and muscle into glucose [33, 34]. In the skeletal muscle, insulin stimulates the uptake of glucose, fatty acids and amino acids from the blood, permitting synthesis and storage of glycogen, carbohydrates, lipids and proteins [34]. In adipose tissue, insulin stimulates glucose uptake, promotes lipogenesis to store substrates and inhibits lipolysis [33] (Table 1). The majority of the glucose (80%) that is taken by peripheral tissues is utilised by muscle with a relatively smaller amount being disposed into fat and liver [35].
Table 1. Summary of the effects of insulin on fuel metabolisms in different tissues responsible for glucose homeostasis of the whole body.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Effects of insulin on glucose metabolism</th>
<th>Effects of insulin on lipid metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Stimulates glycogen synthesis</td>
<td>Stimulates fatty acid synthesis</td>
</tr>
<tr>
<td></td>
<td>Inhibits glycogenolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreases hepatic gluconeogenesis</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Stimulates glucose uptake</td>
<td>Increases triglycerides uptake and synthesis</td>
</tr>
<tr>
<td></td>
<td>Increases glycolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stimulates glycogen synthesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibits glycogenolysis</td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Stimulates glucose uptake</td>
<td>Inhibits lipolysis</td>
</tr>
<tr>
<td></td>
<td>Increase glycolysis</td>
<td>Stimulates fatty acid synthesis</td>
</tr>
<tr>
<td></td>
<td>Stimulates glycogen synthesis</td>
<td>Increases triglycerides uptake and synthesis</td>
</tr>
</tbody>
</table>

In different tissues, the effects of insulin on glucose metabolisms and lipid metabolisms are different, which are due to the different functions of each tissue in the whole body.
Liver - as a major metabolic organ

Liver is an essential organ that plays a pivotal role in metabolic homeostasis in the body which is involved in the regulation of glucose (e.g. gluconeogenesis), fatty acids (e.g. lipogenesis and fatty acid oxidation) and also amino acids metabolism. It is also the primary site of insulin degradation [36]. Liver plays an essential role on immune response due to the constantly exposure to gut-derived bacteria, microbial debris and bacterial endotoxin in the blood from intestines. Similar to spleen, liver is one of the first lines of defences between the host and the external environment [37].

Liver is a major metabolic organ to maintain plasma glucose levels particularly during fasting states by synthesising glucose from a variety of substrates (gluconeogenesis) or releasing glucose from glycogen (glycogenolysis). Insulin resistance in the liver is a major contributor of hyperglycaemia in type 2 diabetes due to a diminished ability of insulin to suppress gluconeogenesis and/or glycogenolysis [38]. Moreover, the whole-body insulin resistance is initiated by impaired hepatic insulin action [39]. Therefore, liver is generally considered as an important site of insulin resistance. The in vivo studies presented in this thesis will focus on metabolism in the liver.

Cellular action of insulin

At the cellular level, insulin action is characterised by diverse effects, including changes in vesicle trafficking [40], stimulation of protein kinases and phosphatases [41], and promotion of cellular growth of gene transcription. Moreover, insulin has a role in hepatocyte
proliferation and 3T3-L1 pre-adipocyte differentiation [42].

**The insulin signalling cascade**

Dietary carbohydrate increases plasma glucose levels and promotes insulin secretion from the pancreatic β-cells. The cellular effects of insulin start with its binding to the insulin receptor (IR) in the target cells, followed by activating a number of intracellular signalling cascades. The activation of the cascades will ultimately lead to important downstream biologic effects which are critical for intracellular functions.

The IR is a transmembrane tetra-meric protein. Insulin binding to IR at the cell surface promotes its activation, which in turn results in the phosphorylation of insulin receptor substrate (IRS). Phosphatidylinositol-3-kinase (PI3K) and p85 regulatory subunit then interact with the tyrosine phosphorylated IRS via their SH2 domain, thereby activating the lipid kinase of the catalytic subunit and catalysing the conversion of phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphoinositide-3, 4, 5-trisphosphate (PIP3) in the plasma membrane. The increased PIP3 is then recognised by the pleckstrin homology (PH) domains of Akt (also known as protein kinase B, PKB) and phosphoinositide-dependent kinases 1 and 2 (PDK), resulting in phosphorylation and activation of Akt at residues Thr 308 and Ser 473. Phosphorylated and activated Akt is a key protein inducing a number of insulin cellular actions. The glycogen synthase kinase 3 (GSK3) proteins, a ubiquitously expressed serine/threonine protein kinase, is one of the principal Akt substrates. GSK 3 has two isoforms α & β (GSK 3α/β). The Akt-induced phosphorylation of GSK 3α/β results in inactivation of
GSK 3α/β and leads to decreased phosphorylation and increased glycogen synthase (GS) activity. Akt can also down-regulate Bcl-2 antagonist of cell death (BAD) to reduce apoptosis. In addition, another identified target of Akt is mammalian target of rapamycin (mTOR) to induce protein synthesis (Figure 5).

In skeletal muscle, insulin binds to its receptor, subsequently phosphorylating and activating IRS1. Insulin increases glucose transportation via translocation of vesicles containing glucose transporter 4 (GLUT4) to the plasma membrane. Once the transporters are in the plasma membrane, glucose enters into the cytoplasm and is immediately converted to glucose-6-phosphate (G6P) by hexokinase (HK), hence promoting glycogen synthesis. In the adipose tissue, insulin suppresses lipolysis and promotes lipogenesis involving the PI3K/Akt pathway (Figure 5).

In the liver, insulin promotes both glycogen synthesis and de novo lipogenesis whereas it inhibits gluconeogenesis through Akt/FoxO1 pathway, resulting in the reduction of glucose production [43]. Briefly, insulin activates the IR, which phosphorylates IRS1 and IRS2, leading to activation of PI3K and ultimately Akt [44]. There are two types of IRS namely IRS 1 and IRS 2 which have complementary roles in the control of hepatic metabolism. IRS 1 is more closely linked to glucose homeostasis and IRS 2 is more closely linked to lipid metabolism [45](Figure 5). The studies presented in this thesis focus on the insulin resistance in relation to glucose metabolism.
Figure 5. Simplified insulin signalling cascade through IRS/PI3K/Akt in the muscle/adipose tissue and liver. It is a synopsis of the key steps that result from insulin action, leading to the regulation of glucose levels in the muscle/adipose tissue (left panel) and in the liver (right panel). The description and abbreviations of the diagram are in the text.
1.2.2 Insulin Resistance

Insulin resistance is defined as reduced responsiveness to normal levels of insulin by target tissues (mainly liver and skeletal muscle) to insulin action [46]. Insulin resistance plays an important role in the pathogenesis of type 2 diabetes, and also it has been recognised as a key link between obesity (particularly visceral adiposity) and type 2 diabetes [47].

Insulin resistance does not appear suddenly, but it is the result of a progressive deterioration of the insulin signalling cascades (Figure 5). At the pre-diabetic stage, which is insulin resistance alone stage, blood glucose level is maintained relatively normal via a compensatory increase in insulin secretion to counteract insulin resistance [48]. In type 2 diabetes, ectopic lipid accumulation impairs insulin signalling. With the accumulation of intracellular lipids, insulin-mediated glucose uptake in the skeletal muscle is impaired resulting in the diversion of glucose to the liver. Increased lipid in the liver also impairs the ability of insulin to regulate gluconeogenesis and glycogen synthesis [33]. In contrast, the unaffected lipogenesis together with the increased delivery of dietary glucose leads to increased lipogenesis and non-alcoholic fatty liver disease (NAFLD) [49]. Impaired insulin action in the adipose tissue causes increased lipolysis promoting re-esterification of lipids in other tissues (such as liver) and further exacerbating insulin resistance. This prolonged insulin resistance may eventually results in pancreatic β-cell failure and the circulating insulin levels become insufficient to control blood glucose level, leading to overt hyperglycaemia [33]. Therefore, improvements of insulin action and insulin secretion are important for the treatment of type 2 diabetes.
Multiple clinical phenotypes including abdominal obesity and polycystic ovary syndrome (POCS) are also associated with insulin resistance. For example, abdominal obesity is associated with hyperlipidaemia and induction of pro-inflammatory cytokines resulting in insulin resistance [50]. Polycystic ovary syndrome is associated with excess androgens, hyperlipidaemia and hyperinsulinaemia but these insults may cause insulin resistance via a different mechanism to that described above [51].

1.3 OBESITY AND LIPID ACCUMULATION

Type 2 diabetes is a complex disease with multiple aetiologies and is usually associated with obesity [52]. About 80% of type 2 diabetic people are reported to be obese. Insulin resistance has been identified as a key factor that could drive the link between obesity and type 2 diabetes [47]. In many cases, type 2 diabetes starts with visceral obesity, leading to insulin resistance and hyperinsulinaemia.

1.3.1 Obesity

Obesity, a metabolic disorder, is a condition that is characterised by abnormal or excessive accumulation and storage of lipids in the body. Obesity has become worldwide epidemics and presents a high risk to health [53]. From 2008, more than 1.4 billion adults (at the age of 20 and older) were overweight. Among them, over 500 million were considered as obese. Obesity is the 5th leading risk of death worldwide and at least 2.8 million adults die each year as a result of being overweight or obese [53].

Obesity develops from the imbalance of energy storage (food intake) and energy expenditure
[54]. For example, increased intake of high energy food such as fructose-rich and fat-rich diets can cause increased calories intake. The excess calories lead to an exaggerated body weight gain which is characterised by an increased fat mass and lipid accumulation, resulting in obesity [55].

There are two main types of obesity, central obesity and peripheral obesity. The level of obesity in adults is determined by the body mass index (BMI, kg/m²). BMI is calculated by using a person’s body weight (in kilograms) divided by the square of one’s height (in metres). A person with a BMI between 25~30 is considered as overweight and with a BMI ≥ 30 is generally classified as obese [53].

Obesity has also been reported as a major health risk factor for a cluster of metabolic diseases, including type 2 diabetes [56], cardiovascular diseases (mainly heart disease and stroke) [57], non-alcoholic fatty liver disease (NAFLD) [58], musculoskeletal disorders (especially osteoarthritis) [59], and some cancers (endometrial, breast and colon) [60] (Figure 6).

**Figure 6. Obesity-related diseases.** Obesity is considered to be a central feature that increases the risk of developing a cluster of metabolic diseases and having degenerative disorders. Modified from Hotamisligli *et al.*, 2006 [56].
1.3.2 Lipid Accumulation

1.3.2.1 Lipid metabolisms

Excessive accumulation of lipids seen in obesity has many negative effects on insulin resistance in type 2 diabetes [33, 61] as discussed in Section 1.2.2. The major aspects of lipid metabolism are involved with lipids mobilisation and oxidation, and lipids synthesis and storage [62].

Fatty acid mobilisation

Lipids play an important role in the cells structure and metabolism. Triglycerides are the major storage form of lipids [62, 63]. At the post-prandial (containing fat) stage, triglycerides are delivered to the liver and lipolysed in lysosomes with the release of fatty acids. During the fasting stage, insulin levels decrease, triglycerides in the adipose tissue can be then hydrolysed to release fatty acids which will be transported to liver. Fatty acids can be also produced from *de novo* lipogenesis in response to a high carbohydrate diet.

Fatty acid oxidation

Fatty acids have to be activated prior to their entry into mitochondria where the enzymes of β-oxidation of fatty acids are located. Activated fatty acids are converted to long-chain fatty acyl CoA (LCACoA). LCACoAs are then actively transported from the cytoplasm into the mitochondria with the help of carnitine palmitoyl transferase 1 (CPT-1). In the mitochondria, LCACoAs are then oxidized to acetyl-CoA through the β-oxidation pathway of fatty acids to generate high energy content molecules (e.g. adenosine triphosphate, ATP) that will be used...
by cells (Figure 7). Four reactions, which occur in repeating cycles, are involved in the β-oxidation of fatty acids. In each cycle, a fatty acid is progressively shortened by two carbons as it is oxidised. Each cycle generates reducing power and captured by electron carries in the form of NADH and FADH₂. At the end of each cycle, one acetyl-CoA with two-carbon unit is released from the end of the fatty acid. The shortened fatty acid then goes through another round of β-oxidation, to continue with oxidisation and shortening even-chain fatty acids until it is entirely converted to acetyl-CoA. Fatty acids with an odd number of carbons in the acyl chain are left at the end with propionyl-CoA (3-carbon unit), that cannot enter another round of β-oxidation. In this case, the produced propionyl-CoA is converted to succinyl-CoA which then enters into the tricarboxylic acid (TCA) cycle.

The acetyl-CoA generated in β-oxidation enters the TCA cycle, in which it will be further oxidized to CO₂ to produce more reduced energy carriers, namely NADH and FADH₂. All the carriers produced in the TCA cycle as well as in the β-oxidation, transfer their electrons to the electron transport chain. In the electron transport chain, they can drive the creation of the proton gradient to support mitochondrial ATP production, which is the cells energy currency molecular. In the liver, another possible destination of acetyl-CoA is the production of ketone bodies that are transported to tissues such as the heart and brain that are able to oxidize them to provide energy.

One of the most attractive mechanisms to promote mitochondrial fatty acid β-oxidation is the activation of AMP-activated protein kinase (AMPK). Activated AMPK phosphorylation
inhibits the activity of acetyl-CoA to reduce malonyl-CoA. This removes the inhibition of malonyl-CoA to the fatty acids transporter CPT1, allowing fatty acids entry into mitochondria for β-oxidation. For this reason, AMPK has been considered as an attractive target for drug discovery to develop new therapeutics for type 2 diabetes and obesity [64].

**Lipid synthesis**

The sequence of reactions involved in the formation of fatty acids is known as lipogenesis. In contrast to the oxidation which occurs in the mitochondria, the synthesis occurs in the cytoplasm building up by the addition of two carbons units.

*De novo* lipogenesis starts with acetyl-CoA generated by metabolisms of non-fat precursors (e.g. glucose and glycerol). Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS), a multi-enzyme complex, then catalysed the synthesis of saturated fatty acids (14:0, a minor end product) and palmitate (16:0, the main end product) from acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids. The whole process is in the presence of NADPH and the productions are substrates for stearoyl-CoA desaturase (SCD). SCD then converts these saturated fatty acids to monounsaturated fatty acids. Triglycerides are synthesised from fatty acyl CoA and glycerol-3-phosphate or dihydroxy acetone phosphate (Figure 7). In the liver, triglycerides may either be stored in cytoplasmic lipid droplets or incorporated into very low-density lipoprotein (VLDL) particles and secreted into the blood.

Increased *de novo* lipogenesis will lead to significantly increase in body weight, especially
when glucose constitutes as an important part of the carbohydrate. Because the elevated de novo lipogenesis can result in lipid accumulation, one of the mechanisms to reduce triglyceride levels is the inhibition of fatty acids synthesis, particularly in the liver and adipose tissue.

1.3.2.2 Mechanisms of lipid-induced insulin resistance

Abnormal lipid accumulation in insulin target tissues (mainly liver, muscle and adipose tissues) is believed to be the common aetiology of insulin resistance of central obesity and dyslipidaemia [33, 58]. Hepatic lipid accumulation can be caused by four different metabolic perturbations: 1) increased fatty acid mobilisation to hepatocytes from lipolysed adipose triglycerides, dietary lipids, or hepatic de novo lipogenesis; 2) decreased hepatic fatty acid oxidation; 3) increased triglycerides synthesis; and 4) inadequate triglycerides secretion in VLDL [62]. The main forms of lipids in these pathways that associated with the development of insulin resistance are fatty acids, long-chain fatty acyl CoAs, diacylglycerols, ceramides and triglycerides. It is now clear that the storage of these major lipid intermediates can interrupt insulin action by multiple mechanisms. Such mechanisms are explained below:

- **Fatty acids (FAs):** the increased intracellular form of FAs from carbohydrate (namely de novo lipogenesis) is a major metabolic pathway leading to lipid accumulation in the liver [65, 66]. The excess of FAs in the tissues could be also due to the reduced fatty acid oxidation leading to a reduction of acetyl-CoA in mitochondria. Acetyl-CoA could suppress the activity of pyruvate dehydrogenase kinase (PDH) to control the rate of
glucose oxidation, and therefore it increases the glucose entry into cells [67].

- **Long-chain fatty acyl CoAs (LCACoAs):** Acyl-CoA carboxylase (ACS) catalyses the conversion of fatty acids to LCACoAs by the addition of the coenzyme A (CoA) to its C-terminal chain. Once fatty acids are activated, LCACoAs can be added to a glycerol molecule to form mono-, di- or tri-glycerides for the long-term storage. The intracellular forms of fatty acids and LCACoAs can inhibit hexokinase and glycogen synthase [65].

- **Diacylglycerol (DAG):** as a second messenger in lipid signalling. DAG is a product from the hydrolysis of phospholipids and triglycerides or from *de novo* biosynthesis, using fatty acids and malony-CoA as precursors. The accumulation of DAGs can interfere with insulin signalling pathways by promoting protein kinase Cs (PKCs)-dependent serine/threonine phosphorylation of IRS-1, which is associated with the development of insulin resistance [68].

- **Ceramides:** is a precursor of sphingosine. Ceramides consist of sphingosine and a fatty acid, and they are found in high concentrations within cell membrane. It has been suggested that ceramides may induce insulin resistance by promoting dephosphorylation of Akt and thus inhibiting its activity [69].

- **Phospholipids:** are a class of lipids involving in many intracellular processes and are important intermediates in lipid biosynthesis [70]. They are also able to bind specifically to different types of proteins [71]. Phospholipids synthetic pathways are regulated at
multiple levels and are sometimes controlled by lipid metabolites or nutrients, such as DAGs and glucose [72]. It has shown that the ratio of unsaturated to saturated fatty acyl chains of phospholipids in the membrane influences the effectiveness of glucose transport by insulin-independent glucose transporters.

- **Triglycerides:** commonly used as an indicator of the accumulation of lipids due to its functions as the major storage of these lipid intermediates [62, 63]. Although they are not directly cause insulin resistance, they are a major source for the production of DAGs as mentioned above. More detailed discussion will be mentioned in Section 1.3.2.3. Triacylglycerol is the alternative diacylglycerol product, catalysed by diacylglycerol acyltransferase (DGAT) [73].

Likewise, these lipid metabolites can also trigger other mechanisms including the production of pro-inflammatory cytokines that exacerbate insulin resistance at the later stage by independent mechanisms [74].
Figure 7. Simplified mechanisms of lipid-induced insulin resistance. Schematic diagram of the proposed mechanisms by which lipid species cause insulin resistance. Mechanisms are explained in the main text. PDH: pyruvate dehydrogenase kinase; LCACoA: long-chain fatty acyl CoAs; CPT-1: Carnitine-palmitoyl transferase 1; ACC: acetyl-CoA; FFA: free fatty acids; PKC: protein kinase C; TCA: tricarboxylic acid cycle, ACS: acyl-CoA carboxylase.
1.3.2.3 Triglyceride

Triglyceride is a major type of neutral lipid. Glycerol-3-phosphate generated from glucose metabolism is acylated by glycerol phosphate acyltransferase (GPAT) and acylglycerolphosphate acyltransferase (AGPAT) and converted to diacylglycerol by phosphatidic-acid phosphohydrolase (PAP). The glycerol-3-phosphate pathway requires NADH and the involvement of glycerol-3-phosphate in the fatty acid esterification is carried out in the mitochondria and endoplasmic reticulum. The alternative pathway involves the acylation of monoacylglycerol (MAG) by MAG acyltransferase (MGAT), which occurs in the endoplasmic reticulum and the peroxisomes. These two pathways together lead to the formation of triglycerides catalysed by diacylglycerol acyltransferases 1 & 2 (DGAT 1/2), which are considered as two major enzymes in this process [75, 76] (Figure 8, black arrow). The most important pathway to triacylglyceride synthesis is from glycerol-3-phosphate which produces more than 90% of liver triglycerides [77].

When fatty acids are required, they are released from the triglycerides mainly by the actions of three enzymes, adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL). ATGL catalyses triglycerides to diacylglycerol, initiating the hydrolysis process of triglycerides. The ATGL is proposed to be the rate-limiting enzyme in monoacylglycerol hydrolysis [78]. Diacylglycerol is then converted to monoacylglycerol by HSL, which is subsequently hydrolysed to glycerol and fatty acids by MAGL [78] (Figure 8, blue arrow).
Figure 8. The pathway of triglyceride synthesis. GPAT, glycerol-phosphate acyltransferase; AGPAT, acylglycerolphosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase. MAGT, monoacylglycerol transferase; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MAGL, monoacylglycerol lipase. This is based on the description in Shi et al., 2004 [75].

Triglycerides by themselves do not directly inhibit to insulin action and their levels are a good indicator of intracellular accumulation of lipids [63]. The cellular levels of triglycerides are determined by its formation of lipid synthesis, influx and export, and lipid utilisation (mainly by fatty acid β-oxidation in mitochondria) as discussed in Section 1.3.2.1. Triglycerides are concentrated primarily in adipocytes as energy storage. The excessive accumulation of triglycerides in adipose tissue as occurring in obesity is a major source of fatty acids to
non-adipose tissues at the post-prandial state. For example, excessive triglyceride deposition in skeletal muscle and the liver is associated with insulin resistance [34, 79, 80]. Although triglyceride accumulation does not directly cause insulin resistance, it releases various lipid metabolites (e.g. DAGs, LCACoAs) which can impair insulin action [81].

Several studies associate increased triglycerides levels with insulin resistance. In animals, accumulation of triglycerides in muscle has been recognized as one of the indicators of whole-body insulin resistance [62]. In humans, intramuscular triglycerides content has been shown to be inversely correlated with insulin sensitivity, independently of adiposity per se [82]. In addition, the hallmark of hepatic steatosis is the presence of triglycerides stored as lipid droplets in the cytoplasm of hepatocytes [79].

1.3.3 Amelioration of Lipid-induced Insulin Resistance

As mentioned in the Section 1.2.2, insulin resistance is a leading factor for type 2 diabetes and the epidemic prevalence of this metabolic disease defect often results from an excessive lipid accumulation (hyperlipidaemia) in the liver and skeletal muscle. Hence, lipids play an important role in the development of insulin resistance. Therapeutic strategies for insulin resistance and its complications aim to reduce (intracellular) lipids availability in insulin sensitive tissues, such as liver and skeletal muscle.

Increased physical activities in overweight and obese people have been shown improving insulin sensitivity, reducing abdominal lipid and triglyceride levels, increasing plasma high density lipoprotein cholesterol (HDL-C) concentrations, as well as improving oxidative
capacity in muscle [83].

Dietary restriction is another treatment for insulin resistance which has been demonstrated in humans. It has been shown that reduced caloric intake associated weight loss as well as increased intake of unsaturated lipids can improve insulin sensitivity [84, 85].

Several pharmacological interventions (e.g. thiazolidinediones, TZDs) are also used in the treatment of lipid-induced insulin resistance [86]. Rosiglitazone and pioglitazone from TZDs exert insulin sensitizing and anti-hyperglycaemic effects and many of the beneficial effects of TZDs have been explained on the basis of lipid re-distribution from ectopic and visceral fat in subcutaneous fat [87] (Section 1.4.3).

Bariatric surgery has been increasingly used over the past decade for the treatment of type 2 diabetes by reducing body weight and food intake [88]. It involves in the treatment of people with severe obesity and requires a variety of procedures performed. Bariatric surgery is achieved on people by reducing the size of the stomach with a gastric band, through removal of a portion of the stomach, or by resecting and re-routing the small intestines to a small stomach pouch [89]. However, the long-term effects of this surgery associated weight loss and the actions of surgery on metabolic control are not clear [90].

In summary, reduction of lipid accumulation is a plausible therapeutic approach to overcome insulin resistance for the treatment of type 2 diabetes. This thesis will focus on the pharmacology intervention for the treatment of type 2 diabetes.
1.4 PHARMACOLOGICAL TREATMENT OF TYPE 2 DIABETES

Diabetes without proper treatments can cause many severe complications as described in Section 1.1.2 as well as the global population with this disease continually to reach new heights in the next decades as discussed in Section 1.1.1. Therefore, prevention and effective treatment of diabetes are important and urgently needed.

Although lifestyle changes (physical activities and dietary restriction) can effectively control hyperglycaemia and delay the need for medications at the early stage, type 2 diabetes requires medications and/or insulin to stabilise blood glucose levels at various stages for most diabetic people [21].

Medications which are used to treat type 2 diabetes aim to reduce blood hyperglycaemia by (1) stimulating secretion of insulin in pancreatic β-cells; (2) slowing the digestion or absorption of carbohydrates in the intestine; (3) inhibiting glucose production in the liver and/or (4) improving sensitivity and responsiveness of insulin target tissues.

Currently, there are 6 classes of oral medications used in Australia to lower blood glucose levels, namely biguanides, sulphonylureas, thiazolidinediones, meglitinides, α-glucosidase inhibitors and dipeptidyl peptidase-4 inhibitors. Additionally, injections with incretin mimetics (insulin releasing agent) and insulin itself are also used for the treatment of type 2 diabetes (Table 2).
1.4.1 Biguanides (Metformin)

Metformin is a biguanide extracted from *French lilac* [91], which has been used to treat diabetes since the middle ages [92]. Metformin has been commonly used in clinic as a glucose lowering drug, although the precise knowledge of the molecular mechanism of its anti-diabetic action is still unclear. A growing body of evidence suggests that metformin decreases blood glucose and hepatic glucose release to the bloodstream mainly by inhibiting gluconeogenesis [93].

Several mechanisms have been proposed to explain this inhibitory action of metformin on hepatic gluconeogenesis including changes in enzyme activities [94], or a reduction in hepatic uptake of gluconeogenic substrates [95]. It has been reported that action of metformin was associated with the activation of AMPK in hepatocytes *in vitro*, increase of fatty acid oxidation, reduction of the expression of the lipogenic transcription factor SREBP-1 (sterol regulatory element-binding protein-1) and decrease of glucose production [96]. Metformin activates AMPK and stimulates glucose uptake in skeletal muscle *ex vivo* [96]. It has been proposed that upstream activator of AMPK, liver kinase B 1 (LKB1) is the possible target of metformin action [97]. However, some people have opposite views on this. Studies from other group suggest that metformin does not directly activate either AMPK or its upstream activator LKB1, because the drug did not influence the phosphorylation of AMPK by LKB1 [98]. Another common proposed mechanism for metformin action is the inhibition of enzymatic activity of Complex I in mitochondrial respiration chain, hence inhibiting hepatic
gluconeogenesis while increasing glucose utilisation in peripheral tissues [99, 100].

However, the limited effects of metformin on improving insulin action in muscle decrease its efficacy as a long-term therapeutic. Metformin treatment has been reported to be associated with possible undesirable effects including nausea, diarrhoea and a metallic taste in the mouth [101].

1.4.2 Sulphonylureas

Sulphonylureas are another common treatment used in clinic. They increase insulin production by shutting the potassium channels in the pancreatic β-cells and helping lower blood glucose levels. Unfortunately, this treatment is only effective when there is sufficient β-cells mass to provide with enough insulin. This specific treatment is inefficient after several years due to the dramatic reduction of β-cells mass. Moreover, several side effects are associated with sulphonylureas including hypoglycaemia, body weight gain, skin rashes, stomach upsets and jaundice [102].

1.4.3 Thiazolidinediones (Glitazones)

The thiazolidinediones (TZDs), also known as glitazones, were introduced in the late 1990s in the treatment of type 2 diabetes, including rosiglitazone, pioglitazone and troglitazone [103]. The beneficial effects of TZDs for the treatment of type 2 diabetes are due to their properties as PPARγ (Peroxisome Proliferator Activated Receptor γ) ligands [104] and stimulating AMPK activity [105] promoting an improvement in glucose tolerance [103] and whole body insulin sensitivity.
Although TZDs can effectively improve insulin action by normalizing blood glucose level, undesirable side effects are associated with these drugs such as body weight gain, increased risk of cardiovascular disease and bladder cancer \[106\]. The first TZD rosiglitazone approved by the Food and Drug Administration (FDA) in the USA was withdrawn in some countries due to the concerns about liver failure and deaths \[107\].

### 1.4.4 Meglitinides

In the meglitinides (glinides) class, Novo Nordisk's repaglinide (Novonorm Prandin) is the main branded drug, which was approved by FDA in 1997 \[108\]. Other drugs in this class include nateglinide (Starlix) and mitiglinide (Glufast). Similar to sulfonylureas, the meglitinides increase insulin secretion to lower blood glucose levels. Meglitinides bind to an adenosine triphosphate (ATP)-dependent potassium channel on the cell membrane of pancreatic β-cells \[109\]. However, they have a relatively weaker binding affinity and faster dissociation from the SUR1 binding site, which could increase the concentration of intracellular potassium and then cause the electric potential over the membrane to become more positive. This membrane depolarization results in opening voltage-gated calcium channels and increase of intracellular calcium levels, which leads to increased fusion of insulin containing vesicles with the cell membrane.

Side effects of meglitinides include body weight gain, hypoglycaemia, gastrointestinal disorders and abnormalities of liver functions. Although the risk of hypoglycaemia is lower than that by sulfonylureas, these are still serious potential side effects that could limit the
usage of meglitinides. One study showed that repaglinide caused an increased incidence in male rats of benign tumours of the thyroid and liver [110].

1.4.5 Alpha-glucosidase Inhibitors

Alpha-glucosidase inhibitors prevent the digestion and absorption of complex carbohydrates and the metabolism of poly-saccharides to mono-saccharides, which results in decreased glucose uptake through the intestine [111]. Unlike the meglitinides or sulfonylureas, these class medicines do not stimulate the pancreas to produce more insulin preventing high levels of blood glucose after meal. Moreover, they will not cause hypoglycaemia unless they are used with other medicines for diabetes or with insulin. The effect of α-glucosidase inhibitors on reducing glucose levels is less than that achieved with metformin or sulfonylurea.

Since α-glucosidase inhibitors prevent the degradation of carbohydrates into glucose, carbohydrates are retained in the intestine, which could cause gastrointestinal inconveniences. Body weight loss, flatulence (wind), bloating and diarrhoea are the common side effects of α-glucosidase inhibitors [112].

1.4.6 Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 (DPP-4) inhibitors (sitagliptin and vildagliptin) are a class of oral hypoglycaemics that inhibit the enzyme DPP-4, therefore enhancing the levels of active incretin hormones. DPP-4 is responsible for the degradation of incretins. These could lower blood glucose levels by increasing insulin secretion and decreasing glucagon secretion for the treatment of type 2 diabetes[113]. DPP-4 inhibitors can only exert their effects when the level
of blood glucose is elevated, hence it will not cause hypoglycaemia [114]. However, vildagliptin may result in swelling of the hands or feet, heartburn, weight gain, or itchy rash [115].

### 1.4.7 Incretin Mimetics

Incretin mimetics mimic the effects of the incretin hormones that originally exist in the body to control blood glucose levels. For example, exenatide belongs to incretin mimetics, which lower blood glucose levels by 1) stimulating the pancreas to release insulin; 2) reducing the amount of glucagon released from the pancreas after a meal; 3) slowing down the process of food from the stomach to the gut, thereby slowing down the absorption of food and 4) increasing the satiety feeling after eating and reducing caloric intake [116]. Side effects of exenatide could include nausea, vomiting and diarrhoea.

### 1.4.8 Combinations

When one medication alone is not sufficient, people with type 2 diabetes may need a second or even a third type of medication to maintain blood glucose levels. For example, metformin plus a sulphonylurea is a common combination [117]. Rosiglitazone plus metformin is another alternative combined medication [118].

Even though the numbers and forms of treatments for type 2 diabetes has been increased in the last decade (Table 2), they are not fully effective in reducing the levels of blood glucose to normal levels [101]. There is still a need of a great medical and novel drug development with improved efficacy and fewer side effects for the treatment of type 2 diabetes.
### Table 2. Anti-diabetic medications prescribed in Australia.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Generic Name</th>
<th>Brand Name</th>
<th>Mode of Action</th>
<th>Major Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanides</td>
<td>Metformin</td>
<td>Diabex, Diabex Xr, Diaformin, Diabex Xr</td>
<td>Suppress glucose production</td>
<td>Gastrointestinal disorders</td>
</tr>
<tr>
<td>Sulphonylureas</td>
<td>Gliclazide</td>
<td>Diamicron, Diamicron MR, Genrx Gliclazide, Gliclazide, Glyade, Mellihexal, Nidem, Oziclide MR</td>
<td>Stimulate insulin secretion</td>
<td>weight gain; Hypoglycemia</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>Daonil, Glimel</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glipizide</td>
<td>Melizide, Minidiab</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glimepiride</td>
<td>Amaryl, Aylide, Diapride, Dimirel, Glimepirdie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Rosiglitazone</td>
<td>Avandia</td>
<td>Improve insulin sensitivity</td>
<td>Weight gain; Expensive; Fluid retention</td>
</tr>
<tr>
<td></td>
<td>Pioglitazone</td>
<td>Actos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Generic Name</td>
<td>Brand Name</td>
<td>Mode of Action</td>
<td>Major Side Effects</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------</td>
<td>------------</td>
<td>-------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Repaglinide</td>
<td>Novonorm</td>
<td>Stimulate insulin secretion</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td>α-glucosidase inhibitors</td>
<td>Acarbose</td>
<td>Glucobay</td>
<td>Slow intestinal glucose absorption</td>
<td>Multiple daily dosing required; Gastrointestinal disorders</td>
</tr>
<tr>
<td>DPP-4 inhibitors</td>
<td>Sitagliptin</td>
<td>Januvia</td>
<td>Enhance insulin secretion</td>
<td>Experimental limited; Expensive</td>
</tr>
<tr>
<td></td>
<td>Vildagliptin</td>
<td>Galvus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incretin mimetics</td>
<td>Exenatide</td>
<td>Byetta</td>
<td>Stimulate insulin secretion</td>
<td>Gastrointestinal disorders</td>
</tr>
<tr>
<td>Combinations</td>
<td>Metformin/Glinclamide</td>
<td>Glucovance</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rosiglitazone/Metformin</td>
<td>Avandamet</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This is a summary of anti-diabetic agents currently used in Australia. Based on the Managing for Type 2 Diabetes, National Diabetes Services Scheme (NDSS), last updated on 12th September 2012 [119].
1.5 DRUG DEVELOPMENT FOR TYPE 2 DIABETES

1.5.1 Small Molecules and Biologics

Small molecules and biologics are the main types of drugs in the current drug development, which are based on their chemical and biological functions.

**Small molecules**

The small molecule drugs (also called new chemical entities, NCE) are chemically manufactured molecules and classic active substances, which account for more than 90% of the drugs in the current drug development. Small molecules have a low molecular weight (< 900 daltons) [120]. To achieve better oral bioavailability, a lower molecular weight (< 500 daltons) has been recommended to select small molecule drug candidates [121]. These small molecules can be processed into tablets or capsules which are easily ingested. The function of small molecule drugs may across a variety of cell types and species (e.g. mice and humans) and predominantly work on G protein-coupled receptors, ion channels, nuclear receptors and enzymes [122]. This advantage of small molecules could lead to the development of new therapeutic agents. The most common anti-diabetic drug metformin (Section 1.4.1) is an example of a small molecule drug.

**Biologics**

Biologics (also known as large molecules) are a class of drugs based on proteins that have a therapeutic effect. Biologics have a large molecular weight (> 150 kilo daltons), and composed of more than 1,300 amino acids [123].
Biologics are becoming increasingly important in clinic because they can bind to specific cell receptors that are associated with the process of disease [124]. For example, monoclonal antibodies used in cancer therapy bind selectively specific to abnormal cells without attacking the healthy cells in this process, so biologics often cause less side effects than classic chemotherapy [125].

Both small molecules and biologics play important role in the current drug development. Compared to biologics, small molecules have little ability to initiate an immune response and remain relatively stable over time [126] (Table 3). The present thesis will emphasise on the drug development from small molecules.

1.5.2 Criteria for the Selection of Compounds

To select candidates, we employed the following criteria: 1) Availability - abundance in natural resources; 2) Structure diversity - in favour of modification; 3) Quantity - sufficient for in vivo studies; 4) Novelty - preliminary study and 5) Drug likeness - the Lipinski’s rule of five is explained as below [127].

- Molecular weight is less than 500 daltons;
- \logP (an octanol-water partition coefficient) is less than 5;
- Hydrogen bond donors are less than 5;
- Hydrogen bond acceptors are less than 5.
Table 3. Characteristics of small molecules vs. biologics.

<table>
<thead>
<tr>
<th></th>
<th>Small molecules</th>
<th>Biologics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>- Small (single molecule)</td>
<td>- Large (mixture molecules)</td>
</tr>
<tr>
<td></td>
<td>- Low molecular weight</td>
<td>- High molecular weight</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td>Simple, well-defined structure, independent of manufacturing process</td>
<td>Complex (heterogeneous), defined by the exact manufacturing process</td>
</tr>
<tr>
<td><strong>Modification</strong></td>
<td>Well defined</td>
<td>Many options</td>
</tr>
<tr>
<td><strong>Manufacturing</strong></td>
<td>- Usually chemical synthetic, organic compounds</td>
<td>- Usually protein or carbohydrate based</td>
</tr>
<tr>
<td></td>
<td>- Predictable chemical process</td>
<td>- Produced in living cell culture</td>
</tr>
<tr>
<td></td>
<td>- Identical copy can be made</td>
<td>- Difficult to control from starting material to final active pharmaceutical ingredient</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Impossible to ensure identical copy</td>
</tr>
<tr>
<td><strong>Characterisation</strong></td>
<td>Easy to characterise completely</td>
<td>Cannot be characterised completely the molecular composition and heterogenicity</td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td>Relatively stable</td>
<td>Unstable, sensitive to external conditions</td>
</tr>
<tr>
<td><strong>Immunogenicity</strong></td>
<td>Mostly non-immunogenic</td>
<td>Immunogenic</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>Cheap</td>
<td>Expensive</td>
</tr>
<tr>
<td><strong>Administration</strong></td>
<td>Typically given orally</td>
<td>Typically given parenterally</td>
</tr>
<tr>
<td><strong>Specific</strong></td>
<td>Potential for off target activity</td>
<td>High selectivity &amp; specificity</td>
</tr>
</tbody>
</table>

The table is summarized the major characteristics of small molecule drugs and biologics as described in the text.
1.5.3 Discovery and Development of New Drugs

There are no consistent rules for early stages of drug discovery. However, when the potential drug is in pre-clinical and clinical phases, specific guidelines must be followed. While the drug development for small molecules might be different for different targets, the processes from initial screening to the final approval as a new drug are similar.

Several years (10~15 years) are required to develop a new clinical drug from the time it is discovered (Table 4). The conventional process typically starts with target identification followed by hits generation with compounds screening, and then leads optimization, drug candidates selection in pre-clinical phase and clinical phases, and ends with the approval for clinical application [128]. Each phase has the right to terminate the project. If the criteria set for the earlier phases are not accomplished, the project cannot be entered in the later phase. The tests are performed in each phase vary depending on the timeline, target, strategy and budget.

Target identification and validation

The development pipeline starts with the identification of the target for a potential new drug. In theory, a good target needs to be specific and functional in relation to pathological conditions. This can be a receptor, an analogue, a gene, protein or RNA involved in a particular disease progression. Therefore, before any potential new drugs can be discovered, biomedical researchers first need to understand the pathology of disease. Further investigations of underlying mechanisms of the disease are required to determine the key
targets involved in the disease. For example, a number of compounds acting at various key steps of the lipogenic pathway have been shown to reduce obesity, hepatic steatosis and/or insulin resistance including specific inhibitors of fatty acid synthase (FAS), C75 and cerulenin [129], ACC inhibitor 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA) [130] and sterol regulatory element-binding protein-1 (SREBP-1c) [131]. Once identified, target need to be tested and confirmed its role in the process of the disease through complicated experiments in vitro and in vivo model of the disease.

**Drug discovery**

Following the process of target identification and validation, a number of compounds (hits) may act on their target to alter the disease will be selected according to the principles of medicinal chemistry for the evaluation of the drug-like properties of a chemical entity, such as Lipinski’s rule of five [127]. To discover the leads from selected compounds, high-throughput screening assays such as a cell-based assay are developed to screen suitable candidates from the entire compound library directly against the drug target or in a more complex assay system [132]. More recently, using pharmacophores and molecular modelling to conduct virtual screening of compound databases are also used as early discovery paradigms [133]. In addition, fragment (molecular weight < 300 daltons) screening also involves in the generation of very small molecular weight compound libraries [134]. Depending on the timeline and target, several different functional assays might be applied. The functional assays are biologically relevant to the target. At this stage, only one or two concentrations of the
compounds is tested to discriminate the compounds and to identify highly promising compounds against particular target. Based on the results, several leading compounds are selected for the further study.

*Lead optimisation*

In this phase, researchers will perform a series of tests to provide an early assessment of the safety of the leading compounds. All the collected results from the initial screening are recorded into compound library. To improve the effectiveness and safety of leading compounds for continuing studies, a number of medicinal chemistry approaches are involved, including intensive structure-activity relationship (SAR) investigations of each core structure of compounds, addition or removal of various chemical groups, or modification of leading compounds to minimise adverse effects. Computer programs may be also applied to simulate the process and to dismiss any unsuitable candidates in silico. The leads will be used for various studies for *in vitro* and *in vivo*, and eventually they will be used in clinical trial for the disease in humans.

*Pre-clinical studies*

At the pre-clinical stage, researchers test the new optimised compounds under *in vitro* and *in vivo* experimental conditions. They study the desired and adverse effects of the leads to determine in details how the compound works and what its safety or toxicity profile is. In addition, the researchers must produce sufficient amount of the compound (candidate) for clinical trials.
Clinical development

The first stage is Phase I clinical trials at which physicians study in humans to test the safety and tolerability of the new drug candidate as well as its pharmacokinetics. These studies are usually conducted within small groups (20~100 people) of healthy volunteers. The goal of Phase I trial is to discover whether the drug is safe in humans. In Phase II clinical trial, physicians evaluate the effectiveness of drug candidate in a small group (100~500 people) of people with the disease. The aim of this Phase is to examine the short-term side effects and to optimise the dosage and schedules for using the drug candidate. In Phase III clinical trial, physicians determine the statistical efficacy and safety of the new drug candidate on a larger group (1000~5000 people) of patient. It also provides the instructions for proper usage of the drug candidate.

New drug application for approval

After demonstrating the effectiveness and safety of the drug candidate during all three phases of clinical trials, the company submits the new drug application and requests the approval of the drug. The expert panels (e.g. FDA panels) review all the information to decide whether it is approval or not.

Phase IV clinical trial

Phase IV clinical trial is a large scale in clinic after the approval to monitor the long-term or low incident adverse events. An approval drug (e.g. rosiglitazone) may be withdrawn from this clinical trial.
Table 4. Key steps and timeline for drug discovery and development.

<table>
<thead>
<tr>
<th></th>
<th>Target Identification</th>
<th>Drug Discovery</th>
<th>Lead Optimisation</th>
<th>Pre-clinical Studies</th>
<th>Clinical Development</th>
<th>Application for Approval</th>
<th>Phase IV Clinical Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Approach</strong></td>
<td>Cellular and Genetic Targets</td>
<td>Synthesis and isolation; Assay development; Library development</td>
<td>SAR studies; In Silico screening; Chemical synthesis</td>
<td>In vitro studies; In vivo studies</td>
<td>Phase I Phase II Phase III</td>
<td>Expert panels</td>
<td>Phase IV</td>
</tr>
<tr>
<td><strong>Expertise</strong></td>
<td>Biomedical researchers</td>
<td>Medicinal/Biological researchers</td>
<td>Medicinal/Biological researchers</td>
<td>Biologists/Medicinal researchers/Toxicologists</td>
<td>Clinical research physicians</td>
<td>Clinical research physicians</td>
<td></td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td>Compounds</td>
<td>Hits</td>
<td>Leads</td>
<td>Drug candidates</td>
<td>Investigational new drugs</td>
<td>Drugs</td>
<td></td>
</tr>
</tbody>
</table>

The table presents the major steps and timeline for drug discovery and development as described in the text.
1.5.4 Natural Products for Drug Development

Natural products have been used as medications for a long time, although many of them have been replaced by conventional pharmaceutical approaches. More recently, natural products such as plant extracts have attracted the attention and the interest of scientific world due to their fruitful source of chemical diversities with potential use for the treatment of chronic diseases including type 2 diabetes [135].

For example, one of the most successful story of the discovery of the anti-diabetic drugs is metformin, which is developed from natural product French lilac [91]. Its parental compound is the natural guanide originally purified from the plant Goat’s Rue [136]. Following the discovery of its anti-diabetic effects, guanide was then chemically modified as a biguanide (metformin) to improve efficacy and reduce toxicity. Currently, metformin is the most commonly used drug for the treatment of type 2 diabetes (Section 1.4.1).

It is estimated that over 30% of all current prescription drugs are derived from natural products. Among the new approved drugs developed over the past 30 years or so, 4% were natural products, 22% were derivatives of natural products, 20% were natural products mimics and 4% were synthetic drugs inspired by natural products [135] (Figure 9).
Figure 9. All new approved drugs 30 years (01/01/1981-12/31/2010). “N” Natural product; “NB” Natural product; “Botanical” (in general these have been recently approved); “ND” Derived from a natural product and is usually a semi-synthetic modification; “S*” Made by total synthesis, but the pharmacophore is/was from a natural product; “NM” Natural product mimic; “S” Totally synthetic drug, often found by random screening/modification of an existing agent; “B” Biological; usually a large (>45 residues) peptide or protein; “V” Vaccine, n=1355. Modified from Newman et al., 2012 [135].

Natural products bring a number of big challenges to researchers for a number of reasons compared with industry drug discovery from synthetic small molecules. For the chemists, not only the process of purification can be slow and expensive, but also the purified natural product compounds are usually difficult to modify and small in quantity. For biomedical researchers (particularly in academic institutions), it is difficult to conduct a large scale of random screenings and tests due to the resource constrains.
1.5.5 Natural Products from Traditional Chinese Medicine

Traditional medicines such as Traditional Chinese Medicines (TCM) have been increasingly regarded as a unique source for drug discovery [137, 138]. For example, artemisinin (known as Qinghaosu) is isolated from the plant *Artemisia annua* and has been widely used for severe malaria in humans [139]. Other clinical studies demonstrated that rocaglamides isolated from the plant *Aglaia* possessed anti-cancer activities [140]. TCM has also been found to be useful in the treatment of obesity and type 2 diabetes. For example, berberine exerts beneficial metabolic effects in diabetic and insulin-resistant states [141, 142]. Triterpenoids compounds extracted from bitter melon improve glucose disposal in insulin resistant models [143]. Another compound, Ginsenoside Rh2 isolated from root *Panax Ginseng*, is reported to prevent obesity possibly via activating the AMPK signalling pathway [144]. Thus, it is likely that using natural products to target the specific receptors is a useful approach for the treatment of type 2 diabetes.

Screening for large and complex small molecule libraries of natural products incurs great expenses and time as well as triggering potential toxicity issues. For this reason, a more targeted approach using TCM with known efficacy on metabolic disorders is recommended. TCM have been used for centuries to treat diseases including diabetes, hence there is already vast literature (including traditional and experimental usage) available for data mining, virtual screening and computer modelling. TCM are manufactured in a large scale overcoming the resource quantity constrains often encountered in research of other natural products. However,
identification of active ingredients from TCM remains a major challenge because of the complex mix. Therefore, the mode of action of TCM is generally not known. In addition, many of the claimed efficacies of TCM are not based on rigorous clinical trials of the modern standard. It is widely believed that the beneficial effects of TCM are due to combinational effects, which results in significant difficulties in isolating the active ingredients from the natural products in TCM. Therefore, designing mechanism-based screening methods become essential to guide the identification and separation of the active molecules from TCM ingredients.

1.6 Summary, Aims and Thesis Focus

Based on the literature and preliminary data, the overall aim of this project is to identify novel anti-diabetic compounds from Traditional Chinese Medicine. In order to address which compounds can have anti-diabetic effects, three specific aims are listed as follows:

**Aim 1**: To establish a cell-based phenotypic assay to determine lipid accumulation as a high-throughput screening tool for the identification of new anti-diabetic and anti-obese compounds (Chapter 3).

As described in Section 1.3.2, excess lipid accumulation is associated with type 2 diabetes. Therefore, screening of compounds for their effects on reducing lipid level is a useful tool for potential treatment of type 2 diabetes.

**Aim 2**: To screen library compounds derived from Traditional Chinese Medicine to identify
compounds with lipid-lowering properties as potential anti-diabetic and anti-obese drugs (Chapter 3).

Natural products used in Traditional Chinese Medicine have been regarded as a unique source for drug discovery due to their chemical diversity (Section 1.5.5). Hence, it is of great importance to explore and identify anti-diabetic properties of the compounds derived from natural products based on vast of literature and virtual screening.

**Aim 3:** To investigate the *in vivo* effects of identified two leading compounds and their molecular mode of action.

Based on the screening results, literature reports as well as criteria of selection compounds in our group (Section 2.3.2), albiflorins and oleanoic acid are selected for further investigations (Chapter 4 & 5).
CHAPTER 2
MATERIALS AND METHODS
2.1 INTRODUCTION

This chapter presents detailed materials, methods and techniques used in the studies of the thesis. Most of the materials such as reagents and solutions were purchased from commercial suppliers. Many of the techniques were either reported by other groups or established within the Molecular Pharmacology for Diabetes Group in the RMIT University, Melbourne, Australia, where all the work was performed. In all the measurements, appropriate controls and/or commercial standards were included with the testing samples, methods of validation and confirmation by assay repetition were employed to ensure the validity of the results. The materials or measurements supported by external collaborators are indicated with the appropriate acknowledgment below.

2.2 IN VITRO

All cell culture experiments were conducted according to the Standard Operating Procedures for Physical Containment Level 2 (PC2) Laboratory area (Module C, Building 223, RMIT University, Melbourne, Australia) using established facilities including Class II Biohazard Safety cabinets and CO₂ incubators.

2.2.1 3T3-L1 Adipocytes

The 3T3-L1 mouse embryonic fibroblast is a cell line derived from mouse 3T3 cells. L1 is a continuous substrain of 3T3 developed through clonal isolation by H. Green and O. Kehinde [145]. 3T3-L1 cells have a fibroblast-like morphology (Figure 10). Under certain conditions, 3T3-L1 cells can undergo a conversion from pre-adipose to adipose-like cells as they are
rapidly dividing to reach confluence and contact inhibited state (Section 2.2.3.1). Therefore, 3T3-L1 cells are commonly used in biological research on adipose tissue.

Figure 10. Representative image for 3T3-L1 mouse embryonic fibroblasts. 3T3-L1 cells have a fibroblast-like morphology. The photo was taken under 20x objective by using the Nikon TS100F inverted microscope and camera (Nikon Instruments Inc., USA).

3T3-L1 mouse embryonic fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and stored in 2 ml cryovials (Corning, Melbourne, Australia) at -80°C freezer (short-term storage) or in the liquid nitrogen (long-term storage). When needed, the cryovials were quickly defrosted at 37°C in a water bath with a constant and moderate agitation until fully thawed. Vials were immediately disinfected with 70% ethanol (Merck, #1.07017.2511) and opened in an UV-disinfected cell culture hood (Thermo Fisher Scientific, Australia) before slowly transferred the thawed cells into a sterile 15 ml tube (Corning, Melbourne, Australia) containing 9 ml pre-warmed normal growth medium. The content were mixed gently by pipetting up and down several times and then centrifuged at 100x g for 5 mins to form a cell pellet. After aspiration of the supernatant, the pellet was gently re-suspended in 10 ml of normal growth medium and transferred into a T75 culture flask (Corning, Melbourne, Australia). Cells were observed under the microscope and cell viability
was assessed. The flask was then placed in an incubator at 37°C in 5% CO₂. After overnight incubation, the cells were re-examined and assessed for viability.

2.2.2 Cell Culture

2.2.2.1 Cell culture solutions

1. Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, #11965): stored at 4°C

2. Fetal Bovine Serum (FBS) (Invitrogen, #10099-141): short-term stored at 4°C, long-term stored at -20°C

3. Penicillin Streptomycin L-Glutamate (PSG) (10⁵ units/ml; 10⁵ µg/ml and 200 mM, respectively, 100x stock, Invitrogen, #10378-016): short-term stored at 4°C, long-term stored at -20°C

4. Trypsin, 0.25% (1x) with EDTA (Invitrogen, #25200056): short-term stored at 4°C, long-term stored at -20°C

5. Insulin (Sigma-Aldrich, #I5500): 4 mg/ml in 0.01N Hydrogen chloride (HCl), 2000x stock. Sterilized by filtration through a 0.22 µm filter and stored at 4°C

6. Dexamethasone (Sigma-Aldrich, #D4902). 1 mg/ml in ethanol. 10,000x stocks. Sterilised by filtration through a 0.22 µm filter and store at 4°C

7. 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, #I15879): 500 mM in Dimethyl sulfoxide (DMSO, Sigma-Aldrich, #D4540) 1000x stock and stored at -20°C

8. Biotin (Sigma-Aldrich, #B4639): 0.1 mg/ml in dH₂O. 1000x stock. Sterilized by filtration through a 0.22 µm filter and stored at 4°C

9. 10x phosphate buffered saline (PBS): 0.2 M Sodium chloride (NaCl, Sigma-Aldrich, #S3014), 10 mM Sodium phosphate dibasic dihydrate (Na₂HPO₄, Sigma-Aldrich,
#71633), 3 mM Potassium chloride (KCl, Sigma-Aldrich, #P3911) and 2 mM Potassium phosphate monobasic (KH₂PO₄, Sigma-Aldrich, #P5655), pH 7.4, autoclaved and stored at 4°C

### 2.2.2.2 General cell culture procedures

3T3-L1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin-glutamine (PSG) at 37°C in 5% CO₂. The cell culture medium was changed every two or three days. Cell passages number 5~20 were used for all experiments. Briefly, cells were subcultured at 1 in 5 dilutions when cells reached 70%~80% of confluence. Subculture was made by rinsing the cells first with 1x phosphate buffered saline (PBS) buffer, and then adding 1~2 ml of 1x trypsin-EDTA solution to detach the cells from the T75 flask (usually within 1 min). Cells were then centrifuged at 100x g for 5 mins and re-suspended in ~5 ml of fresh growth medium and the desired amount of cells was transferred into a new T75 flask containing fresh growth medium.

### 2.2.2.3 Freezing down cells for long-term storage

A T75 flask containing adherent cells was first washed in 1x PBS to remove all content of growth medium. This was subsequently removed and 1~2 ml of 1x trypsin-EDTA solution added for 1 min. A firm tap to the side of the flask successively detached the cells and the enzymatic action of trypsin-EDTA was stopped with the further addition of 5 ml of growth medium. This cell suspension was centrifuged at 100x g for 5 mins. After removal of the supernatant, the remaining cell pellet was re-suspended in 0.5 ml of DMEM / 20% FBS / 15%
DMSO / 1% PSG to re-suspend cells, and then 1 ml aliquots were prepared in each cryovial. Cryovials were then wrapped in a clean paper towel and transferred to a Nalgene® cryo 1°C freezing container (Thermo Fisher Scientific, Australia) filled with 100% isopropanol (Sigma-Aldrich, #I9516), and stored overnight at -80°C. One of the frozen vials was defrosted in the following day and tested for cell viability whereas the rest vials were placed in liquid nitrogen for long-term storage.

2.2.2.4 Counting of cells number

Cell numbers were estimated by adding 10 µl of cells suspension between a glass coverslip and the surface of a haemocytometer (Grace Davison Discovery Sciences, Australia) marked as depicted in Figure 11. Cells were viewed using microscope (10x or 20x magnification) and the cell numbers in each area were then calculated (Figure 11, Area 1~4). To find the mean number of cells per Area (4 x 16 corner squares), the sum of Areas 1 to 4 was divided by 4, and this value was then multiplied by $10^4$ to give an estimated number of cells per ml.

Figure 11. Formula of counting cells number. Counted cells number = Cells number of Sum (Area 1 + Area 2 + Area 3 + Area 4). Cells number/ml = Counted cells number/4 x $10^4$. 
2.2.3 Cell Treatments

2.2.3.1 Differentiation of 3T3-L1 pre-adipocytes

When cells reached the desired confluence, they were used in the experiments described as follows. 3T3-L1 pre-adipocytes, under appropriate conditions, can be differentiated into adipocyte-like cells. Differentiated 3T3-L1 cells display increased synthesis and accumulation of triglycerides, and they present the round droplets appearance of adipose cells. These cells are also sensitive to lipogenic and lipolytic hormones as well as the drugs, including epinephrine, isoproterenol and insulin [146].

To induce differentiation, 2-day post-full confluent 3T3-L1 pre-adipocytes were incubated in differentiation medium which contained DMEM, 10% FBS, 1% PSG, 2 µg/ml insulin, 100 ng/ml dexamethasone, 500 µM IBMX and 10 ng/ml biotin. Cells were incubated in this media for 3 days before switching to a post-differentiation medium which contained DMEM, 10% FBS, 1% PSG and 2 µg/ml insulin for an additional 3 days (Table 5, Figure 12).

Table 5. Process of differentiation of 3T3-L1 cells. As described in the text. Cells photos were taken under 20x objective.

<table>
<thead>
<tr>
<th>Base medium</th>
<th>Differentiation medium</th>
<th>Post-differentiation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day -2</td>
<td>Day -1 Day 0</td>
<td>Day 1 Day 2 Day 3 Day 4 Day 5 Day 6</td>
</tr>
<tr>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
<td><img src="image3.jpg" alt="Image" /></td>
</tr>
<tr>
<td>Lipid droplets (Triglyceride)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12. Process of 3T3-L1 cells differentiation. **Left panel:** 3T3-L1 pre-adipocytes can be stimulated and differentiated to adipocytes by addition of cocktail, including IBMX, dexamethasone, biotin and insulin in the DMEM. IBMX increases intracellular level of cAMP, dexamethasone binds to the glucocorticoid receptor (GCR) and insulin binds to the insulin receptor. These three pathways promote the expression of the master adipogenic transcription factors, PPARγ and C/EBP family genes (C/EBP β and δ). **Right panel:** 3T3-L1 adipocytes contain PPARγ and C/EBPa, which activate adipocyte specific genes encoding secreted factors, insulin receptor, and proteins involved in the synthesis and binding of fatty acids that compose oil red O stainable lipid droplets (triglyceride). Adapted from Merck Millipore - adipogenesis assay [147].
2.2.3.2 Screening compounds using 3T3-L1 cells model

Undifferentiated 3T3-L1 cells were treated with compounds, which are from our unique compound library, for 6 days and triglyceride content was then measured using a novel triglyceride extraction method described in Section 3.2.3. The tested concentration of compounds was 1 µM or 10 µM unless indicated elsewhere.

To assess the effects on lipid accumulation, compounds were dissolved in 100% DMSO and then diluted 1 in 1000 of medium to give the final test concentration indicated in the corresponding figure. Compounds were presented throughout the whole period of differentiation (Figure 13). 3T3-L1 cells were cultured in normal growth medium (DMEM with 10% FBS and 1% PSG) with 0.1% DMSO used as a vehicle control for all experiments. Cells were monitored and photographed using the Nikon TS100F inverted microscope and camera (Nikon Instruments Inc., USA).

![Figure 13. Treatment timeline for screening compounds in 3T3-L1 cells.](image)

3T3-L1 cells were incubated in normal growth medium for 3 days before change to differentiation medium plus compounds. After 3 days of differentiation, cells were cultured in post-differentiation medium plus compounds. DMSO was used as a vehicle control. After 3-day differentiation, fresh compounds were added into post-differentiation medium for another 3 days.
2.2.4 *In Vitro* Measurements

2.2.4.1 Intracellular triglyceride extraction and determination

The level of triglycerides is commonly used as an indicator of lipid accumulation [62, 63]. The determination of triglycerides was based on using a lipoprotein lipase from microorganisms, which can hydrolyse triglyceride to glycerol and fatty acids. Glycerol is then oxidised to dihydroxyacetone phosphate and hydrogen peroxide by glycerol kinase and glycerolphosphate oxidase. The produced hydrogen peroxide then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidise to form a red dyestuff 4-(p-benzoquinone-monoimino)-phenazone, which is an endpoint reaction. The colour intensity of the red dyestuff can directly indicate the proportion of triglyceride concentration and can be also measured photometrically at 485 nm [148] (Figure 14).

\[
\text{Triglyceride} + 3\text{H}_2\text{O} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + 3\text{Fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{Glycerolphosphate oxidase}} \text{Dihydroxyacetone} + \text{Phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + 4\text{-chlorophenol} \xrightarrow{\text{Peroxidase}} 2\text{H}_2\text{O} + \text{HCl} + \text{Dye}
\]

**Figure 14. Biochemical reactions for the determination of triglycerides.** As described in the test.

At the end of the 3-day post-differentiation medium incubation period (Table 5, Day 6), the culture medium was removed and the cells were washed twice with ice-cold 1x PBS buffer, PBS was then removed before cells were subjected to dry in a 37°C incubator (Ratek...
Instruments, Australia) until cells were totally dried. Cells were then lysed by a novel or a conventional extraction method. The novel method involved the addition of potassium hydroxide (KOH, Sigma-Aldrich, #221473, final concentration 50 mM, 25 μl/ well for 96-well plate) for 10 mins at 60°C followed by centrifugation at 4000x rpm for 5 mins. The conventional extraction method involved the addition of chloroform/methanol (C/M, 2:1) [149], immediately followed by cell scraping. Chloroform was purchased from Sigma-Aldrich (#319988) and methanol was obtained from Merck (#1.00921.2500). The cell lysate produced by C/M method was then mixed with 1 ml of 0.6% NaCl solution, completely mixed and centrifuged at 2000x rpm for 10 mins to separate the aqueous from the organic phases. The lower organic layer containing triglycerides 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.

The total triglycerides content from both KOH and C/M extractions was determined by using a commercial Peridochrom triglyceride GPO-PAP reagent (Roche Diagnostics, #11730711) where glycerol (Precimat® glycerol, Roche Diagnostics, #166588) acted as a reference. Usually, 15 μl of cell lysates were added to 300 μl triglyceride reagent and then incubated at 37°C for 10 mins. The absorbance was measured at 485 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany). Internal standards (varying dilutions of glycerol standard) were included to create a standard curve between the range of 0 μg/well to 1.050 μg/well.
2.2.4.2 Oil red O staining

Oil red O (ORO) staining is commonly used in combination with light microscopy to assess lipid accumulation in adipocytes [150]. Briefly, the treated differentiated 3T3-L1 cells were washed twice with ice-cold 1x PBS buffer and incubated with 10% neutral formalin for 5 mins before fixing in fresh 10% neutral formalin (Sigma-Aldrich, #F5554) overnight. The plate containing cells was wrapped to prevent from drying and covered with aluminium foil. The next day, cells were washed with 60% isopropanol (Sigma-Aldrich, #I9516) and completely dried followed by the staining with a 0.35% (w/v) ORO solution (Sigma-Aldrich, #O0625) for 10 mins. ORO was dissolved in 100% isopropanol and stored at -4°C until used. Excess stain was then removed by rinsing the cells with distilled water and then dried before microscopic examination. For quantitative analysis, ORO was eluted with 100% isopropanol for 10 mins and the absorbance was measured at 485 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany).

2.2.4.3 Lactate dehydrogenase release determination

Cytotoxicity was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) released from the damaged cells into the culture medium. After cells were treated with selected compounds for 6 days, the medium was collected and centrifuged at 1000x rpm at 4°C for 10 mins to remove detached cells. To measure the intracellular LDH activity, 3T3-L1 cells were lysed by two freeze-thaw cycles (chilling the plate at -80°C for approximately 30 mins followed by thawing at 37°C for 15 mins) after washing with ice-cold 1x PBS buffer.
The cell lysate was then centrifuged at 1000x rpm for 4 mins, and the supernatant was stored at 4°C until ready to use. The enzyme activity in the whole cell lysate and medium were determined by the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, #G1780) according to the manufacturer's protocol. Usually, 50 µl of the diluted cell medium (1:5) or cell lysate (1:10) was transferred into a 96-well plate before the addition of 50 µl reconstituted substrate mixture. The plate was then covered with foil and incubated at room temperature for 30 mins. 50 µl of stop solution was added to each well before measuring absorbance. The absorbance of the samples was read at 490 nm using a Polarstar Optima micro-plate reader (BMG Lab Technologies, Germany). The released LDH into the medium (cells viability) was expressed as a percentage of the total LDH activity (ratio of LDH in lysate and in the medium).

### 2.3 ANIMALS

All experiments were carried out with the approval of the Animal Ethics Committee of RMIT University following guidelines issued by the National Health and Medical Research Council of Australia (Approval No. #1012 & #1208). 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-hour light/dark cycle with free access to food and water. During the acclimatisation period, all mice were fed ad libitum with a standard chow diet (12% calories from fat, 65% calories from carbohydrate and 23% calories from protein) (Meat free rat and mouse diet, Specialty Feeds, Perth, Australia).
2.3.1 Diet Treatments

2.3.1.1 High-fat or high-fat cholesterol diet to induce insulin resistance

After one-week acclimatisation, mice were randomly assigned to receive either chow diet (CH group) as normal control or a high-fat diet (HF group) to induce insulin resistance. Chow-fed animals were maintained on the standard CH diet throughout the whole study with free access to food and water whereas the HF group received a diet that contained high fat (45% calories from fat, 35% calories from carbohydrates and 20% calories from protein) with equal quantities of fibre, vitamins and minerals as the standard diet. In some studies, 0.2% cholesterol (Sigma-Aldrich, #C75209) was added to the high-fat diet (HFC group) to produce non-alcoholic steatohepatitis (NASH) phenotype [151]. The main source of fat in the diet was lard (Allowrie, Melbourne, Australia). This diet was prepared weekly from the ingredients presented in Table 6 and kept in -20°C until used. Animals were fed for 8 weeks with or without selected compounds in HF or HFC diet for 2~4 weeks.

Table 6. Composition of the high-fat diet.

<table>
<thead>
<tr>
<th>Prepared Ingredients</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (MPD Dairy Products, Acidcasein)</td>
<td>215.6</td>
</tr>
<tr>
<td>Sucrose (Sigma-Aldrich, #84097)</td>
<td>294</td>
</tr>
<tr>
<td>Starch from corn (Sigma-Aldrich, #S4126)</td>
<td>173</td>
</tr>
<tr>
<td>AIN-76 Mineral Mix (MP Biomedicals Australia, #290545502)</td>
<td>51</td>
</tr>
<tr>
<td>Trace Minerals (MP Biomedicals Australia, #296026401)</td>
<td>14.8</td>
</tr>
<tr>
<td>Bran (Hudson Pacific Corporation, #BC20KGA)</td>
<td>63</td>
</tr>
<tr>
<td>DL-Methionine (Sigma-Aldrich, #M9500)</td>
<td>2.8</td>
</tr>
</tbody>
</table>
## 2.3.1.2 High-fat diet with streptozotocin injections to induce type 2 diabetes

After one week acclimatisation, the mice were fed *ad libitum* with a standard lab chow (CH) diet (12% calories from fat, 65% calories from carbohydrate and 23% calories from protein) or high fat (HF) diet (45% calories from fat, 35% calories from carbohydrates and 20% calories from protein, Table 6) for 10 weeks [152]. HF-fed mice were then injected with vehicle (saline, Sigma-Aldrich, #P3813) or multiple low dose of streptozotocin (STZ, Sigma-Aldrich, #S0130, 40 mg/kg/day) for 5 consecutive days to partially damage the pancreatic β-cells, a typical animal model of type 2 diabetes [113, 153, 154]. Diet was prepared weekly from the ingredients listed in Table 6 and kept in -20°C until required.

## 2.3.2 Administration of Test Compounds

To select candidates for *in vivo* experiments, we based on the criteria as described in Section 1.5.2. In the albiflorin (AF) study, AF, at a dose of 100 mg/kg/day [155], was mixed in the
high-fat or high-fat cholesterol diet. In the oleanolic acid (OA) study, OA (100 mg/kg/day [154]) was mixed in the high-fat diet.

2.3.3 Measurements of Plasma Parameters

2.3.3.1 Plasma collection and storage

During the study, blood samples were collected from the tail tip using a heparin capillary tube (Hirschmann Laboratory, Germany) to prevent clotting and haemolysis. The blood samples were placed in 1.5 ml eppendorf tubes containing an equivalent volume of sterile normal saline and then put in the ice. The absolute volumes used were depended on the experimental requirements and were given with the relevant study. Immediately after the collection, the samples were centrifuged at 13,000x g for 1 min to separate the plasma, which were then transferred to a clean eppendorf tube and stored at -80°C until use.

2.3.3.2 Glucose determination

At the end of the study, animals were fasted for 5~7 hours. Plasma was extracted from blood samples collected from the tail tip and stored at -80°C as described above. Intraperitoneal glucose tolerance tests (ipGTT, glucose load 1.0~2.0 g/kg body weight) were performed where blood glucose levels were measured at designed time points (0, 15, 30, 60 and 90 mins) using an Accu-Chek glucometer (Roche Diagnostics, Australia).

2.3.3.3 Triglyceride determination

Plasma triglyceride levels were determined by a commercial enzymatic method using a Peridochrom triglyceride GPO-PAP reagent where glycerol acted as a reference according to
the manufacturer’s instructions (Roche Diagnostic, Australia). Usually, 5 µl of plasma samples were added to 300 µl triglyceride reagent and then incubated at 37°C for 10 mins. The absorbance was measured at 485 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany). Internal standards (varying dilutions of glycerol standard) were included to create a standard curve between the range of 0 µg/well to 1.050 µg/well.

### 2.3.3.4 Aspartate transaminase/Alanine transaminase determination

Aspartate aminotransferase (AST) and alanine transaminase (ALT) are transaminases and are commonly included in diagnostic tests to examine liver function. ALT is more specific to the liver, as AST is also found in cardiac muscle, skeletal muscle and red blood cells. Plasma AST and ALT levels were determined by a commercial enzymatic kit (Laboratory Diagnostics Pty. Ltd., #ST2920-500) according to the manufacture’s instruction. 20 µl of each plasma sample was added into 200 µl working reagent (5 parts buffer I to 1 part buffer II). Solutions were mixed and incubated at 37°C, continuously recorded the absorbance every 1 min up to 10 mins. Distilled water was as blank. The absorbance was measured at 340 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany).

### 2.3.3.5 Cholesterol determination

Cholesterol is an important component of mammalian cell membranes where it functions in intracellular transport, cell signalling, and maintaining membrane fluidity. Within the blood, cholesterol circulates as both of the free acid and cholesterol esters. Controlling serum cholesterol plays an important therapeutic role as elevated cholesterol levels are associated
with the development of pathologies of several diseases including diabetes, cardiovascular disease [156] and non-alcoholic steatohepatitis (NASH) [151]. Recent evidence suggests a disturbance of cholesterol homeostasis contributes to the development of a chronic inflammatory state [157]. Plasma cholesterol levels were determined by a commercial enzymatic kit (Roche Diagnostics, #11491458-216) according to the manufacture’s instruction. Usually, 10 µl of plasma samples were added to 200 µl cholesterol reagent and then incubated at 37°C for 10 mins. The absorbance was measured at 485 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany). Internal standards (varying dilutions of cholesterol standard) were included to create a standard curve between the range of 0 mg/ml to 1.0 mg/ml.

2.3.3.6 Insulin determination

Plasma samples were collected at 0, 15, 30 and 60 mins during ipGTT. Plasma insulin was determined using a commercial rat insulin radioimmunoassay kit (Merck Millipore, #SRI-13K) according to the manufacturer’s instructions. The method is based on a double antibody technique, using ^125^I-labelled insulin and a specific rat insulin antiserum. 100 µl of plasma sample was added to 100 µl of hydrated ^125^I-insulin, followed by the addition of 100 µl insulin antibody overnight at 4°C. The next day, 1 ml of cold precipitating reagent was added to each tube and incubated at 4°C for 20 mins after vortexing. All the samples were then centrifuged at 2000x g for 20 mins at 4°C. After centrifugation, the supernatant was immediately discarded and the pellet was dried. All samples were counted in a gamma
scintillator counter (Perkin Elmer, USA) for 1 min. Total count tubes were as reference. The coefficient of variation was less than 10% within and between assays.

2.3.4 Measurements of Tissue Parameters

2.3.4.1 Mitochondria isolation and respiration measurement

Fresh rat liver tissue (400~800 mg) was homogenized in 8 ml medium containing 250 mM Sucrose, 10 mM Tris-HCl (Trizma base and HCl), 1 mM Ethylene glycol-bis (2-aminoethylether)-N, N’, N’-tetraacetic acid (EGTA, Sigma-Aldrich, #E3889), 1% BSA (fatty acid free, Sigma-Aldrich, #A7030), pH 7.4. The homogenates were centrifuged at 2500x rpm for 5 mins and supernatant was transferred to new Eppendorf tubes, followed by another centrifugation at 12,000x rpm for 10 mins. White fluff was removed around brown pellet (mitochondria). Mitochondria were then re-suspended in isolation medium and centrifuged at 10,000x rpm for 10 mins and pellets were re-suspended in respiration medium without BSA (2 ul/mg tissue).

Mitochondrial respiration was measured at 37°C with a Clark-type oxygen electrode (Strathkelvin Instruments, Motherwell, Scotland) in a respiration medium that contained 225 mM D-Mannitol (Sigma-Aldrich, #M9647), 75 mM Sucrose, 10 mM Tris-HCl, 10 mM KH₂PO₄, 10 mM KCl, 0.8 mM Magnesium chloride (MgCl₂, Sigma-Aldrich, #M0631), 0.1 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, #E6758) and 0.3% BSA (fatty acid free), pH 7.0. Dose-dependent effects of different compounds on mitochondrial respiration were determined in the presence of 2.4 mM Adenosine 5’-diphosphate sodium salt.
(ADP, Sigma-Aldrich, #A2754), using substrate combinations targeting either respiratory
Complex I including 5 mM Pyruvate (Sigma-Aldrich, #P8574) plus 2 mM D-Malic acid
(malate, Sigma-Aldrich, #M0750) or respiratory Complex II including 10 mM Succinic acid
(succinate, Sigma-Aldrich, #S3647) plus 4 µM Rotenone (Sigma-Aldrich, #R8875) [142, 158].
The results were expressed against the protein level of each sample.

Protein concentration in each sample was measured through use of commercially available
colorimetric bi-cinchoninic acid (BCA) protein kit (Sigma-Aldrich, #B9643). 1 µl of diluted
tissue lysate (1 part lysate to 19 µl dH2O) was added to 200 µl BCA reagent mix (50 parts
reagent A to 1 parts reagent B). Solutions were mixed and incubated at 37°C for 30 mins prior
to the determination of absorbance at 562 nm using a Polarstar Optima microplate reader
(BMG Lab Technologies, Germany). In every BCA assay, varying dilutions of BSA protein
were included to create a standard curve between the ranges of 0 µg/ml to 2.0 µg/ml.

2.3.4.2 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-weighted sample of tissue (30~40 mg liver)
was homogenized in 4 ml of chloroform/methanol (C/M) (2:1) using a glass pestle tissue
grinder (VWR, Australia). After transferring the homogenate to a clean 15 ml tube, the
homogenizer was rinsed with another 2 ml of C/M 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, completely
mixed and centrifuged the tubes at 2000x rpm for 10 mins 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, #11730711). Usually, 5 µl tissue lysate was added to 1 µl tissue lysate was added to 19 µl dH2O, and then 200 µl triglyceride reagent was added. Solutions were mixed and incubated at 37°C for 10 mins prior to the determination of absorbance by spectrophotometry at 485 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany). The triglyceride content of samples was determined against a standard curve between the range of 0 µg/well to 1.050 µg/well (varying dilutions of glycerol standard). This method has been used in the previous publications from this laboratory [159, 160].

2.3.4.3 Western blotting

Reagents / Buffers

1. **RIPA buffer**: 65 mM Trizma® base (Tris, Sigma-Aldrich, #T1503), 150 mM NaCl, 5mM EDTA, 1% Nonide-P 40 Substitute (NP-40, Sigma-Aldrich, #74385), 0.05% Sodium-deoxycholate (Sigma-Aldrich, #D6750), 0.1% (w/v) Sodium dodecyl sulphate (SDS, Sigma-Aldrich, #L4390), 10% Glycerol (Sigma-Aldrich, #49770), pH 7.5 and stored at 4°C

2. **Lysis buffer**: 10 mM Sodium fluoride (NaF, Sigma-Aldrich, #S7920), 1 mM Sodium orthovanadate (Na₃VO₄, Sigma-Aldrich, #S6508), 1 mM Phenylmethanesulfonyl fluoride (PMSF, dissolved in 100% Ethanol, Sigma-Aldrich, #78830), and 10 µl/ml
Protease/Phosphatase inhibitor (Sigma-Aldrich, #P5726) in RIPA buffer

3. **4x Laemmli’s buffer** (100ml): 8.2 g SDS, 40ml Glycerol, 50 ml 0.5 M Tris, 500 µl 1% Bromo-phenol blue (Sigma-Aldrich, #114391) in dH2O, pH 6.8 and stored at -20°C. Added 6.2 mg DL-Dithiothreitol (DTT, Sigma-Aldrich, #D9779) before use

4. **Running gel**: 1.5 M Tris Buffer (pH 8.8), 30% Acrylamide/Bis-acrylamide (Sigma-Aldrich, #A3574), 10% (w/v) SDS, dH2O, 10% Ammonium persulfate (APS, Sigma-Aldrich, #A9164), Tetramethylethylene diamine (TEMED, Sigma-Aldrich, #T9281)

5. **Stacking gel**: 0.5 M Tris Buffer (pH 6.8), 30% Acrylamide/Bis-acrylamide, 10% (w/v) SDS, dH2O, 10% APS and TEMED

6. **10x Running buffer** (1L): add 30 g Tris, 144 g Glycine (Sigma-Aldrich, #G8898) and 10 g SDS in dH2O, pH 8.8 and stored at room temperature

7. **10x Transfer buffer** (1L): add 30 g Tris, 144 g Glycine in dH2O and stored at room temperature

8. **10x TBS** (1L): add 24.2 g Tris and 80 g NaCl in dH2O, pH 7.6 and stored at room temperature

9. **1x TBS-Tween** (TBST, 1L): 100 ml 10x TBS buffer and 500 µl Tween® 20 (Sigma-Aldrich, #P9416) in 900 ml dH2O and stored at room temperature

10. **Blocking buffer**: 3% (g/100ml) Bovine Serum Albumin (BSA, Sigma-Aldrich, #A9418) in 1x TBST and stored at 4°C

11. **Stripping buffer** (1L): 6.25% 1 M Tris-HCl (pH 6.7), 10% (w/v) 20% SDS in dH2O and stored at room temperature. Added 100 mM 2-Mercaptoethanol (Sigma-Aldrich, #M7154) before use
**Tissue homogenisation**

Freeze-clamped liver tissues were homogenised with a pestle mixer in ice-cold RIPA lysis buffer. The resulting homogenate was solubilised for 2 hours in a cold room and then centrifuged at 15,000x rpm for 10 mins at 4°C to eliminate debris. The supernatant was transferred to an eppendorf tube and stored at -80°C.

**Protein quantification**

Protein concentrations were determined through use of commercially available colorimetric bi-cinchonnic acid (BCA) protein kit (Sigma-Aldrich, #B9643). Usually, 1 µl tissue lysate was added to 19 µl dH₂O to dilute lysate, then 200 µl BCA reagent mix (50 parts reagent A to 1 parts reagent B) was added to detect concentration of protein in the each sample. Solutions were mixed and incubated at 37°C for 30 mins prior to the determination of absorbance by spectrophotometry at 562 nm using a Polarstar Optima microplate reader (BMG Lab Technologies, Germany). In every BCA assay, varying dilutions of BSA protein were included to create a standard curve between the ranges of 0 µg/ml to 2.0 µg/ml.

**SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis)**

8%, 10%, 12% and 14% Tris-glycine based acrylamide gels were prepared for separation of proteins according to their molecular weights (Table 7). Polymerisation of the gel was catalysed by the addition of both APS and TEMED. After polymerisation of the running gel, stacking gel was poured on the top of running gel.
Table 7. Composition of running gel and stacking gel.

<table>
<thead>
<tr>
<th></th>
<th>Running Gel (in ml) (for two gels)</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5M Tris Buffer, pH 8.8</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>30% Acrylamide/Bis-acrylamide</td>
<td>5.3</td>
<td>6.7</td>
<td>8.0</td>
<td>9.3</td>
</tr>
<tr>
<td>3</td>
<td>10% SDS</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>dH₂O</td>
<td>9.5</td>
<td>8.1</td>
<td>6.8</td>
<td>5.5</td>
</tr>
<tr>
<td>5</td>
<td>10% APS</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>TEMED</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Stacking Gel (in ml) (for two gels)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5M Tris Buffer, pH 6.8</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>30% Acrylamide/Bis-acrylamide</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>dH₂O</td>
<td>7.0</td>
</tr>
<tr>
<td>5</td>
<td>10% APS</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>TEMED</td>
<td>0.02</td>
</tr>
</tbody>
</table>

An aliquot of tissue lysate was dissolved in the Laemmli’s buffer to receive the equal amount of protein levels for all samples. The samples were heated at 37°C for 30 mins prior to SDS-PAGE.

Equal amounts of protein were loaded into the gels and run alongside protein ladders (Bio-Rad Laboratories Inc., Australia) of known molecular weight markers. The gels were run at 100 V for 30 mins which was then increased to 130 V until the markers above or below the protein of interest were well separated.
**Immunoblotting**

Following the separation of protein by SDS-PAGE, proteins were then transferred from the gels to methanol-activated polyvinylidene fluoride (PVDF) transfer membranes (Bio-Rad Laboratories Inc., USA) at 90 V for 90 mins. Non-specific binding was blocked with 3% BSA in 1x TBST for 1 hour at a room temperature. The membranes were then incubated in the appropriate primary antibodies for 2 hours at the room temperature or overnight at 4°C.

The next day, the membranes were washed with 1x TBST for 6 times (10 mins each time) to remove any unbounded antibody. Subsequently the membrane was incubated with an appropriate secondary antibody (1:3000 dilution in 1x TBST) for 2 hours at the room temperature. The membranes were again washed with 1x TBST for 6 times (10 mins each time) to remove excess unbounded secondary antibody. The bound antibody was detected using a chemiluminescence system with Western Lighting Ultra Solution (Perkin Elmer, #NEL113001EA). The membranes were exposed to ChemiDoc (Bio-Rad Laboratories Inc., USA) capturing images for a sufficient time. Quantitative densitometry analysis of interested bands was performed using Image Lab software (Bio-Rad Laboratories Inc., USA). Relative protein expression was normalised by the band intensity of GAPDH, as the internal control.

All the methods have been used in the previous publications from this laboratory [154, 160].

**2.3.4.4 Quantitative real-time PCR**

**Isolation of RNA from animal tissues**

Mice liver tissues (20~30mg) were homogenized in 1 ml TRIZOL® reagent (Invitrogen,
with a power homogenizer. Chloroform (200 µl, VWR, #22711324) was added to each homogenate and the mixture was capped securely and vigorously hand inverted for 15 seconds followed by incubation at room temperature for 5 mins. The homogenates were subsequently centrifuged at 13,000x rpm for 15 mins at 4°C. The mixture was separated into a lower red, phenol chloroform phase containing protein, an interphase containing DNA, and a colourless upper aqueous phase containing RNA. Only the RNA-containing upper aqueous phase was transferred to a set of fresh micro-centrifuge tubes, and mixed with 500 µl of isopropanol (Sigma-Aldrich, #I9516) before another centrifuge at 13,000x rpm for 20 mins at 4°C. The RNA precipitate formed a gel-like pellet on the bottom side of the tube after centrifugation. The supernatant was removed and the pellet was washed twice with 500 µl 75% ethanol (Merck, #1.07017.2511). The sample was then mixed by vortexing and centrifuged at 13,000x rpm for 5 mins at 4°C. The supernatant (ethanol) was removed and the RNA pellet was air dried. At the end of the procedure, the pellet was dissolved in Ambion® DEPC-treated water (Invitrogen, #AM9916, 100 µl for each sample) for RNA concentration determination.

**Measurement of RNA concentration**

RNA concentration was quantified by using a NanoDrop Spectrophotometer (Eppendorf Thermo Scientific, Australia) at an absorbance of 260/280 nm (A260/280). The NanoDrop Spectrophotometer was initialized by 1.0 µl of DEPC water, which was also used as a blank. Each RNA sample (1.0 µl) was loaded onto the sampling platform for the measurement of RNA concentration.
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 manufacturer’s instructions. RNA in each of samples was diluted to the same final concentration (1 µg/8 µl) by using DEPC water in a sterile 1.5 ml eppendorf tube on ice. To remove the original DNA in each sample, RNA and primer master mix was prepared (1 µg of RNA templates, 1 µl Dnase I reaction buffer and 1 µl Dnase I amplification grade) (Invitrogen, #11904-018) in a sterile 1.5 ml eppendorf tube and incubated at room temperature for 15 mins. 1 µl of 25 mM EDTA was then added and incubated for 10 mins at 65°C. RNA (2 µl from the above sample mix) and a reverse transcription master mix (1x reverse transcription buffer, 2x dNTP mix, 1x random primers, 1 µl reverse transcriptase (Invitrogen, #18064-014), DEPC water to a final volume of 18 µl and they were added to each reaction tube to give a final reaction volume of 20 µl. The tubes were then placed in a controlled-temperature heat block and first equilibrated at 25°C for 10 mins, then 37°C for 2 hours, then 85°C for 5 seconds and finally held 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 (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,
#170-8882), 500 nM primers forward 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 samples in a sterile 96-well plate. The plate was placed in a controlled-temperature heat block equilibrated at 50°C for 2 mins, 95°C for 3 mins 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). All reactions were performed on the iQ™ 5 Real-time PCR Detection System (Bio-Rad Laboratories Inc., USA). Primers used for specific genes are listed in Section 4.2.3 and 5.2.6. All the methods have been used in the previous publications from this laboratory [154, 161].

2.3.4.5 Tissue immunohistochemistry

Tissue processing

Mice liver tissues were placed in a tissue embedding cassette and kept in 10% netural formalin solution at 4°C overnight. The tissues were then transferred to 70% ethanol and were processed by a tissue processor (Leica, Wetzlar, Germany). The processed liver tissues were then embedded in paraffin wax prior to paraffin microtome sectioning. Embedded tissues were cut to a 5 µm thickness and were mounted on “+” marked slides (Thermo Fisher Scientific, Australia) with 4 sections per slides with 50 mm intervals between sections. Paraffin section slides were incubated at 37°C overnight prior to staining.

Hematoxylin and eosin (H & E) staining

Paraffin section slides were de-paraffinised by incubating with xylene (Grale Scientific Pty.
Ltd., #XL005/10) for 5 mins. This was followed by two 1 min washes in 100% ethanol and then repeatedly (5 times) submerging the sections into 70% ethanol. Slides were then placed into distilled water for 2 mins for rehydration. Slides were stained with Mayer’s haematoxylin (Grale Scientific Pty. Ltd., #MH-1L) for 2 mins and rinsed with tap water until the colour of dye change to blue. At this stage, nuclei were clearly stained, with nuclear details visible and there was no cytoplasmic staining. The slides were then briefly rinsed with tap water for 2 mins before the addition of 1% Aqueous eosin (Grale Scientific Pty. Ltd., #EOA-1L) for 90 seconds. Slides were then dehydrated by repeatedly submerging (10 times) first in 70% ethanol and then in 100% ethanol. The 100% ethanol step was then repeated to ensure that the slides were fully dehydrated. Finally, slides were immersed in xylene and mounted using a dibutylphthalate polystyrene xylene (DPX) mounting medium (Grale Scientific Pty. Ltd., #3197).

**Histological ballooning scoring criteria for liver cell injury**

To distinguish the steatohepatitis (a state characterised by the presence of hepatocyte injury) from simple steatosis, hepatocyte ballooning is the most important diagnostic criteria [162]. Hepatocyte ballooning occurs in many forms of hepatic and cholestatic liver diseases [163], resulting from intracellular fluid accumulation and other toxic cell injury. It is characterised by swelling of hepatocytes with rarefied cytoplasm. Evaluation of ballooned hepatocytes is limited to three categories (none, few, and many) (Table 8) [162, 164]. After H & E staining, each section was taken 6 photos at 20x magnification and scored in line with the double-blind
procedure. The total score of all the samples from the same group was summed up and calculated in the average score.

**Table 8. Scoring criteria of ballooning.**

<table>
<thead>
<tr>
<th>Item</th>
<th>Definition</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ballooning</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Few balloon cells</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Many cells/prominent ballooning</td>
<td>2</td>
</tr>
</tbody>
</table>

Ballooning classification: few indicates rare but definite ballooned hepatocytes as well as case that are diagnostically borderline. Based on Kleiner *et al.*, 2005 [164]. Examples are shown in Figure 15.

**Figure 15. Assessment and scoring of ballooning for liver injury.** The ballooning hepatocytes are the enlarged hepatocytes with a central nucleus surrounded by white cytoplasm. (A) Mild steatosis but no ballooning degeneration. This case was scored as “0” for ballooning injury; (B) Several scattered balloon cells that are not larger than the surrounding steatotic hepatocytes but with the same cytoplasmic characteristics as more obvious balloon cells seen in panel C. This case was scored as “1” ballooning injury; (C) A contiguous patch of hepatocytes showing prominent ballooning injury, sharply contrasted against the more normal hepatocytes in the field. This case was scored as “2” ballooning injury. These cells were examined by H & E staining and original 600x objective. Adapted from Kleiner *et al.*, 2005 [164].
2.4 STATISTICAL ANALYSIS

Throughout the thesis, results are presented as means ± SEM and analysed by Prism software.

For the comparison of only two groups, a Student’s t test was used. For the comparison of more than two groups, one-way analysis of variance (ANOVA) was performed followed by the Dunnett’s multiple comparisons test. Differences at p<0.05 were considered to be statistically significant.
CHAPTER 3
NOVEL METHOD FOR
TRIGLYCERIDE EXTRACTION
To identify the compounds with potential anti-diabetic properties by reducing lipid accumulation, a novel biochemical screening assay was developed using 3T3-L1 adipocytes and triglyceride content was assessed as readout of cellular lipids level. Potassium hydroxide (KOH) was used in this newly-developed assay to overcome the obstacles of conventional chloroform/methanol (C/M) method in triglyceride extraction. This greatly simplified the procedures and enhanced the efficiency of the assay as a relatively high-throughput screening tool. By using this assay, I have screened more than 200 candidates selected from Traditional Chinese Medicine after careful evaluation of the relevant indications and computer-based modelling. Out of these compounds, I have identified 76 hits from more than 10 different classes in terms of the reduction of triglyceride content by either >50% or >75% at tested concentrations. These results indicated that this approach is highly effective in identifying the potential candidates with 38% successful rate.

3.1 INTRODUCTION

As described previously (Chapter 1), excess lipid accumulation is a major cause of insulin resistance in type 2 diabetes and obesity [33, 61]. Compounds that eliminate excess lipid accumulation (in liver and muscle) can improve glucose homeostasis by reversing lipid-induced insulin resistance [86, 165]. Similarly, reducing lipid accumulation in adipose tissue can alleviate obesity [55]. The levels of triglycerides are commonly used as an indicator of intracellular accumulation of lipids [62, 63]. The first study of this project aimed to establish a phenotypic assay in cells to determine triglyceride accumulation as a
high-throughput screening tool for the identification of new anti-obese and anti-diabetic compounds. The rational for this functional assay was to identify compounds that act at various cellular targets in the upstream pathways of lipid metabolism which either increase fatty acid oxidation (energy expenditure) or inhibit de novo lipogenesis (Figure 16).

This new screening assay was based on using potassium hydroxide (KOH) due to its ability to lyse cells and hydrolyse triglyceride [166]. It also overcame the obstacles encountered with the conventional organic solvent extraction. After validation of this assay by a series of biochemical and pharmacological tests, I have used this assay to screen more than 200 candidates selected from >10 different classes of compounds derived from Traditional Chinese Medicine. Our results identified over 70 potential compounds with significant lipid-lowering effects. These findings provide strong support for the application of this screening assay for the discovery of novel anti-diabetic and anti-obese drugs.
Figure 16. Illustration of the concept for screening compounds on lipid accumulation to identify anti-obese or anti-diabetic drugs. Reducing ectopic lipid accumulation can reverse insulin resistance in the liver and muscle and can alleviate obesity in adipose tissue. The design of the study was establish a novel biochemical assay as a high-throughput screening tool to determine intracellular triglyceride levels, capable of capturing lipid-lowering compounds derived from our unique Tradition Chinese Medicine compounds library via different mechanisms [167].
3.2 EXPERIMENTAL DESIGN AND METHODS

3.2.1 Reagents, Chemicals and Test Compounds

Insulin, dexamethasone, DMSO, biotin, IBMX, 5-aminoimidazole-4-carboxamide
1-β-D-ribofuranoside (AICAR, Sigma-Aldrich, #A9978), berberine (BBR, Sigma-Aldrich, #B3251), oleanolic acid (OA), arctigenin (ATG, Sigma-Aldrich, #SMB00075), betulin (Sigma-Aldrich, #B9757), C75 (Sigma-Aldrich, #C5490), cerulenin (Sigma-Aldrich, #C2389), 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA, Sigma-Aldrich, #T6575), rosiglitazone (RSG, Sigma-Aldrich, #R2408), fructose (Sigma-Aldrich, #F0127), genipin (≥98.0%, Sigma-Aldrich, #G4796), bile acids including cholic acid (Sigma-Aldrich, #C1129), deoxycholic acid (Sigma-Aldrich, #D4297) and chenodeoxycholic acid (CDCA, Sigma-Aldrich, #614122) were used in this study.

The test compounds were kindly supplied via or by Prof. Lihong Hu and Prof. Yang Ye of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China) and Prof. Jun Xu of the School of Pharmaceutical Sciences, Sun Yat-sen University (Guangzhou, China). Specifically, albiflorin (98%) was obtained from Baoding Yunxin Trade (Hebei, China) and oxymatrine (98%) from Hangzhou Tianlong Biotechnology (Hangzhou, China). Tiliroside (>98%) was obtained from Meryer Chemical Technology (Shanghai, China). The derivatives of albiflorin, oxymatrine, berberine, genipins, tiliroside, endoplasmic reticulum (ER) stress inhibitors, genipin and danshen were developed at Shanghai Institute of Materia Medica by modifying their chemical structures. Triterpenoids were obtained from the purification of the
extracts of bitter melon as we recently described [165]. Hispidin derivatives, farnesoid X receptor (FXR) agonists and other bile acid analogues were developed at Sun Yat-sen University by Prof. Jun Xu. Other test compounds were obtained from the chemical library of Sun Yat-sen University after computer-based virtual screening against selected targets involved in lipid metabolism [168].

To improve the likelihood of success and to minimize the numbers for screening, we selected the candidates with a wide range of chemical diversity by one of the two following virtual screening processes [142, 165, 168]. The first virtual screening was based on careful evaluation of documented (or reported) effects implicated for diabetes or related conditions. Our recent identification of BBR and its derivatives from *Golden Seal* [142] and cucurbitane triterpenoids from *Bitter Melon* [165] was achieved by using this approach. The second process involved computer modelling of possible binding test compounds to cellular targets, such as the FXR (a regulator of lipid metabolism [169]), using the software available to us by Prof. Jun Xu. Similar approach was used to identify compounds targeting the liver X receptor (LXR) [168].

### 3.2.2 Cell Culture

#### 3.2.2.1 Differentiation of 3T3-L1 pre-adipocytes

3T3-L1 fibroblasts undergo a pre-adipose to adipose-like conversion as they progress from rapidly dividing to a confluent and contact inhibited state (Figure 12). The 3T3-L1 pre-adipocytes were seeded into 48- or 96-well microplates and allowed to reach 100%
confluence. They were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PSG at 37°C in 5% CO₂. To induce adipocytes differentiation (Figure 12), 2-day post-confluent cells were incubated in the differentiation medium (DMEM containing 10% FBS, 1% PSG, 2 µg/ml insulin, 100 ng/ml dexamethasone, 500 µM IBMX and 10 ng/ml biotin) for 3 days before switching to the post-differentiation medium (DMEM with 10% FBS, 1% PSG and 2 µg/ml insulin) for additional 3 days (Table 5).

3.2.2.2 Compounds treatment in 3T3-L1 cells

To assess effects of compounds on lipid accumulation, the test compounds were dissolved in 100% DMSO and supplemented at the concentrations indicated in the figure throughout the course of the differentiation process. Test compounds were made as stocking solutions (1000x) and stored at -20ºC in aliquots. They were added on day 1 in the differentiation medium (for 3 days) and replaced with another aliquot in post-differentiation medium on day 4 (for another 3 days) (Figure 13). 3T3-L1 cells cultured in DMEM supplemented with 0.1% DMSO were used as a vehicle control for all experiments. Cells were monitored and photographed using the Nikon TS100F inverted microscope and camera (Nikon Instruments Inc., USA).

3.2.3 Potassium Hydroxide-based Triglyceride Extraction

This newly-developed screening assay was based on using potassium hydroxide (KOH) due to its ability to lyse cells and hydrolyse triglyceride [166]. In parallel, triglycerides were extracted by the classic chloroform/methanol (C/M) mixture compare with the novel KOH-based method. After cells were treated with compounds for 6 days, triglyceride content
from cells were extracted and determined as described in Section 2.2.4.1.

To evaluate the influence of potassium hydroxide (KOH) on the triglyceride enzymatic assay, the triglyceride assay was performed in the presence of 50 mM KOH (used for triglyceride extraction) at various concentration of intralipid (Sigma-Aldrich, #I141) as a triglyceride donor [66, 170]. The recovery rate of triglyceride extraction from 3T3-L1 cells was assessed by adding known amount of triglyceride from the intralipid before the extraction either by KOH or the C/M mixture (Section 2.2.4.1).

3.2.4 Oil red O Staining

Cells were stained with oil red O solution after 6-day treatment of individual compound. The detailed methods are described in Section 2.2.4.2.

3.2.5 Lactate Dehydrogenase Release Determination

After cells were treated with compounds for 6 days, the cell medium and lysate were collected to measure the activity of lactate dehydrogenase (LDH) released into the culture medium from cells. The detailed methods are described in Section 2.2.4.3.

3.2.6 Statistical Analysis

Data were presented as means ± SEM and analysed by Prism software. Unpaired student test was performed for comparison of relevant groups. Pearson’s two-sided correlation was used for correlation calculation. Differences at p<0.05 were considered to be statistically significant.
3.3 RESULTS

3.3.1 Validation of the Novel Method for Triglyceride Extraction

The conventional method for the extraction of triglyceride from tissues with organic solvents requires a series of steps including the separation of organic and aqueous phases [149]. A one-step method was designed to extract triglyceride with the use of KOH based on its ability to lyse cells and hydrolyse triglyceride [166] (Figure 17).

Figure 17. Design of KOH extraction for biochemical assay of triglyceride content in 3T3-L1 cells for high-throughput drug screening. (A) Oil red O (ORO) staining for the quantification of cell triglyceride (TG) content. Multiple procedures involved in the ORO method which usually required two days. (B) Biochemical determination of TG using the conventional extraction with chloroform/methanol (C/M). Multiple steps involved including separation, drying and reconstitution which usually requires one full day. (C) Designed method for TG extraction and cell lysis with KOH. This new method intended to avoid multiple steps, shorten the time to ~2 hours and allow miniaturization of the biochemical assay of TG for high-throughput drug screening.

To test whether or not KOH may influence the triglyceride determination biochemical assay, a comparision experiment was performed. Figure 18 shows that at the concentration (50 mM) used for triglyceride extraction, KOH did not have any significant influence on the
quantification of triglyceride extraction assay. The obtained results in the presence of KOH were almost identical to those obtained in the absence of KOH as indicated by a high degree of linear correlation (\(Y = 1.1416 \times R^2 = 0.9978, p < 0.001\)) (Figure 18).

**Figure 18. Influence of KOH in cell-free biochemical assay per se.** Various concentrations of triglyceride (TG) from intralipid were measured with an enzyme-based TG kit under cell-free conditions in the presence or absence of KOH (50 mM).

I next examined whether KOH could extract a similar amount of triglycerides from cells, by adding 1.0 or 2.5 µg triglyceride (50%~100% of triglyceride from 3T3-L1 adipocytes) to a cell culture plate for 5 mins. As shown in Figure 19, the recovery rates of TGs extracted with KOH at these two concentrations of added triglyceride were 87.8% ± 4.6% and 95.1% ± 0.5%, respectively. These recovery rates were approximately four times higher of those obtained by C/M extraction (12.1% ± 8.9% and 18.0% ± 8.4%, respectively, p < 0.001). Furthermore, approximately 90% of added triglyceride was recovered with KOH extraction method (0.88 ± 0.05 µg from 1.0 µg and 2.4 ± 0.01 µg from 2.5 µg) (Figure 19).
Figure 19. Validation of the KOH extraction method for triglyceride measurement. Extraction of triglyceride (TG) from 3T3-L1 pre-adipocytes using KOH as compared with the chloroform/methanol (C/M) solvent. 3T3-L1 cells were seeded into a 12 well-plate and cultured in DMEM containing 10% FBS and 1% PSG. Two days after 100% confluence, cell medium was removed and washed twice with ice-cold 1x PBS. After cells were dried, intralipid was added for 5 mins. TG was then extracted using KOH (50 mM) or C/M (2:1) mixture methods. The recovery rate of TG extraction was calculated from values obtained in the presence or absence of TG (1.0 or 2.5 µg from intralipid). Data are means ± SEM from three independent experiments. †† p < 0.01 KOH vs. C/M extraction method.

3.3.2 Validation of the Novel Extraction Method by Using Pharmacologic Agents Known to Alter Triglyceride Levels

In order to verify the ability of this new assay as an endpoint measurement able to capture the effects on lipid accumulation induced by different upstream mechanisms, we tested a number of pharmacological agents well known to promote fatty acid oxidation, inhibit or enhance lipogenesis in our newly-developed assay system. As expected, the AMPK activators AICAR [171], BBR [142], oleanolic acid (OA) [165], and arctigenin (ATG) [172] all reduced the
triglyceride levels significantly (p < 0.01) at 1 µM and/or 10 µM (Figure 20). Similar results were observed after the incubation of 3T3-L1 cells with C75 and cerulenin (specific FAS inhibitors [130]), betulin (specific SREBP-1c inhibitor [131]), and TOFA (specific ACC inhibitor [130]) (all p < 0.05, Figure 20). In contrast, incubation with 20 mM fructose (Fru, lipogenic substrate [173]) or 1 µM and 10 µM rosiglitazone (RSG, specific PPARγ agonist [174]) increased triglycerides accumulation by more than 150% (p < 0.05 for both, Figure 20). Detailed examination revealed a dose-dependent efficacy for these agents (Figure 21).

Figure 20. Assessment of the ability of the new screening assay to detect intracellular lipid accumulation. 3T3-L1 cells were seeded into a 48 well-plate and cultured in DMEM. Two days after reaching 100% confluence, cells were incubated with differentiation medium (DMEM containing 10% FBS, 1% PSG, 2 µg/ml insulin, 100 ng/ml dexamethasone, 500 µM IBMX and 10 ng/ml biotin) for 3 days. Differentiated cells were cultured for additional 3 days in DMEM with 2 µg/ml insulin (post-differentiation medium) with modulators affecting lipogenesis and/or lipolysis. These lipogenesis/lipolysis modulators included i) AMPK activators - AICAR (AIC, 500 µM), berberine (BBR), oleanolic acid (OA) and arctigenin (ATG); ii) lipogenic inhibitors - betulin (SREBP-1 inhibitor), C75 (FAS inhibitor), cerulenin
(Ceru, FAS inhibitor), TOFA (ACC inhibitor); and iii) lipogenic enhancers - rosiglitazone (RSG, PPARγ agonist) and fructose (Fru, 20 mM). Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. Data are means ± SE from three independent experiments. † p < 0.05, †† p < 0.01 vs Veh.

Figure 21. Dose-response effects of representative compounds on lipid accumulation. 3T3-L1 pre-adipocytes were differentiated and treated with compounds (BBR, Betulin, C75,
TOFA and RSG) with different concentrations (0.01~10 µM in X axis) for 6 days. Triglyceride levels were measured using validated KOH method. Data are presented as means ± SEM from three independent experiments and for each of experiment triplicates were performed.

To exclude a possibility that the decrease in triglycerides content was resulting from cytotoxic effect of the compounds, we determined LDH activity in the cultured medium, as an indicator of cell damage. All agents caused no significant elevation of LDH levels at 1 µM concentration with significant reduction of triglyceride. At 10 µM, betulin and BBR have moderate elevation of LDH activity (Figure 22). Consistent with the results of LDH, the cells displayed a normal morphology in response to these treatments (Figure 23).

**Figure 22. Assessment of lactate dehydrogenase release for compounds acting at different sites of lipid metabolism.** The cells medium and lysates were collected 6 days after the incubation with test compounds. Lactate dehydrogenase (LDH) release was determined by its activity in the medium relative to the cell lysate and corrected with the vehicle (Veh) control. Quantified results by measuring the optical density of LDH release after the incubation with lipogenesis/lipolysis modulators. Data are means ± SEM from three
independent experiments. †† p < 0.01 vs Veh.

Figure 23. Representative images of differentiated 3T3-L1 cells treated with different compounds. 3T3-L1 pre-adipocytes were differentiated and treated with compounds (BBR, Betulin, C75, cerulenin (Ceru), TOFA, Rosiglitazone (RSG) and Fructose (Fru)) with different concentrations (1 and 10 μM) for 6 days. Cells were photographed using the Nikon TS100F inverted microscope and camera under 10x objective.

3.3.3 Comparison with Results Obtained from Oil Red O Staining

Oil red O (ORO) staining is widely used to screen the changes in lipid content in cultured cells [150]. We compared the values of intracellular lipid content obtained from KOH
extraction method with the semi-quantitative ORO method. As shown in Figure 24, the ORO staining method confirmed all the responses we detected using our new screening assay. The obtained values of triglyceride were all significantly reduced by BBR, lipogenesis inhibitors betulin, C75, cerulenin and TOFA (>40%). As expected, ORO staining was increased by fructose and rosiglitazone (>130%) under the experimental conditions (all $p < 0.01$). The triglyceride results obtained from the new triglyceride assay and ORO staining were significantly correlated ($Y = 1.0906X, R^2 = 0.9032, p < 0.001$) (Figure 25).

![Graph showing ORO density results](image)

**Figure 24. Comparison with results obtained from oil red O staining method.** 3T3-L1 cells were cultured and incubated under the same conditions as described in the legend of Figure 20. At the end of the experiment, cells were stained with oil red O (ORO) for the quantification of triglycerides content. Quantified results by measuring the optical density of ORO stained cells after the incubation with lipogenesis/lipolysis modulators. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. †† $p < 0.01$ vs Veh.
Figure 25. Correlation of KOH method vs. oil red O staining method. Correlation of the results from the new assay using the new KOH-enzyme method versus the oil red O (ORO) staining is presented. Data are means ± SEM from three independent experiments.

3.3.4 Screening Compounds Altering Triglyceride Accumulation in 3T3-L1 Adipocytes

Following the biochemical and pharmacological validations as described above, we applied this new screening assay in 3T3-L1 cells cultured in 48-well plates in an attempt to identify potential lipid-lowering compounds from Traditional Chinese Medicine. Among more than 200 candidates selected for the screening, we detected 76 compounds with efficacy in reducing triglyceride accumulation by > 50% at 1 µM or > 75% at 10 µM. These compounds belong to several classes of molecules with different core chemical structures, including derivatives of albiflorin (Figure 26), tiliroside (Figure 26), bile acid analogues (Figure 27), endoplasmic reticulum (ER) stress inhibitors (Figure 28), genipin (Figure 29), BBR (Figure 30), oxymatrine (Figure 31), hispidin derivatives (Figure 32), danshen derivatives (Figure 33), triterpenoids (Figure 34), FXR agonists (Figure 35), and other compounds belongs to different classes (Figure 36).
**Albiflorin** (an analogue of paeoniflorin) has been suggested as a key ingredient of *Paeonia Radix* which has been shown to exert acute effect in reducing blood glucose in diabetic mice [175] as described in Section 4.1.1.

**Tiliroside** is a glycosidic flavonoid, extracted from several dietary plants, such as rose hips, strawberry and raspberry. Tiliroside has been reported it possesses anti-inflammatory [176], anti-oxidative [177], hepato-protective [178], anti-obese and anti-diabetic [177] activities.

![Figure 26. Albiflorins and tilirosides identified from the new high-throughput screening.](image)

3T3-L1 cells were differentiated and treated with test compounds at 1 and 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were used as vehicle (Veh) control. The effects of albiflorins and tilirosides on triglyceride levels as well as their core chemical structures are presented respectively. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 1 hit out of total 3 compounds from albiflorins class was identified. 2 hits out of total 6 compounds from tilirosides class were identified.
Bile acids are steroid acids and are major cholesterol metabolites synthesised in the liver and released into the small intestine postprandial. Bile acids play important roles in the regulation of cholesterol homeostasis via cholesterol degradation. Recent studies have indicated that bile acid homeostasis is altered in type 2 diabetes [179]. Bile acids also activate the farnesoid X receptor (FXR) and play a major role in regulating lipid, glucose, and energy metabolism [180].

Figure 27. Bile acid analogues identified from new high-throughput screening. 3T3-L1 cells were differentiated and treated with test compounds at 1 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of bile acid analogues on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. No hit was identified.
**Endoplasmic reticulum (ER)** serves a variety of functions in the cells. Disruption of ER, termed ER stress, leads to the accumulation of misfolded proteins in the ER [181]. ER stress has been proposed as a key mediator of lipogenesis, inflammation and insulin resistance in the liver [182, 183]. Several studies have been reported a closely relationship between ER stress and diabetes [181, 184]. Mechanistic studies have revealed that ER stress promotes a JNK-dependent serine phosphorylation of IRS-1 and inhibit insulin action in cultured liver cells [185]. Activation of key molecules of ER stress signalling has also been shown to enhance lipogenesis and alleviate hepatic steatosis and insulin resistance [186].

![Figure 28](image.png)

**Figure 28.** ER stress inhibitors identified from the new high-throughput screening. 3T3-L1 cells were differentiated and treated with test compounds at 1 and or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of ER stress inhibitors on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. No hit was identified in this class.
Genipin is an aglycon of geniposide and a natural product presenting in the fruit of Gardenia jasminoides [187]. Genipin exhibits diverse pharmacological functions such as anti-tumour [188], anti-inflammation [189], immunosuppression [190] and anti-sepsis [191]. Moreover, it has been reported genipin has beneficial effects on cardiovascular system [192]. Several studies have also shown the protective effects of genipin on the treatment of type 2 diabetes [193, 194].

![Figure 29. Genipins derivatives identified from the new high-throughput screening.](image)

3T3-L1 cells were differentiated and treated with test compounds at 1 and or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of genipins class compounds on triglyceride levels as well as their core chemical structure are presented respectively. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 6 hits out of total 7 compounds were identified.
**Berberine** (BBR) is an alkaloid and can be found in several plants, such as *Coptis chinensis* (Huang Lian in Chinese). In China, BBR is commonly used as a non-prescription oral medication to treat gut infections and diarrhoea with few side effects. Recently, BBR has been reported to have beneficial effects for the treatment of type 2 diabetes and dyslipidaemia [195] in humans. Further investigations have revealed that BBR activates AMPK via inhibition of respiratory complex I of the mitochondrion [141, 142].
Chapter Three – Novel Triglyceride Extraction Method
Figure 30. BBR and its derivatives identified from the new high-throughput screening. 3T3-L1 cells were differentiated and treated with test compounds at 1 and 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh.) control. (A-F) The effects of BBR and its derivatives on triglyceride level; (G) The core chemical structure of presented compounds. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 28 hits out of 84 total compounds are identified.
**Oxymatrine** (OM) is also an alkaloid and is rich in *Sophora flavescens*. Oxymatrine has been reported several pharmacological effects, including anti-tumour [196] and anti-inflammatory effects [197]. It is used in humans as a safe oral drug in treatment of hepatic fibrosis [198]. Additionally, it has been suggested oxymatrine may affect lipid metabolism [199].

![Diagram of oxymatrine derivatives](image)

**Figure 31. Oxymatrine derivatives identified from the new high-throughput screening.** 3T3-L1 cells were differentiated and treated with oxymatrine and its derivatives at 1 and or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of oxymatrine and its derivatives on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 8 hits out of 18 total compounds are identified.
Hispidin can be found in mushroom *Inonotus xeranticus* [200] or *Phellinus* [201]. It can be also synthesised [202]. Hispidin has been showed its potential effects on anti-oxidation [200, 201], anti-cancer [202] and anti-hepatotoxicity [203].

**Figure 32. Hispidin derivatives identified from the new high-throughput screening.** 3T3-L1 cells were differentiated and treated with hispidin and its derivatives at 1 and or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of hispidin and its derivatives on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 2 hits out of 12 total compounds were identified.
Danshen (Salvia miltiorrhiza) is isolated from the root of genus Salvia. In China, it has been used in clinic for the treatment of cardiovascular [204], kidney injury [205] and hyperlipidaemia [206].

Figure 33. Danshen derivatives identified from the new high-throughput screening. 3T3-L1 cells were differentiated and treated with danshen and its derivatives compounds at 1 and or 10 μM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of danshen and its derivatives on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 1 hit out of total 10 compounds was identified.
Triterpenoids are saponin compounds and can be derived from many plants [207]. They possess interesting pharmacological properties, including the anti-oxidative, microbicide, anti-diabetic, anti-inflammatory, hypolipidaemic and anti-atherosclerotic actions [165, 208, 209] as describe in Section 5.1.1.
Figure 34. Tetracyclic and pentacyclic triterpenoids identified from new high-throughput screening. 3T3-L1 cells were differentiated and treated with test compounds at 1 and or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. The effects of triterpenoids and its derivatives (41-57) on triglyceride levels (A-C) as well as their core chemical structure (D) are presented. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 5 hits out of total 26 compounds were identified.
**Farnesoid X receptor** (FXR), one of bile acid receptors, can maintain bile acid homeostasis by regulating the expression of key enzymes in bile acid synthesis and transport in the liver and intestine [210]. It can also control lipid metabolism [211], regulates glucose homeostasis and insulin sensitivity [212, 213]. The expression of FXR is decreased in the liver of diabetic animal and is regulated by insulin and glucose in hepatocytes [214].

**Figure 35. FXR agonists identified from new high-throughput screening.** 3T3-L1 cells were differentiated and treated with test compounds at 1 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of FXR agonists on triglyceride levels as well as their core chemical structure are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 3 hits out of total 16 compounds were identified.
Figure 36. **Other classes compounds identified from new high-throughput screening.** 3T3-L1 cells were differentiated and treated with test compounds at 1 µM or 10 µM. Berberine (BBR) or rosiglitazone (RSG) were used as the standard (STD) positive and negative controls. Fully differentiated 3T3-L1 cells were as vehicle (Veh) control. The effects of compounds on triglyceride levels are presented. Data are means ± SEM from one independent experiment and for each of experiment triplicates were performed. 20 hits out of total 42 compounds were identified.
3.4 DISCUSSION

Excess accumulation of lipids in cells is an indicator of obesity and insulin resistance [33, 61]. Reduction of elevated cellular lipid levels is an effective approach for the treatment of these pathological conditions [66, 170]. Based on this concept, we established a biochemical assay to determine cellular triglyceride levels as a targeted high-throughput screening tool by designing a new lipid extraction method in order to identify compounds with potential efficacy for obesity and associated metabolic diseases.

Oil red O staining has been commonly used in adipocytes to screen the efficacy on lipid content [150], particular for drugs promoting adipocytes differentiation such as PPARγ agonists [174]. However, this method involves multiple processes which are time consuming and can be labour intensive when used at a large scale. Furthermore, there are concerns about the low specificity and the inaccuracy of the results obtained from the oil red O staining [150]. Therefore, it would be advantageous to develop a high-throughput screening for the cell triglyceride levels based on a biochemical assay of triglyceride, which is widely used for in plasma and tissue samples. To overcome the technical obstacles in applying the biochemical assay for high-throughput screening, we designed a method of triglyceride extraction using KOH based on its ability to lyse cells and hydrolyse triglycerides [166]. This new method provides advantages over the organic extraction that requires multiple steps, allowing the biochemical assay performed in a micro-plate for high-throughput drug screening. Importantly, KOH extraction produced an almost full recovery of triglyceride (88%~95%), which is far
more superior to the 12%~18% of recovery obtained by the C/M method. Our new procedure also substantially improves the efficiency of whole triglyceride assay (approx. 2 hours) compared to the oil red O staining and the C/M extraction method, which usually require much longer time (Table 9).

**Table 9. Comparison of conventional methods and novel established method.**

<table>
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<th>Methods</th>
<th>Step(s)</th>
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<th>Recovery rate</th>
<th>High-throughput</th>
<th>Targeted</th>
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<td>yes</td>
<td>-</td>
<td>not suitable</td>
<td>no</td>
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<tr>
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<td>high</td>
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</table>

For the quantification of cell triglyceride content, 1) Oil red O (ORO) staining method requires multiple procedures which usually required two days and is not suitable for high-throughput screening; 2) Chloroform/methanol (C/M) involves multiple steps which usually requires one full day and is not suitable for high-throughput screening with low recovery rate; 3) The novel designed method with KOH avoids multiple steps, shorten the time to ~2 hours and allow for high-throughput screening with high recovery rate. This method is more targeted than others.

Cellular triglyceride level can be regulated by either the lipogenic and fatty acid oxidative pathways. The assay established with the new extract method was able to capture the expected effects of well-defined pharmacological agents in lipid metabolism. Among them, AICAR, BBR, bitter melon triterpenoids and ATG have been demonstrated to activate AMPK in previous studies including ours [142, 165, 171, 172]. Activated AMPK can reduce cellular lipid accumulation by promoting fatty acid oxidation [215] and inhibiting lipogenesis [216].
Decreased triglyceride was also evident, as predicted, in the presence of lipogenic inhibitors acting at the site of SREBP-1c (botulin [131]), ACC (TOFA [130]) or FAS (C75 and cerulenin [130]). Furthermore, our results also showed that our screening system was sensitive in detecting increased triglyceride accumulation in response to lipogenic substrates (indicated by fructose [173]) and PPARγ agonists (indicated by RSG [174]). These results verify the suitability of this high-throughput screening to capture potential anti-diabetic drugs which alter lipid metabolisms by acting at different cellular sites.

Recently, our group has identified two anti-diabetic compounds such as BBR and cucurbitane triterpenoids from the plants used in Traditional Chinese Medicine for the treatment of diabetes and related conditions [142, 165]. Compared with a random screening, natural products from Traditional Chinese Medicine has been a much more fruitful resource as it allows us to reach vast available bibliography and perform careful analysis [142, 165], as well as computer analysis [168] to reduce the large numbers of compounds to a handful of highly selected candidates for biological screening. Using this unique and targeted approach, we selected candidates representing a wide range of chemical diversity (for new discovery) or different structures of the same class of compounds (e.g. BBR derivatives) for the screening of novel anti-diabetic drugs using the method established in this study. Apart from obtaining detailed information for the new derivatives from BBR and triterpenoids which would allow for detailed structure-activity analysis, our results also confirmed a number of compounds which have been suggested to affect lipid metabolism including tiliroside[177], oxymatrine
and FXR agonists [168]. Collectively, these data demonstrate that the screening of the efficacy on cellular lipid accumulation is an effective tool to identify novel compounds with anti-diabetic properties.

With this newly-developed screening tool, I have screened more than 200 compounds selected from two Traditional Chinese Medicine banks based on extensive evaluation of Traditional Chinese Medicine database [142, 165] and/or computer modelling[217] [168]. Among them, 76 showed an efficacy in reducing triglyceride by > 50% at 1 μM or > 75% at 10 μM, which we regarded as identified hits (Figure 37).

In summary, the present study established a new phenotypic assay to determine triglyceride accumulation in 3T3-L1 cells as a reliable high-throughput screening tool capable of identifying of novel drugs with anti-obese and anti-diabetic properties by reducing lipid accumulation via different mechanisms. As illustrated in Figure 16, these results provide the proof of concept for the use of this approach which could be extended to other cell types (such as HepG2 hepatocytes or L6 myocytes) to examine the effect on cell-specific lipid metabolism. Also, it serves as a base for further investigation of the effect on fatty acid oxidation, uptake, synthesis and transport.
Figure 37. Overview of compounds identified (namely hits) from different classes. A hit is defined as a compound with the efficacy in lowering triglycerides for > 50% at 1 μM or > 75% at 10 μM. Results were obtained from three independent experiments.
CHAPTER 4

ASSESSMENT OF METABOLIC EFFECTS OF ALBIFLORIN IN VIVO
Albiflorin (AF) has been implicated as containing potential anti-diabetic properties. In the first animal study, I investigated the efficacy of albiflorin in insulin resistant mice induced by high-fat/high-fat cholesterol diet, a commonly used animal model which resembles lipid-induced insulin resistance in humans. Administration of albiflorin significantly reduced triglyceride and cholesterol levels in the liver of high-fat cholesterol fed mice. Interestingly, further examinations of the liver samples showed significant reduction in the mRNA expression of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) by the treatment of albiflorin. These results suggest that albiflorin may have potential therapeutics for hepatic steatosis and associated liver metabolic conditions, such as non-alcoholic steatohepatitis (NASH). To further test the effects of albiflorin on insulin resistance, we examined its efficacy in a more established insulin resistant mice model induced by high-fat diet. Albiflorin-treated mice significantly improved glucose intolerance in insulin resistant mice. However, further studies are required with more sensitive and reliable technique, such as hyperinsulinaemic-euglycaemic clamp with glucose tracers, to confirm its therapeutic potential for insulin resistance.

4.1 INTRODUCTION

Chapter 3 demonstrated that we have established a useful cellular triglyceride-based high-throughput screening assay that captured the lipid lowering efficacy of compounds acting at different pathways of lipid metabolisms. Albiflorin significantly reduced triglyceride levels in fully differentiated 3T3-L1 cells (Figure 26). Based on this screening result along
with the analysis for its drug-like properties mentioned in Section 1.5.2, albiflorin may have potential efficacy on the treatment for obesity or type 2 diabetes. Thus, I investigated whether albiflorin have anti-obese or anti-diabetic effects in vivo by using high-fat fed mice, a well-recognised animal model of insulin resistance.

4.1.1 Albiflorin

Albiflorin is a major component in herbal remedies such as *Paeoniae Radix* (Chi Shao Yao in Chinese). Among the ingredients of *Paeoniae Radix*, monoterpenoid glucosides are usually considered to be the main bioactive compounds and albiflorin is one of the main bioactive monoterpenoid glucosides that contains an unique cage-like pinane skeleton [218] (Figure 38).

![Figure 38. Structure of albiflorin. Albiflorin is a monoterpene glucoside containing a unique cage-like pinane skeleton. Albiflorin has a good absorption property and pharmacokinetics in vivo [219]. Albiflorin has been approved to use clinically in Traditional Chinese Medicine for its beneficial pharmacological actions with little adverse effects [220], such as anti-biotic, anti-inflammatory [221-223], anti-oxidant [224], anti-allergic [225], anti-thrombosis [226], treatment of liver injury [227] and analgesic [228].

In terms of the anti-diabetic actions, albiflorin analogue has been reported to lower blood
glucose in hyperglycaemic mouse models without increasing pancreatic insulin secretion [229]. It also exerts effects on kidney protection [230] and acute effect in reducing blood glucose [175] in diabetic animals. These effects have been proposed be related to the inhibition of glucose absorption [231], stimulation of peripheral glucose uptake [229], and the reduction of hyperlipidaemia [232]. However, the mechanisms of albiflorin action in reducing lipid accumulation in the liver have not been investigated. Therefore, the present study examined the effect of albiflorin on hyperglycaemia, hyperinsulinaemia and hepatic hyperlipidaemia in insulin resistant mice. We also further investigated its possible action on inflammatory pathway.

### 4.1.2 Insulin Resistance Mice Induced by High-fat or High-fat Cholesterol Feeding

Type 2 diabetes is characterised by insulin resistance and the inability of the β-cells to produce sufficient insulin compensate insulin resistance [47]. Therefore, animal models of type 2 diabetes tend to include models of insulin resistance and/or models of β-cells failure. A large body of evidence related to the mechanisms of diabetes and obesity has been derived from mouse studies [151]. Many animal models of type 2 diabetes are obese, reflecting the human condition where obesity is closely linked to the development of type 2 diabetes. Obesity in rodents can be the result of naturally occurring mutations or genetic manipulation. Alternatively, obesity can be induced by lifestyle intervention such as high-fat or high-fat cholesterol feeding.
High-fat feeding in rodents is a widely used model of obesity, dyslipidaemia and insulin resistance for many decades although this model does not induce diabetes or diabetic complications as observed in humans. Chronic high-fat feeding in rats and mice increased body weight compared to standard chow-fed mice [233, 234]. High-fat feeding in mice induced lipid accumulation in the muscle and liver eventually leading to hyperglycaemia and impaired glucose tolerance [152, 235].

Cholesterol accumulated within lipid droplets increases with the proportion of triglyceride content [236]. It has been showed that addition of cholesterol to a high-fat diet could aggravate insulin resistance, dyslipidaemia, inflammation and atherosclerosis in mice [237, 238]. Recent reports proposed that dietary cholesterol is a critical factor in the development of hepatic steatosis and progression to steatohepatitis in animal models [239]. Further investigations showed that high-fat plus cholesterol diet induced greater body weight gain, hepatic lipid accumulation, plasma levels of liver enzymes, adipose tissue inflammation and histological fibrosing streatohepatitis compared to the high-fat alone diet [151].

4.2 MATERIALS AND METHODS

4.2.1 Animal Models

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-hour light/dark cycle with free access to food and water. All animal experiments were approved by the Animal Ethics Committee of RMIT University (Approval No. #1208)
following the guidelines issued by the National Health and Medical Research Council of Australia.

4.2.1.1 High-fat cholesterol feeding induced insulin resistant mice model

After one week of acclimatisation, animals were randomly assigned to receive a standard lab chow (CH) diet or high-fat cholesterol (HFC) diet for 9 weeks to generate insulin resistance [157, 237]. Animals in HFC group were then fed for 4 weeks with or without albiflorin (AF, 100 mg/kg/day in HFC diet) (Figure 39). Body weight and food intake were monitored on weekly basis.

![Figure 39. Study design of albiflorin treatment in high-fat cholesterol induced insulin resistant mice model.](image)

Insulin resistant mice model was induced by 9-week high-fat cholesterol feeding. One week before albiflorin (AF) treatment (week 8), after 1 week (week 10) and 3 weeks (week 12) of AF treatment (100 mg/kg/day, in high-fat diet) in HFC-fed mice, glucose tolerance test (glucose load 1.5 g/kg bodyweight, i.p.) was performed after 5~7 hours fasting.

4.2.1.2 High-fat feeding induced insulin resistant mice model

After one week acclimatisation, animals were randomly assigned to receive either a standard
chow (CH) or high-fat (HF) diet to generate insulin resistance [165, 240]. Animals were fed for 8 weeks with or without 100 mg/kg/day albiflorin within the diet for the final 2 weeks (Figure 40).

Figure 40. Study design of albiflorin treatment in high-fat diet induced insulin resistant mice model. Insulin resistant mice model was induced by 8-week high-fat feeding with or without albiflorin (AF) for the final 2 weeks. AF (100 mg/kg/day) was mixed in high-fat diet. Glucose tolerance test (glucose load 2.0 g/kg bodyweight, *i.p.*) was performed after 5~7 hours fasting on week 7.

4.2.2 Measurements of Plasma Parameters

Plasma glucose, insulin, triglycerides and cholesterol level were determined following the assay methods described in Section 2.3.3.

4.2.3 Quantitative Real-time PCR

Total RNA was extracted from liver tissues as described in Section 2.3.4.4. Real-time PCR was carried out using the IQ SYBR Green Supermix for tumor necrosis factor alpha (TNFα) (Genework, Australia). The gene expression from each sample was analyzed in duplicates and normalised against the housekeeper gene 18S. The primer sequences (5’ to 3’) of 18S were:
CGCCGCTAGAGGTAAATTCT (sense) and CGAACCTCCGACTTTCGTCT (antisense); TNFα: CACAAGATGCTGGACAGTGA (sense) and TCCTTGATGGGACGTGATGA (antisense). All reactions were performed on the iQ™ 5 Real-time PCR Detection System (Bio-Rad Laboratories Inc., USA).

4.2.4 Statistical Analysis

Data were presented as means ± SEM. Unpaired student test was performed for comparison of relevant groups. Pearson’s two-sided correlation was used for correlation calculation. Differences at p<0.05 were considered to be statistically significant.

4.3 RESULTS

4.3.1 Effects of Albiflorin on Body Weight and Food Intake in HFC-fed Mice

To examine the effects of albiflorin, an insulin resistant mice model by high-fat cholesterol (HFC) feeding for 9 weeks was induced. After establishing an insulin resistant mice model, half of the HFC-fed mice were received albiflorin (HFC-AF, at a dosage of 100 mg/kg/day in HFC diet) for the last 4 weeks of the 13-week feeding regime. As shown in Table 10, HFC mice did not gain much body weight compared to CH mice, while 4 weeks of albiflorin-treated mice significantly increased body weight gain in HFC mice without affecting caloric intake.

Table 10. The effects of albiflorin on body weight and food intake in insulin resistant mice induced by high-fat cholesterol feeding.
## Chapter Four

### Albiflorin

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>HFC</th>
<th>HFC-AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prior AF treatment (g)</td>
<td>29.3±0.7</td>
<td>30.0±0.6</td>
<td>33.2±1.4##</td>
</tr>
<tr>
<td>After AF treatment (g)</td>
<td>30.8±0.7</td>
<td>30.5±0.5</td>
<td>35.5±1.7#†</td>
</tr>
<tr>
<td><strong>Body weight gain (g)</strong></td>
<td>1.3±0.3</td>
<td>0.5±0.2*</td>
<td>2.0±0.5††</td>
</tr>
<tr>
<td><strong>Caloric intake (kcal/mouse/day)</strong></td>
<td>7.9±0.5</td>
<td>7.8±0.1</td>
<td>8.7±0.3</td>
</tr>
</tbody>
</table>

Body weight and daily food intake for C57BL/6J mice fed 13 weeks on either standard chow (CH), high fat cholesterol diet (HFC), or HFC supplemented with albiflorin (AF, 100 mg/kg/day) (HFC-AF) for the last 4 weeks. Data are shown as means ± SEM of 6-8 mice of each group. * p < 0.05 HFC vs. CH; † p <0.05, †† p <0.01 HFC-AF vs. HFC; # p < 0.05, ## p < 0.01 HFC-AF vs. CH.

### 4.3.2 Albiflorin Reduced Hepatic Triglyceride and Cholesterol Levels in HFC-fed Mice

Ectopic lipid accumulation in insulin-target tissues is believed to be closely linked to insulin resistance [33]. To investigate whether albiflorin has effects on lipid accumulation in insulin-target tissues, we measured triglyceride and cholesterol contents in the liver which is a major organ response to insulin action. HFC feeding markedly increased triglyceride level by >3 fold compared to CH-fed mice. Albiflorin treatment resulted in an apparent reduction (~50%) of triglyceride level in the liver of HFC mice (Figure 41A). Similarly, cholesterol level of HFC mice were significantly increased by >4 fold in the liver compared to CH mice. 4-week albiflorin treatment significantly reduced cholesterol level in the liver of HFC mice by >50% (Figure 41B).
Figure 41. Albiflorin reduced hepatic triglyceride and cholesterol levels in HFC mice. C57BL/6J mice were fed a HFC diet for 13 weeks and albiflorin (100mg/kg/day in HFC diet, HFC-AF group) was given for the last 4 weeks. Triglyceride and cholesterol levels in the liver were measured. Data are shown as means ± SEM of 7-8 mice of each group. ** p < 0.01 HFC vs. CH; †† p < 0.01 HFC-AF vs. HFC.

4.3.3 Albiflorin Reduced Plasma AST Level in HFC-fed Mice

To determine whether the albiflorin-mediated reduction in hepatic lipid accumulation is associated with the improvement of liver function induced by chronic HFC feeding, we next measured the levels of plasma AST and ALT, common markers for liver function. As shown in Figure 42A, HFC-fed mice showed significantly increased AST level compared to the CH-fed mice, while 4-week administration of albiflorin decreased the AST level in HFC-fed mice. The plasma ALT levels, however, had no significantly statistics changes among each group (Figure 42B).
Figure 42. Albiflorin reduced plasma AST level in HFC-fed mice. Plasma samples were collected after 13 weeks of CH diet, HFC diet, or HFC diet with albiflorin treatment (HFC-AF). (A) Plasma AST and (B) ALT levels were analysed. Data are presented as mean ± SEM of 7-8 mice of each group. * p < 0.05 HFC vs. CH, † p < 0.05 HFC-AF vs. HFC.

4.3.4 Albiflorin did not show improvement in liver morphology in HFC-fed Mice

Lipid accumulation (hepatic steatosis) is strongly associated with insulin resistance in the liver [241, 242], and people with benign hepatic steatosis might progress to more severe steatohepatitis [243]. To distinguish the steatohepatitis from simple steatosis, hepatocyte ballooning is the most important diagnostic criteria [162], involving hematoxylin and eosin (H & E) staining [164]. HFC feeding resulted in a dramatic increased hepatocellular swollen and rarefied cytoplasm (ballooning). However, albiflorin did not improve the severe hepatocytes ballooning in HFC-fed mice after 4 weeks of its administration (Figure 43, Table 11).
Figure 43. Albiflorin did not improve liver injury in HFC-fed mice. Liver sections of mice fed with 13 weeks of standard chow (CH), high-fat cholesterol (HFC) diet with or without AF administration were subjected to H & E staining for evaluating the liver status. Images were captured using a light microscope and shown at 20x objective. The ballooning score for each is listed in Table 11. n=6-8 mice of each group.

Table 11. Scoring of ballooning liver injury in HFC-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>HFC</th>
<th>HFC-AF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0±0.0</td>
<td>8.6±4.0**</td>
<td>8.9±4.1##</td>
</tr>
</tbody>
</table>

This is based on the scoring criteria mentioned in Section 2.3.4.5. Data was presented as mean ± SEM. ** p < 0.01 HFC vs. CH, ## p < 0.01 HFC-AF vs. HFC. n=6-8 mice of each group.

4.3.5 Albiflorin Reduced the Hepatic Expression of TNFα

Albiflorin has been reported to have beneficial effects on inflammatory conditions [221-223], I next determined whether the albiflorin-mediated reduction in hepatic lipid accumulation may result in the reduced inflammation, the gene expression level of pro-inflammatory cytokine TNFα in the liver was measured. HFC diet led to a marked elevation (~40% increase compared to CH-fed mice) in hepatic expression of the TNFα, while 4-week administration of albiflorin in HFC-fed mice significantly restored the gene expression of TNFα (Figure 44).
Figure 44. Albiflorin reduced the hepatic gene expression of TNFα in HFC-fed mice. TNFα mRNA expression level of mice fed with 13 weeks of CH or HFC -/+ 4-week administration of albiflorin was determined by quantitative real-time PCR and normalised by 18S mRNA. Data are shown as fold change ± SEM against control mice, * p < 0.05 vs. CH, † p < 0.05 vs. HFC. n=7-8 mice of each group.

4.3.6 Effects of Albiflorin on Glucose Metabolism in HFC-fed Mice

Inflammation are closely associated with obesity, insulin resistance and type 2 diabetes chronic [56, 244]. As albiflorin has shown beneficial effect on TNFα, a marker of pro-inflammatory signalling pathways, we next examined the effects of albiflorin on glucose metabolism. As shown in Table 12, mice fed with HFC showed a ~50% increase of fasting blood glucose compared to chow-fed mice (CH). Albiflorin-treated HFC mice had significantly lower fasting blood glucose to the level of the CH mice. Consistently with the glucose level, HFC-fed mice displayed a significant increase of basal plasma insulin level compare to CH-fed mice (Table 12). One week before albiflorin administration (week 8), mice fed with HFC (HFC-pre-AF) group displayed significantly higher plasma insulin level
than the CH-fed mice (p<0.05). 4-week administration of albiflorin in HFC-fed mice (week 13) largely reduced basal plasma insulin level compare to HFC group (Table 12).

To assess the effect of albiflorin on glucose homeostasis, mice were subjected to an ipGTT at pre-treatment and during treatment periods. HFC-fed mice exhibited significantly glucose intolerance (all p<0.01) compared to CH mice throughout the experimental period. One week before albiflorin administration (week 8), mice fed with HFC (HFC-pre-AF) group displayed significant glucose intolerance compared to the control chow diet (CH) mice (40%~50% increase in iAUC, p<0.01) (Table 12, Figure 45). After administration of albiflorin for 3 weeks (week 12), HFC-AF group still showed significantly glucose intolerance compare to CH group (Table 12, Figure 45).

Table 12. The effects of albiflorin on blood glucose and insulin levels in insulin resistant mice induced by high-fat cholesterol feeding.

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>HFC</th>
<th>HFC-AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior AF treatment (mM)</td>
<td>10.1 ± 0.3</td>
<td>10.6 ± 0.3</td>
<td>11.3 ± 0.5†</td>
</tr>
<tr>
<td>After AF treatment (mM)</td>
<td>8.7 ± 0.6</td>
<td>12.8 ± 0.8**</td>
<td>9.4 ± 0.5††</td>
</tr>
<tr>
<td><strong>Blood insulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior AF treatment (mM)</td>
<td>91 ± 16</td>
<td>167 ± 21**</td>
<td>168 ± 35#</td>
</tr>
<tr>
<td>After AF treatment (mM)</td>
<td>88 ± 14</td>
<td>153 ± 16*</td>
<td>73 ± 19†</td>
</tr>
<tr>
<td><strong>iAUC (90 mins)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior AF treatment (min.mM)</td>
<td>370.0 ± 40</td>
<td>728.9 ± 48**</td>
<td>845 ± 88††</td>
</tr>
<tr>
<td>After AF treatment (min.mM)</td>
<td>343 ± 51</td>
<td>779 ± 71**</td>
<td>904 ± 107††</td>
</tr>
</tbody>
</table>

Fasting blood glucose and insulin levels for C57BL/6J mice fed 13 weeks on either standard chow (CH), high fat cholesterol diet (HFC), or HFC supplemented with albiflorin (AF, 100
mg/kg/day) (HFC-AF) for the last 4 weeks. Glucose tolerance test (GTT, 1.5 g glucose/kg body weight, i.p.) was performed at different treatment period (at week 8 and 12) and glucose levels were measured at indicated time point. ipGTT results were quantified by calculating the area under the incremental AUC. Data are shown as means ± SEM of 6-8 mice of each group.

* p < 0.05, ** p < 0.01 HFC vs. CH; † p < 0.05, †† p < 0.01 HFC-AF vs. HFC; # p < 0.05 HFC-AF vs. CH.

Figure 45. Albiflorin didn’t improve diet-induced glucose intolerance in HFC fed mice. C57BL/6J mice were fed a HFC diet for 13 weeks and AF (100mg/kg/day in HFC diet) was given for the last 4 weeks. Glucose tolerance test (GTT, 1.5 g glucose/kg body weight, i.p) was performed at different treatment period (week 8 and 12) and glucose levels were measured at indicated time point. Data are shown as means ± SEM of 7-8 mice of each group.

* p<0.05, **p<0.01 HFC vs CH; # p < 0.05, ## p < 0.01 HFC-AF vs. CH.

4.3.7 Albiflorin Improved Glucose Intolerance in HF-fed Mice

As the results were not consistent between the basal levels of glucose (insulin) and the ipGTT in HFC-fed mice model, further studies were conducted in high-fat (HF) diet induced insulin resistant mice model. HF-fed mice were received albiflorin for the last 2 weeks of the 8-week feeding regime. As shown in Table 13, mice fed with HF diet showed a significant increase of
fasting blood glucose compared to chow-fed mice (CH). Albiflorin-treated HF mice (HF-AF) had lower fasting blood glucose to the similar level of the CH-fed mice (p=0.06).

**Table 13. The effects of albiflorin on blood glucose levels in insulin resistant mice induced by high-fat feeding.**

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>HF</th>
<th>HF-AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting glucose (mM)</strong></td>
<td>6.3 ± 0.2</td>
<td>7.3 ± 0.2*</td>
<td>6.6 ± 0.1</td>
</tr>
</tbody>
</table>

Fasting blood glucose levels for C57BL/6J mice fed 8 weeks on either standard chow (CH), high-fat diet (HF), or HF supplemented with albiflorin (AF, 100 mg/kg/day) (HF-AF) for last 2 weeks. Data are shown as means ± SEM of 7-10 mice of each group. * p < 0.01 HF vs. CH.

ipGTT was then performed in the high-fat diet induced insulin resistant mice model. As shown in Figure 46, HF-fed mice showed significantly glucose intolerance compared to CH-fed mice, while mice treated with albiflorin displayed significantly improved glucose tolerance than the HF-fed mice (~50% decrease in iAUC, p<0.01) (Figure 46).

**Figure 46. Albiflorin improved glucose intolerance in HF-fed mice.** C57BL/6J mice were fed a HF diet for 8 weeks and albiflorin (AF, 100mg/kg/day in HF diet) was given for the last
2 weeks. Glucose tolerance test (GTT, 2.0 g glucose/kg body weight, i.p.) was performed at different treatment period and glucose levels were measured at indicated time point (A). ipGTT results were quantified by calculating the area under the incremental AUC (iAUC) (B). Data are shown as means ± SEM of 7-10 mice of each group. * p<0.05, ** p < 0.01 HFC vs CH; † p < 0.05, †† p < 0.01 HFC-AF vs. HFC.

4.4 DISCUSSION

Albiflorin is an active ingredient found in herbal remedies (e.g. Paeonia Radix) with a good oral absorption property and pharmacokinetics in vivo [219]. Albiflorin is widely used in Traditional Chinese Medicine for its anti-biotic, anti-inflammatory and anti-allergic properties with little adverse effects [220]. In addition, albiflorin has been reported to have anti-diabetic effects [229]. The present study investigated the effect of albiflorin, a monoterpene glycoside, on hyperlipidaemia and hyperglycaemia in insulin resistant mice induced by chronic high-fat or high-fat cholesterol feeding. The results showed that albiflorin was able to reverse the elevated hepatic triglyceride and cholesterol levels in high-fat cholesterol (HFC) induced insulin resistant mice and markedly attenuated liver damage. Apart from the correction of hyperlipidaemia, albiflorin did not affect hyperglycaemia in HFC-induced insulin resistant mice, but it was able to reverse hyperglycaemia in high-fat (HF) induced insulin resistant mice.

Since previous screening results showed albiflorin was able to reduce intracellular lipids (Figure 26), along with the analysis for its drug-like properties, I first investigated the effects of albiflorin on hyperlipidaemia in insulin resistant mice generated by chronic HFC feeding.
HFC feeding in mice has been used as a model of insulin resistance induced by lipid accumulation [237, 238]. As expected, HFC-fed mice significantly increased triglyceride and cholesterol levels in the liver (Figure 41). Oral administration of albiflorin to the HFC-fed insulin resistant mice significantly reduced liver triglyceride and cholesterol levels within 4 weeks (Figure 41). These results are consistent with the screening data (Figure 26).

Aspartate aminotransferase (ASL) and alanine aminotransferase (ALT) are commonly measured as a part of a diagnostic evaluation of hepatocellular damage in clinical work. Studies have demonstrated that AST and ALT levels independently predict type 2 diabetes [245]. These aminotransferases have been shown to be positively associated with indirect measurements of insulin resistance including fasting insulin levels and HOMA-IR [246]. I therefore examined whether albiflorin-mediated reduction in hepatic lipid accumulation is associated with the improvement of liver function induced by chronic HFC feeding. The results showed that the addition of cholesterol in HF diet caused a significant elevated plasma AST level, while 4-week treatment of albiflorin markedly reduced the increased plasma AST level induced by HFC feeding.

It has been reported that inflammation is associated with obesity and type 2 diabetes [56, 244]. Moreover, albiflorin analogues have been proposed to exert the beneficial effects on inflammatory conditions [221-223]. I next investigated the effects of albiflorin on inflammation in HFC-induced insulin resistant mice. Albiflorin significantly reduced the hepatic TNFα gene expression which is one of the pro-inflammatory cytokines [247]. These
findings indicate that albiflorin may improve the non-alcoholic steatohepatitis (NASH) induced by HFC diet. NASH is a type of liver disease and characterised by inflammation with elevated lipid accumulation in the liver. This may also contribute to its reported efficacy for liver fibrosis because hepatic steatosis is an early event of steatohepatitis [248].

Insulin resistance is closely related with increased accumulation of lipids in peripheral tissues [33, 61]. A reduction of lipid content in these tissues is an effective means to improve insulin sensitivity. It has been reported that albiflorin derivatives can reverse insulin resistance and hyperglycaemia during treatment period [175, 229]. The present study observed that HFC-AF mice displayed significantly lower fasting blood glucose and insulin levels. However, albiflorin-treated HFC mice did not attenuate glucose intolerance as shown by glucose tolerance test. The reversal of HFC-diet induced insulin resistance but not glucose intolerance during albiflorin treatment might be associated with the effect of albiflorin on decreasing insulin secretion from β-cells [249]. I further assessed the effects of albiflorin on glucose homeostasis in a well-establishing insulin resistant mice model induced by HF diet [165, 240]. Albiflorin attenuated the HF-induced increased fasting glucose levels and improved glucose intolerance consistent with previous findings [229, 231].

In summary, the present study investigated the effects albiflorin on insulin resistant mice induced by lipid over supply (high-fat or high-fat cholesterol feeding). Albiflorin is one of the identified leads from our newly established screening based on its ability of lowering intracellular lipid accumulation. Albiflorin demonstrated its ability of reducing triglyceride
and cholesterol levels in the liver, which strongly validates and supports our newly established screening assay *in vivo*. The reduction of lipids level indicated the effect of albiflorin on improvement in hepatic steatosis and inflammation in insulin resistant mice induced by chronic high-fat cholesterol feeding. Furthermore, attenuation of glucose intolerance in HF-fed mice suggests the potential therapeutics for type 2 diabetes. Taken together, these results show the potential therapeutic effect of albiflorin on hepatic inflammation, type 2 diabetes and associated complications. Further investigations are definitely needed, such as plasma triglyceride and cholesterol levels. To confirm the effects of albiflorin on insulin resistance, a more sensitive and reliable technique are required, such as hyperinsulinaemic-euglycaemic clamp combined with glucose tracers [66, 250].
CHAPTER 5
INVESTIGATION OF
OLEANOLIC ACID AND
MOLECULAR MODE OF ACTION
IN THE LIVER
Our recent study showed that the triterpenoid oleanolic acid (OA) was able to produce a sustained correction of hyperglycemia beyond treatment period in type 2 diabetic (T2D) mice with liver as a responsible site [154]. To follow up the previous observations, the present study investigated the possible role of acetylation of FoxO1 and associated events in this therapeutic memory by charactering the pathways regulating the acetylation status during and post-OA treatments. OA treatment (100 mg/kg/day for 4 weeks, during OA treatment) reduced hyperglycemia in T2D mice by ~87% and this effect was largely (~70%) maintained even 4 weeks after the cessation of OA administration (post-OA treatment). During OA treatment, the acetylation and phosphorylation of FoxO1 were markedly increased (1.5 to 2.5-fold) while G6Pase expression was suppressed by ~80%. Consistent with this, OA treatment reversed pyruvate intolerance in high-fat fed mice. Histone acetyltransferase 1 (HAT1) content was increased (>50%) and histone deacetylases (HDACs) 4 and 5 (not HDAC1) were reduced by 30-50%. The OA-induced changes in FoxO1, G6Pase, HAT1 and HDACs persisted during the post-OA treatment period where the increased phosphorylation of AMPK, SIRT1 content and reduced liver triglyceride had subsided. These results confirmed the ability of OA to control hyperglycemia far beyond treatment period in T2D mice. Most importantly, in the present study we demonstrated acetylation of FoxO1 in the liver is involved in OA-induced memory for the control of hyperglycemia. Our novel findings suggest that acetylation of the key regulatory proteins of hepatic gluconeogenesis is a plausible mechanism by the triterpenoid to achieve a sustained glycemic control for T2D.
5.1 INTRODUCTION

Type 2 diabetes is a major disease with serious consequences [251]. As type 2 diabetes is not curable with the available medications at the present time, the major goal for the treatment of type 2 diabetes is to achieve effective control of hyperglycaemia to prevent/delay the onset and progression of its complications [252].

Triterpenoids class compounds have been suggested to be a valuable source for the discovery of new efficacious anti-diabetic drugs [165, 253]. The screening results presented in Chapter 3 showed that oleanolic acid (OA) from triterpenoids class significantly reduced triglyceride level in fully differentiated 3T3-L1 cells (YC5-4 in Figure 34). Moreover, our recent results demonstrated that OA is able to reduce hyperglycaemia and such effect is maintained even 4 weeks after the cessation of administration [154]. However, the underlying effects of OA on sustained anti-hyperglycaemia are still not clear. Therefore, the aim of the study in this Chapter sought to investigate the mechanisms responsible for the sustained glycaemic control after the cessation of OA treatment in the liver of type 2 diabetic mice.

5.1.1 Oleanolic Acid

Oleanolic acid (OA) is a pentacyclic triterpenoid abundantly available for drug discovery as more than 20,000 of them are known to exist in the plant kingdom [207]. OA and related triterpenoids possess interesting pharmacological properties, including the anti-oxidative, microbicide, anti-diabetic, anti-inflammatory, hypolipidaemic, and anti-atherosclerotic actions [208, 209]. They interfere in the development of different types of cancer [209] and
neurodegenerative disorders [254].

Several studies including ours have indicated that triterpenoids compounds may emerge as potential anti-diabetic drugs with distinct therapeutic properties [255]. Our acute studies in mice showed that triterpenoids are able to reduce glucose intolerance in insulin resistant high-fat fed mice after a single injection [165]. Moreover, we found that triterpenoids isolated from bitter melon have potent efficacy in stimulating GLUT4 translocation in L6 myotubes and 3T3L1 adipocytes, along with the activation of the AMP-activated protein kinase (AMPK) pathway [165]. AMPK has been reported as a major cellular target of several anti-diabetic small molecules, namely metformin [96], thiazolidinedione (pioglitazone and rosiglitazone) [256] and berberine (BBR) [142]. Interestingly, our recent studies showed that these triterpenoids may activate AMPK by a mechanism entirely different from these other anti-diabetic small molecules [143]. Additionally, as well as activating AMPK, OA has been found to suppress mitochondrial ROS, inflammation and glucose-6-phosphatase (G6Pase) expression in the liver of db/db mice [253].

On this note, OA has been used in humans for its potential therapeutic application for cancer [207] and one of OA analogues has demonstrated promising therapeutic effects in humans for treating diabetic complications such as nephropathy [257] where prolonged hyperglycaemia is a major culprit [8]. Moreover, OA and its analogues have been shown to lower hyperglycaemia in STZ-treated rodents [258], high-fat fed, or db/db mice [259], protect against diabetic nephropathy [258] and pancreatic islets [260]. Our recent results
demonstrated that OA reduced hyperglycaemia and this effect was sustained even 4 weeks after the cessation of its administration [154]. However, the underlying mechanisms have not been reported. Therefore, the present study aimed to investigate the mechanisms of OA for the sustained control of hyperglycaemia in a mouse model of type 2 diabetes.

**5.1.2 Type 2 Diabetic Mice Model Induced by High-fat Feeding plus STZ Injections**

Chronic high-fat feeding in rodents is a widely used model of insulin resistance induced by lipid accumulation in muscle and liver [152, 235]. However, high-fat feeding alone is insufficient to cause diabetes due to the compensatory capacity of pancreatic β-cells to increase insulin secretion to overcome insulin resistance [261]. Multiple low doses injections of streptozotocin (STZ) has been shown to induce hyperglycaemia by restricting the ability of pancreatic β-cells to increase insulin secretion as previously reported [113, 153]. Therefore, in the present study, the mice model of type 2 diabetes were generated by chronic high-fat feeding (to generate insulin resistance) plus multiple low doses of STZ (to block the compensatory secretion of insulin) [153, 154].

**5.2 MATERIALS AND METHODS**

**5.2.1 Animal Experiments and Procedures**

Ten-week old male C57BL/6J mice (the Animal Resources Centre, Perth, Australia) were used for this study. The mice were housed at a temperature-controlled room (22 ± 1°C) on a 12-hour light/dark cycle with free access to water and food. All experiments were carried out
with the approval of the Animal Ethics Committees of RMIT University (Project #1012) in accordance with the guidelines of the National Health and Medical Research Council of Australia.

After one week of acclimatisation, the mice were fed *ad libitum* with a standard lab chow (CH) diet (12% calories from fat, 65% calories from carbohydrate and 23% calories from protein) or high fat (HF) diet (45% calories from fat, 35% calories from carbohydrates and 20% calories from protein) as described previously [159] for 10 weeks. HF-fed mice were then injected with vehicle (saline) or low doses of streptozotocin (STZ, 40 mg/kg/day) for 5 consecutive days to generate a diabetic mouse model (type 2 diabetic mice, T2D mice) [113, 153, 154]. Once hyperglycaemia (fasting blood glucose >14 mM) was developed, one group of T2D mice received oleanolic acid (OA, 100 mg/kg/day) in the diet [165] for 4 weeks (during treatment, T2D-OA); another group of T2D mice received OA (100 mg/kg/day) in the diet for 2 weeks before switching back onto the HF diet for another 4 weeks (post-treatment, T2D-OA) before tissues collection, while the rest of the mice remained on either the CH or HF diet (Figure 47). Body weight, food intake and blood glucose levels were monitored weekly.

![Figure 47. Study design of oleanolic acid treatment in high-fat diet with streptozotocin](image-url)
injections induced type 2 diabetic mice. Type 2 diabetic (T2D) mice model was induced by 10-week high-fat feeding plus low doses of streptozotocin (STZ, 40 mg/kg/day) for 5 consecutive days. One week after STZ injection, (1) During treatment: one group of T2D mice received oleanolic acid (OA, 100 mg/kg/day) in the diet for 4 weeks before tissue collection; (2) Post-OA treatment: another group of T2D mice received OA for 2 weeks before switching back onto the high-fat diet for another 4 weeks before tissues collection.

5.2.2 Measurement of Oxygen Consumption Rate in Isolated Mitochondria

Mitochondria play a pivotal role in cellular function, not only as a major site of ATP production, but also regulation of energy expenditure. ADP-dependent oxygen consumption directly reflects coupled respiration or oxidative phosphorylation. Therefore, the functional measurements of oxygen consumption rates from isolated mitochondria provide useful and valuable information to evaluate mitochondrial dysfunction and disease. Moreover, altered mitochondrial function is reported to be a key underlying mechanism of insulin resistance [262]. Mitochondria from rat liver were isolated by specific isolation medium under conditions detailed in Section 2.3.4.1. Oxygen consumption rate was determined by Clark-type oxygen electrode as described in Section 2.3.4.1.

5.2.3 Assessment of Blood Glucose, Blood Insulin and Liver Triglyceride

Blood glucose was measured using a glucometer. Insulin was determined by radioimmunoassay as described in Section 2.3.3. At the end of the study, mice were killed by
cervical dislocation and the relevant tissue samples were immediately freeze-clamped.

Triglyceride level in plasma and liver were determined by a Peridochrom triglyceride GPO-PAP kit as explained in Section 2.3.4.2.

### 5.2.4 Assessment of Glucose and Pyruvate Tolerance

Intraperitoneal glucose tolerance tests (ipGTT, 1.5 g glucose/kg body weight) were performed after 5-7 hours fasting after 2 weeks of OA administration and 2 weeks after the cessation of OA administration. Blood glucose levels were measured at 0, 15, 30, 60 and 90 mins and plasma insulin levels were sampled at 0, 15, 30 and 60 mins. The effect of OA on hepatic glucose production from gluconeogenesis was examined in another set of experiment. Briefly, mice fed the HF diet for 8 weeks were treated with OA at the same dose for the last 10 days for a pyruvate tolerance test (PTT). The PTT was performed with an intraperitoneal injection of sodium pyruvate (2.0 g/kg in 1x PBS) after overnight fasting. Blood glucose levels were measured at 0, 15, 30, 60 and 90 mins.

### 5.2.5 Western Blot Analysis

Activation of key insulin signalling proteins and levels of lipogenic and gluconeogenic enzymes were examined by immunoblotting as described in Section 2.3.4.3. Specific antibodies were used including total- and phospho (Thr 172)-AMPK (Cell Signalling, #2532, #2535), total- and phospho (Ser 79)-acetyl-CoA carboxylase (ACC, Cell Signalling, #3662, #3661), sirtuin 1 (SIRT1, Millipore, #07-131), sterol regulatory element-binding protein (SREBP-1c, Santa Cruz, #sc367), stearoyl-CoA desaturase 1 (SCD-1, Cell Signalling, #2794),
fatty acid synthase (FAS, Cell Signalling, #3180), total- and phospho (Ser 473)-Akt (Cell Signalling, #9271, #9271), total- and phospho (Ser 9)-GSK3β (Cell Signalling, #9315, #9331), total- and phospho (Ser 256)-forkhead box protein O1 (FoxO1, Cell signalling, #9454, #9461), HAT1 (Abcam, #ab12163), total- and phospho (Ser 632)-HDAC4 (Cell Signalling, #2072, #3424), total- and phospho (Ser 498)-HDAC5 (Cell Signalling, #2082, #3424), HDAC1 (Santa Cruz, #sc8410), and acetyl (lys 259, 262 and 271)-FoxO1 (Santa Cruz, #sc49437). Quantitative densitometry analysis was performed using Image Lab software (Bio-Rad Laboratories Inc., USA).

5.2.6 Quantitative Real-time PCR

Total RNA was extracted from liver tissues as desried in Section 2.3.4.4. Real-time PCR was carried out using the IQ SYBR Green Supermix for phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Genework, Australia). The gene expression from each sample was analysed in duplicates and normalised against the housekeeper gene 18S (Genework, Australia). All reactions were performed on the iQ™ 5 Real-time PCR Detection System (Bio-Rad Laboratories Inc., USA). The primer sequences (5’ to 3’) were described in Table 14.

Table 14. The primer sequences of PEPCK and G6Pase.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>CGCCGCTAGAGGTGAAATTCT</td>
<td>CGAACCTCCGACTTTTCGTTCT</td>
</tr>
<tr>
<td>PEPCK</td>
<td>CCACAGCTGCTGCAGAACA</td>
<td>GAAGGTCGATGCGAAA</td>
</tr>
<tr>
<td>G6Pase</td>
<td>AACGCCTTCTATGTCCCTTTTC</td>
<td>GTTGCTGTAGTAGTCGCGTCC</td>
</tr>
</tbody>
</table>
5.2.7 Statistical Analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for comparison of relevant groups. When significant variations were found, the Dunnett’s multiple comparisons test was applied. Differences at p<0.05 were considered to be statistically significant.

5.3 RESULTS

5.3.1 Sustained Correction of Hyperglycaemia Induced by OA Treatment

We first confirmed the effects of OA treatment on glucose and lipid metabolism in a model of T2D mice [154]. HF-feeding combined with low-dose STZ injections induced the typical characteristics observed at the late stage of type 2 diabetes, indicating hyperglycaemia (>2 fold, p<0.05) and hypertriglyceridaemia (~80%, p<0.05) due to the inability of the pancreas to increase blood insulin levels to compensate for the HF-induced insulin resistance (Table 15, left hand panel). As expected, there was a trend of mild weight loss without significant changes in calorie intake. Liver triglyceride levels of T2D mice were also significantly elevated (2.2-fold, p<0.01) compared to chow-fed control (CH) mice. OA treatment of T2D mice (T2D-OA) restored fasting blood glucose and plasma triglycerides to the levels of the control animals. Body weight and liver triglyceride levels were reduced by 9% and 33% respectively; in OA-treated mice compared to untreated T2D animals. The T2D mice also displayed improved glucose tolerance (30%, p<0.01) and slightly greater blood insulin
availability during ipGTT in response to OA treatment. No significant effects on caloric intake or fasting blood insulin levels were found in the OA-treated T2D mice.

Upon the cessation of treatment, some T2D-OA mice were switched back to the normal HF diet for a further four weeks. During this period, these mice regained body weight and their liver triglyceride levels had returned to comparable levels to the T2D group (Table 15, right hand panel). Intriguingly, the OA-treated T2D mice were able to maintain normal fasting glycaemia and improved glucose tolerance even when OA was removed from the diet. This occurred without any significant alterations in caloric intake. Despite the rebounded body weight and liver triglyceride content, these mice had lowered plasma levels of triglyceride, while no detectable differences were found with blood insulin levels when compared to the T2D group.
Table 15. Metabolic responses during and post-OA treatments in HF-STZ induced type 2 diabetic mice.

<table>
<thead>
<tr>
<th></th>
<th>During treatment</th>
<th>Post-OA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
<td>T2D</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>29.5±0.4</td>
<td>27.8±0.2</td>
</tr>
<tr>
<td>Caloric intake (kcal/mouse/day)</td>
<td>13.6±0.7</td>
<td>12.2±0.6</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>9.6±0.2</td>
<td>21.3±0.4**</td>
</tr>
<tr>
<td>ipGTT AUC (mM x 90 mins)</td>
<td>1442±38</td>
<td>2902±71**</td>
</tr>
<tr>
<td>Blood insulin (pg/ml)</td>
<td>361±37</td>
<td>306±45</td>
</tr>
<tr>
<td>Blood insulin (5-60 mins, pg/ml)</td>
<td>417±37</td>
<td>181±13**</td>
</tr>
<tr>
<td>Plasma triglyceride (mM)</td>
<td>1.2±0.1</td>
<td>2.1±0.2**</td>
</tr>
<tr>
<td>Liver triglyceride (µmol/g)</td>
<td>7.6±0.9</td>
<td>16.8±0.9**</td>
</tr>
</tbody>
</table>

**During treatment:** 4 weeks of administration of oleanolic acid (OA, 100 mg/kg/day in high-fat diet). Parameters were measured between in the last 2 weeks of while mice were under the OA treatment. **Post-OA treatment:** OA was removed from the diet after 2 weeks of administration and fed an OA-free HF diet for another 4 weeks. Assessments were conducted in the last 2 weeks of the post-treatment period. CH, chow fed mice (normal control); T2D, High fat-fed mice with STZ injections (untreated T2D); T2D-OA, T2D with OA treatment. AUC, area under the curve of blood glucose during an ipGTT. Data are shown as means ± SEM. * p < 0.05, ** p < 0.01 vs. corresponding CH; † p <0.05, †† p <0.01 vs. corresponding T2D, n=7-8 per group.
5.3.2 Changes in the AMPK Pathway and SIRT1 During and Post-OA Treatments

Triterpenoids have been previously shown to activate AMPK [165]. Activated AMPK can suppress hepatic gluconeogenesis [64, 263] by phosphorylating/acetylating FoxO1. The possible role of AMPK and its downstream targets in the metabolic response following OA treatment was examined. The phosphorylation of AMPK was reduced in T2D mice compared to non-diabetic CH mice (1.00 ± 0.13 vs. 2.08 ± 0.24 of CH mice, p<0.01, n=5-8). OA administration restored the phosphorylation of AMPK (2-fold, p<0.05) and its downstream effector ACC (1.7-fold, p<0.05, Figure 48A). However, the effect of OA on the phosphorylation of AMPK and ACC were not maintained after cessation of treatment (Figure 48B).

The expression of SIRT1 was then examined, which is a down-stream target of AMPK that is responsible for regulating gluconeogenesis [264]. The expression of SIRT1 was reduced in the liver of T2D mice compared to non-diabetic CH mice (1.00 ± 0.05 vs. 1.48 ± 0.10 of CH mice, p<0.05, n=5-8). Treatment of OA increased the expression of SIRT1 (1.5-fold, p<0.05, Figure 48A) compared to the T2D group, however it was not maintained after cessation of treatment (Figure 48B).
Figure 48. Changes in the AMPK pathway and SIRT1 in the liver during and post-OA treatments. Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of phosphorylated (p-) or total (t-) AMPK and ACC and SIRT1 for during OA treatment (at the end of 4-week OA treatment) (A) and post-OA treatment (4 weeks after the removal of OA) (B). Data are mean ± SEM. * p < 0.05 vs. T2D, n = 7-8 per group.

5.3.3 Effects of OA on Mitochondrial Respiration

Activation of AMPK via the inhibition of mitochondrial respiration (particular in Complex I) is a well-characterised mechanism shared by a number of anti-diabetic agents, such as metformin, rosiglitazone and berberine [99, 100, 142]. We reasoned whether OA might be
activating AMPK via a similar mechanism. As shown in Figure 49, berberine (BBR) attenuated mitochondrial respiration in a dose-dependent manner via inhibition of Complex I (pyruvate and malate) but not Complex II (succinate in the presence of rotenone). Unlike BBR, OA treatment had no detectable effects on mitochondrial respiration regardless of substrates supplied. These results suggest that OA may exert a mechanism to activate AMPK that is different to metformin, berberine and rosiglitazone.

![Chemical structure of oleanolic acid and its effect on mitochondrial respiration.](image)

**Figure 49. Chemical structure of oleanolic acid and its effect on mitochondrial respiration.** Oleanolic acid (OA) is a pentacyclic triterpenoid which has a different chemical structure from berberine (BBR). Mitochondrial oxygen consumption was measured at 37°C using a Clark-type oxygen electrode with substrates/inhibitor for Complex I (5 mM pyruvate and 2 mM malate) or Complex II (10 mM succinate in the presence of 4 µM rotenone). Data are mean ± SEM. ** p < 0.01 vs. vehicle control (0 µM, DMSO), n=3 per group.
5.3.4 Changes in the Hepatic Lipid Metabolism During and Post-OA Treatments

Reducing lipid accumulation in insulin target tissues is an important mechanism for the anti-diabetic effect of AMPK activators [66] and the triterpenoids have been shown to activate AMPK [165]. To verify the metabolic effects of OA-induced activation of AMPK in T2D mice, the expressions of key lipogenic proteins that are subjected to the regulation of AMPK were examined. The expression of the mature form of SREBP-1c (mSREBP-1c), SCD-1 and FAS were increased in T2D mice compared to non-diabetic mice (1.00 ± 0.16 vs. 0.80 ± 0.06 of CH mice; 1.00 ± 0.29 vs. 0.51 ± 0.19 of CH mice, p<0.05; 1.00 ± 0.14 vs. 0.88 ± 0.15 of CH mice, all n=5-8 mice per group, respectively). Consistent with the effect on AMPK and ACC, the expression of mSREBP-1c, SCD-1 and FAS were significantly reduced during the period of OA treatment (by 58%, 61% and 50%, respectively, all p<0.05 vs. T2D mice) (Figure 50A). Coincided with the subsided activation of AMPK, the levels of these lipogenic proteins of mSREBP1c, FAS and SCD-1 returned to similar levels as the T2D group after the cessation of OA treatment (Figure 50B).

A  During OA treatment

![Graphs showing expression levels of mSREBP-1c, SCD-1, and FAS during and after OA treatment.](image)
Figure 50. Changes in AMPK- and/or SIRT1-regulated key lipogenic proteins in the liver during and post-OA treatments. Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of mature form of SREBP-1c (mSREBP-1c), SCD-1 and FAS for during OA treatment (A) and post-OA treatment (B). Data are mean ± SEM. * p < 0.05, ** p < 0.01 vs. T2D, n = 5-8 per group.

5.3.5 Changes in the Activities of Akt, FoxO1 and Gluconeogenic Enzymes During and Post-OA Treatments

The Akt-FoxO1 axis has been reported to have prominent implications in the regulation of hepatic gluconeogenesis [265], hence understanding the nature of this observation is a paramount step in explaining the sustained glycemic control beyond the treatment period. To do so, the activities of Akt and FoxO1 during and after cessation of OA treatments were compared. Our previous data demonstrated an increase in the phosphorylation of Akt and FoxO1 in the liver of T2D mice after cessation of OA treatment [154]. It is however unclear whether such observation was a sustained consequence of OA administration or a secondary effect that manifested after the cessation of treatment. T2D mice exhibited reduced phosphorylation of Akt (1.00 ± 0.06 vs. 1.97 ± 0.09 of CH mice, p<0.01, n=5-8 per group)
and of its downstream effector, GSK3β (1.00 ± 0.08 vs. 1.60 ± 0.21 of CH mice, p<0.05, n=5-8 per group) compared to non-diabetic mice throughout the course of experiment. The phosphorylation of Akt and GSK3β were restored in response to OA treatment (Figure 51A) and sustained beyond the cessation of OA treatment (Figure 51B).

**Figure 51. Changes in the insulin signalling pathway in the liver during and post-OA treatments.** Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of phosphorylated (p-) or total (t-) Akt and GSK3β for during OA treatment (A) and post-OA treatment (B). Data are mean ± SEM. * p < 0.05, ** p < 0.01 vs. T2D, n = 5-8 per group.
In line with this, T2D mice showed decreased phosphorylation of FoxO1 (1.00 ± 0.07 vs. 1.43 ± 0.10 of CH mice, p<0.05, n=5-8 per group) and increased total content of FoxO1 (1.00 ± 0.13 vs. 0.75 ± 0.12 of CH mice, n=5-8 per group) compared to non-diabetic CH mice. The phosphorylation of FoxO1 in the liver was augmented by 1.7-fold, meanwhile the total content was reduced by 50% both during (all p<0.01, Figure 52A) and after the cessation of the treatment (all p<0.05, Figure 52B). Along with this, the mRNA expression of G6Pase, which is a rate-limiting regulator for gluconeogenesis, was also found to be up-regulated in the T2D mice (1.00 ± 0.17 vs. 0.72 ± 0.15 of CH mice, during OA treatment period; 1.00 ± 0.27 vs. 0.59 ± 0.21 of CH mice post-OA treatment, all n=5-8 per group). The expression of G6Pase was down-regulated in the OA-treated mice throughout the course of experiment (~80% during treatment, ~50% after cessation of treatment, all p<0.05 vs. T2D mice). However, no significant suppression was observed for PEPCK, which is another rate-limiting regulator for gluconeogenesis.
Figure 52. Changes in the phosphorylation of FoxO1 in the liver during and post-OA treatments. Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of phosphorylated (p-) and total (t-) FoxO1 for during OA treatment (A) and for post-OA treatment (B). RT-PCR analysis of G6Pase and PEPCK gene expression in liver from during OA treatment (A) and post-OA treatment (B), data was normalised by 18S. Data are mean ± SEM. * p < 0.05, ** p < 0.01 vs. T2D, n = 5-8 per group.

5.3.6 Changes in Histone Acetyltransferases and Deacetylases in the Liver During and Post-OA Treatments

A growing body of evidence suggests that post-translational modifications, such as acetylation of key transcription factors, can maintain an induced phenotype even after the triggering cause is no longer present [266, 267]. Protein acetylation is a dynamic process regulated by the equilibrium of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [268]. Therefore, the expression and activity status of these acetylation-modifying enzymes were examined to investigate how the treatment of OA might alter such equilibrium.

No detectable difference was found in the expression of HAT1 protein between T2D and CH
mice (1.00 ± 0.08 vs. 0.80 ± 0.04 of CH mice, p>0.05, n=5-8 per group). However, the total content of two class IIa HDACs, namely HDAC4 (1.00 ± 0.11 vs. 0.70 ± 0.03 of CH mice, p<0.05, n=9) and HDAC5 (1.00 ± 0.25 vs. 0.57 ± 0.08 of CH mice, p<0.05, n=5-8 per group) were elevated in the liver of T2D mice. In addition, the phosphorylation of HDAC4 (1.00 ± 0.09 vs. 0.87 ± 0.07 of CH mice, n=5-8 per group) and HDAC5 (1.00 ± 0.05 vs. 1.19 ± 0.08 of CH mice, n=5-8 per group) were reduced in the liver of T2D mice.

OA treatment of T2D mice induced an 80% increase in hepatic HAT1 content (p<0.05 vs. T2D mice), while the expression of HDAC4 and HDAC5 were markedly reduced (50%, p<0.05 vs. T2D mice, Figure 53A). The reduced expression of these HDACs was associated with a concomitant increase in their phosphorylation at specific serine residues (1.5-3-fold, p<0.01 vs. T2D mice, Figure 53A), which are indicative of protein de-activation [269]. These data suggested that OA increased the availability of HAT1 while decreasing the activity of HDACs, hence a possible shift in equilibrium more favourable for acetylation events. Removal of OA from these treated-mice did not alter the pattern of change of these acetylation-modifying enzymes (Figure 53B) indicating the shift in equilibrium initiated by OA was sustained.
A  During OA treatment

![Graphs showing expression levels of different proteins during OA treatment.]

B  Post-OA treatment

![Graphs showing expression levels of different proteins post-OA treatment.]

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Chapter Five – Oleanolic Acid
Figure 53. Changes in Class IIa HDACs in the liver during and post-OA treatments. Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of HAT1, phosphorylated (ac-) and total (t-) HDAC4 and HDAC5 for during OA treatment (A) and post-OA treatment (B). Data are mean ± SEM. * p < 0.05, ** p < 0.01 vs. T2D. n = 5-8 per group.

5.3.7 Changes in the Acetylation of Hepatic FoxO1 During and Post-OA Treatments

Acetylation of FoxO1 has been reported to reduce the expression of key enzymes for gluconeogenesis [266, 270, 271]. We reasoned whether or not the sustained equilibrium initiated by OA might result in the long-term acetylation of FoxO1. The acetylation of FoxO1 was markedly reduced in T2D mice compared to non-diabetic CH mice (1.00 ± 0.07 vs. 1.48 ± 0.08 of CH mice, p<0.05, n=5-8 per group). Indeed, treatment of OA restored the acetylation of FoxO1 at the specific residues of lysine 259, 262 and 271 (1.7-fold, p<0.01 vs. T2D) while concurrently reducing the total content of this protein in the liver (50%, p<0.01 vs. T2D, Figure 54A). The removal of OA did not impact on the increased acetylation or the reduced protein content of FoxO1 established by the treatment (Figure 54B). This indicates
that the sustained shift in equilibrium initiated by OA may result in the chronic acetylation of FoxO1 which in turns may down-regulate the gluconeogenic program in the liver.

Figure 54. Changes in the acetylation of FoxO1 in the liver during and post-OA treatments. Liver lysates from mice were immunoblotted with indicated antibodies. Representative immunoblots and quantifications of acetylated (ac-) and total (t-) FoxO1 and GAPDH for during OA treatment (A) and post-OA treatment (B). Data are mean ± SEM. * p < 0.05, ** p < 0.01 vs. T2D, n = 5-8 per group.

5.3.8 Effect of OA on Hepatic Glucose Production from Pyruvate

To determine whether OA effects on hepatic glucose output are shown in vivo, the effect of
OA on pyruvate tolerance in HF-fed mice was examined. As shown in Figure 55, HF feeding significantly caused pyruvate intolerance compared with CH mice with a 50% increase in the area under the curve of blood glucose (p<0.05). This increase was reduced to the normal levels in response to OA treatment. These results provide further evidence that the anti-hyperglycaemic properties of OA are most likely due to the inhibition of hepatic glucose production.

**Figure 55. Effect of OA treatment on pyruvate tolerance test in HF-fed mice.** CH: chow fed mice, HF: high fat fed mice, HF-OA: high-fat fed mice treated with OA (100 mg/kg in diet for 2 weeks). (A) Pyruvate tolerance test, intraperitoneal injection of sodium pyruvate (2.0 g/kg in 1x PBS) after overnight fasting. Blood glucose levels were measured at 0, 15, 30, 60 and 90 mins. (B) Incremental glucose area under the curve (iAUC). * p < 0.05, vs. HF, † p < 0.05 vs. CH. n = 6-8 per group.

**5.4 DISCUSSION**

The pentacyclic triterpenoid oleanolic acid (OA) has been shown to be effective in treating
various diseases such as diabetic nephropathy [257] and cancer [207] in humans. OA has shown significantly reduced triglyceride level in fully differentiated 3T3-L1 cells in Chapter 3 (YC5-4 in Figure 34). Moreover, our group have recently found that OA ameliorates hyperglycaemia in T2D mice beyond the cessation of its administration with the liver as a major target site [154]. However, the molecular mechanisms underlying the sustained anti-hyperglycaemic effects (metabolic memory) of OA remained unclear.

To investigate the mechanism underlying this persistent effect, the present study examined changes in key regulator on FoxO1 in the liver during the period of OA administration in diabetic mice in comparison with post-OA treatment. OA treatment (100mg/kg/day for 4 weeks) reduced hyperglycaemia in diabetic mice by ~87% and this effect was largely (~70%) maintained even 4 weeks after the cessation of OA administration. During the OA treatment, the acetylation and phosphorylation of FoxO1 in the liver were markedly increased (1.5 to 2.5-fold) while G6Pase mRNA was suppressed by ~80%. Histone acetyl-transferase 1 (HAT1) content was increased (>50%) and histone deacetylases (HDACs) 4 and 5 (not HDAC1) were reduced by 30%~50%. Consistent with this, OA treatment reversed pyruvate intolerance in high-fat fed mice. The OA-induced changes in FoxO1, G6Pase, HAT1 and HDACs persisted during the post OA treatment period where the increased phosphorylation of AMPK, SIRT1 content and the reduced liver triglyceride had subsided.

Hepatic gluconeogenesis is under the direct regulation of FoxO1 which mediates the expression of key genes of the gluconeogenic pathway including G6Pase [272]. G6Pase is a
rate-limiting enzyme controlling hepatic glucose production and this enzyme is largely regulated at the level of mRNA expression [273]. The transcriptional activity of FoxO1 is regulated by post-translational modifications which determine its subcellular location, molecular half-life, and/or DNA-binding activity [274]. Amongst these modifications, both phosphorylation and acetylation have been reported to dampen the transcriptional activity of FoxO1. Phosphorylation at serine 256 has been demonstrated to suppress FoxO1 transactivation by promoting its nuclear-to-cytosol shuttling [275]. On the other hand, acetylation at the various lysine residues has been found to attenuate the DNA binding activity of FoxO1 along with an increased sensitivity to the serine phosphorylation mediated by Akt [276, 277]. We found that OA treatment triggered a marked and persistent acetylation of FoxO1 at lysine 259, 262 and 271 residues. As FoxO1 is subjected to ubiquitination dependent proteasome degradation after its translocation into the cytoplasm [278], this may explain the reduced expression of this protein in response to OA treatment.

Acetylation is a dynamic process controlled by the enzymatic activity of HATs and HDACs [279], and this is crucial in the regulation of non-histone proteins, particularly members of the FoxO family [280]. Intriguingly, the increase in FoxO1 acetylation was sustained after the cessation of OA treatment and there was a matching increase in HAT1 expression as well as the serine phosphorylation of two class IIa HDACs, namely HDAC4 and HDAC5. It has been proposed that the phosphorylation of these HDAC proteins potentially provides docking sites for the chaperone proteins 14-3-3 which in turns promotes their nuclear export into the
cytosolic compartments where they remain inactive [281, 282]. Additionally, HATs may play a direct role in regulating FoxO1 independent of HDACs. For example, increased HAT activity acetylates (thus represses) FoxO-mediated responses in C2C12 cells in response to dexamethasone and starvation [283], which are known to increase hepatic gluconeogenesis. The concomitant increase in HAT1 expression and inhibition of HDACs by OA are likely to trigger a shift in the equilibrium of FoxO1 modification towards enhanced protein acetylation. Importantly, this shift in acetylation of FoxO1 is sustained beyond the period of OA treatment. Thus, our findings suggest acetylation may act in concert with phosphorylation to constitute a metabolic memory on FoxO1, repressing its transcriptional activity on gluconeogenic genes leading to the long-lasting glycaemic control in the OA-treated mice.

One of the most intriguing observations in the present study is that the changes in HDACs, HAT1, FoxO1 and G6Pase appeared to be memorized long after the direct action by OA per se. Although the sustained serine 256 phosphorylation of FoxO1 strongly correlates with the increased activity of Akt as well as the persistent improvement of glycaemic control, these changes are not due to increased blood levels of insulin during and post-treatment period of OA. HDACs have been suggested to play a regulatory role in physiological insulin action including the suppression of glucose uptake and glucose transporters expression in skeletal muscles [284, 285], and the reduction of the acetylation of the insulin receptor substrate, which interferes with the proper signal transduction of insulin in the liver [286]. Thus, this mechanism may explain the effects on glycaemic control during the period of OA treatment.
While the shifted equilibrium of HAT1 and HDACs activities might be the underlying mechanism, the exact cause for this shifted equilibrium is currently unknown. It is clear that the activated AMPK signalling was subsided following the cessation of OA treatment, which suggests that these effects may occur by a mechanism independent of chronic activation of the AMPK pathway. However, we cannot rule out that the activation of AMPK is only a prerequisite for the initiation of the metabolic memory but not required for sustained effects [267]. To support this hypothesis, activated AMPK has been reported to mediate the phosphorylation/acetylation of FoxO1 and the subsequent suppression of hepatic gluconeogenesis [64, 263]. Moreover, recent studies have shown that activation of AMPK can lead to acetylation and inhibition of FoxO1 via phosphorylation of HDAC 4 and 5, resulting in decreased gene expression of G6Pase [271, 287].

In the present study, the activation on AMPK per se had clearly subsided following the cessation of OA treatment as indicated by the phosphorylation AMPK and ACC together with the lipogenic enzymes under the regulation of AMPK. These results consistently indicate that the sustained changes in HAT1 and class IIa HADCs after the cessation of OA treatment do not require the simultaneous presence of chronic activation of AMPK. Thus far, we are not aware of similar report for other anti-diabetic agents, which activate AMPK by different mechanisms. Further studies are needed to investigate whether the activation of AMPK is a prerequisite for the initiation of the metabolic memory as reported for its effect on viral infection [267]. Hence, the exact role of AMPK activation, as well as the possible effects of
other AMPK-activators in the initiation of such metabolic memory warrants for further investigation.

Akt is another key regulator of FoxO1-induced suppression of hepatic gluconeogenesis [265]. OA-induced increase in Akt phosphorylation was also sustained for 4 weeks after the cessation of OA treatment. This suggests that the sustained activation of Akt may also mediate the suppression of FoxO1 and G6Pase expression. It has been reported that the phosphorylation of Akt can be increased by chronic inhibition of HDAC [288, 289]. The inhibition of HDAC4 and 5 (increased phosphorylation) in the present study is consistent with this notion.

In summary, the findings of the memorized changes in class IIa HDACs, acetylation and phosphorylation of FoxO1 provide novel insight into the mechanisms underlying the persistent anti-hyperglycaemic effects observed post cessation of OA treatment. These modifications constitute a metabolic memory at the post-translational level leading to a suppression of hepatic gluconeogenesis via FoxO1 inhibition. A proposed role of the hepatic HDACs/FoxO1 axis in the sustained reduction of hyperglycaemia post-OA treatment is presented in Figure 56. Although the proposed mechanism requires further study, these results suggest a potential of pentacyclic triterpenoid class of drugs as a long-lasting therapeutic of type 2 diabetes. In a broader sense, these mechanistic data on OA also provide a basis for targeting class IIa HDACs and FoxO1 in the gluconeogenic pathway for the sustained treatment of type 2 diabetes.
Figure 56. Proposed role of the hepatic HDACs/FoxO1 axis in the sustained correction of hyperglycaemia. **No treatment:** High-fat diet plus STZ injections induced typical stage of type 2 diabetes. Increased lipid accumulation leads to impaired insulin signalling pathway indicated by dephosphorylation of Akt, GSK3β and FoxO1. These dephosphorylations result in the increased expression of G6Pase causing hyperglycaemia; **During treatment:** Administration of oleanolic acid (OA) stimulates AMPK activity which may lead to an increased inhibitory phosphorylation of class Ila HDACs. The suppression of class Ila HDACs (possible reduction in the nucleus) induces acetylation and phosphorylation of FoxO1 (via IRS-1/Akt pathway) leading to its inactivation and this decreased the expression of G6Pase, resulting in reduced hepatic glucose production; **Post-OA treatment:** After removal of OA, the sustained inactivation of FoxO1 either by its acetylation and/or phosphorylation may contribute to the persistent anti-hyperglycaemic effect of OA on HF-STZ induced type 2 diabetic mice. The mechanism for the sustainability of these effects in the absence of AMPK activation during the period of post-OA treatment is yet to be determined.
CHAPTER 6
GENERAL DISCUSSION
AND FUTURE DIRECTIONS
This Chapter will summarise the major findings of the studies performed for this thesis, and reach the overall conclusions from these findings. I will conduct a critical review of my studies in comparison to the reports from the literature. From this analysis, this Chapter will highlight the novelties arising from this thesis and identify the gaps need to be addressed. Finally, based on the studies in this thesis, I will make proposal for future directions of this research.

6.1 MAJOR FINDINGS

The epidemic of type 2 diabetes has been increasing at an alarming rate worldwide [3] and insulin resistance is a fundamental metabolic disorder of this disease [47]. Therefore, the reversal of insulin resistance is critical for the treatment of type 2 diabetes and its associated complications. A high energy intake (particular in the form of fat) and low energy expenditure are major risk factors leading to insulin resistance and type 2 diabetes [47]. It has been widely recognised that the excessive lipid accumulation, particularly in the liver and muscle, is the common cause of insulin resistance [56]. Therefore, reducing tissue lipids, such as by dietary control, exercise and some anti-diabetic drugs, can improve insulin sensitivity as an effective treatment of type 2 diabetes [61].

As reviewed in Chapter 1, current medications for the treatment of type 2 diabetes are insufficient either due to the lack of long-term efficacy or various undesired adverse effects, such as cardiovascular disease and gastrointestinal disorder. It is therefore important to identify novel drugs to improve the treatment of type 2 diabetes. This thesis set to target the
most common form of insulin resistance - lipid-induced insulin resistance - for the studies of potential anti-diabetic therapeutics.

Natural products have been recently rejuvenated as an important source for the discovery of anti-diabetic drugs [137, 138] due to their rich source and chemical diversities [135]. This thesis focused on the natural products commonly used as the major ingredients in Traditional Chinese Medicine. This targeted approach was based on the following premises. Firstly, Traditional Chinese Medicine have been used for centuries to treat diseases including diabetes, hence there are already vast literatures available for data mining [290]. Secondly, there is less concern about toxicity of Traditional Chinese Medicine because they have been used in humans [135]. Thirdly, the natural products in Traditional Chinese Medicine can be screened against the relevant target or database by high-performance computer modelling [217]. Therefore, rather than screening for complex compositions, which causes great expenses and time as well as potential toxicity issues, it is essential to design a more mechanism and target-based screening method to guide the identification and separation of the active molecules from combined ingredients.

Based on the role of lipid accumulation in insulin resistance, I proposed that inhibition of the \textit{de novo} lipogenesis or enhancement of fatty acid oxidation can be an alternative approach to attenuate insulin resistance by reducing lipid accumulation. The general aim is to identify compounds from natural products in Traditional Chinese Medicine with lipid-lowering capacity may become potential candidates of novel anti-diabetic drugs.
6.1.1 Establishment of a Novel High-throughput Screening Assay

The first study presented in this thesis (Chapter 3) established a novel biochemical assay as a phenotypic screening tool to identify novel compounds with lipid-lowering efficacy in cells. This novel screening assay is capable of capturing compounds with anti-obese and anti-diabetic properties via different mechanisms. Effective compounds may act at various cellular targets in the upstream pathways of lipid metabolism which either increase fatty acid oxidation (energy expenditure) or inhibit \textit{de novo} lipogenesis.

Currently, target-based and phenotypic screenings are the main approaches for drug discovery. Targeted-based screening involves identifying a single molecular target against thousands of small molecules. The advantage of this screening relies on the existing knowledge of molecular and chemical to investigate specific molecular target, and it is often suitable for high-throughput screening [291]. However, target-based screening has extremely high attrition rate and it is limited by the complexity of diseases [292]. Therefore, this screening may not be sufficient to capture the pathophysiology of the disease. Phenotypic screening is cell-based approach which can overcome many of the limitations. The advantage of this screening is its potential to select compounds by modifying endpoint biological response rather than individual targets \textit{per se} [293]. Additionally, phenotypic screening yields higher successful rate. In the present study, we opted to develop a mechanism-based phenotypic screening assay to determine lipid accumulation in 3T3-L1 adipocytes.

As triglycerides are commonly used as an indicator of lipid levels in cells [62, 63], the
newly-developed method used potassium hydroxide (KOH) to extract triglycerides as an endpoint measurement. The triglyceride extraction methods most comparable to the present study are probably oil red O (ORO) staining [150] and chloroform/methanol (C/M) method [149]. Both of them are conventional and widely used methods for measurement of lipid content. ORO staining is commonly used to measure lipid content in adipocytes [150, 174]. However, there are concerns about the low specificity (semi-quantitative measurement) and inaccuracy (high background) of the results obtained from the ORO staining [150]. C/M method is another conventional method for triglyceride extraction, which is more accurate and quantitative than ORO staining. However, the recovery rate of triglyceride produced by this method is only 12%~18% (Figure 19). Furthermore, both of the conventional methods involve multiple processes which could lead to be time-consuming and intensive labour when used at a large scale (Figure 17). Therefore, based on the C/M method, we designed a simplified triglyceride extraction assay by using KOH based on its ability to lyse cells and hydrolyse triglycerides [166]. It has shown that the KOH extraction method is simple processes and time-saving (fewer steps), faster/less labour intense and higher recovery rate (88%~95%, Figure 19).

It is great of interest to discovery new anti-diabetic drugs by using high-throughput screening method [294]. While conventional high-throughput screening typically produces between 0.1%~1.0% success rate when a general compound library is tested against a particular molecular target or functional biological assay [295]. To improve the screening efficiency, we
created our unique library of selected compounds based on the available information from preliminary studies, computer modelling and function-structure relationship (conducted by Prof. Jun Xu) in a similar way as described [217, 296]. In terms of high-throughput screening, this screening method also allows us to measure a number of compounds simultaneously with multiple plates (48- or 96-well plate). This high-throughput screening has produced ~40% success rates of the identified hits in which compounds will be considered for further studies 

in vivo after analysing additional information (e.g. drug ability and novelty) (Figure 57).

**Figure 57. Schematic diagram for the drug discovery process from Traditional Chinese Medicine.** This diagram illustrates the drug discovery process by using the newly-developed high-throughput screening in 96-well plates for the identification of anti-diabetic leads by targeting Traditional Chinese Medicine. It also illustrates the consideration of additional information for the selection of promising compounds (leads) for further studies in vivo.

After validating the high-throughput screening assay by a series of biochemical and pharmacological tests, we applied this approach to screen more than 200 candidates selected from different classes compounds derived from our unique Traditional Chinese Medicine
library. Among them, 76 hits (>30% successful rate) were identified in reducing intracellular triglyceride. To test whether this new in vitro screening system may guide the identification of new leads with anti-diabetic and/or anti-obese efficacy in vivo, the therapeutic effects of two selected candidates (albiflorin and oleanolic acid) were examined in well-recognised mice models of insulin resistance and type 2 diabetes, respectively (as presented in Chapter 4 & 5).

In summary, the newly established phenotypic screening assay is able to accurately determine the intracellular triglycerides level in a high-throughput screening scale. Also, it serves as a base for further investigations of the identified compounds’ effects on fatty acid oxidation, uptake, synthesis and transport.

### 6.1.2 Potential Anti-diabetic and Anti-inflammatory Effects of Albiflorin

Insulin resistance has been recognized as a fundamental cause of type 2 diabetes [47]. In order to investigate the role of identified compound albiflorin in the treatment of type 2 diabetes, a lipid-induced insulin resistant mice model was employed for the first in vivo study which is presented in this thesis (Chapter 4). In the study, the high-fat (HF) and high-fat cholesterol (HFC) were used to produce insulin resistance and hepatic steatosis with inflammation, respectively [151, 152, 235].

Albiflorin, a monoterpenic glycoside, has been suggested to be one of the main bioactive constituents of Paeoniae Radix with a good oral absorption property and pharmacokinetics in vivo [219]. Albiflorin has been widely used in Traditional Chinese Medicine for its anti-biotic,
anti-inflammatory and anti-allergic properties with little adverse effects [220].

Several reports related to the present study have shown the beneficial effects of *Paeoniae Radix* for the treatment of type 2 diabetes. The most studied compound is paeoniflorin, one of the albiflorin analogues. Paeoniflorin significantly reduced cholesterol level in the hyperlipidaemic rats induced by HFC feeding [232]. In their study, they found that 4-week administration of paeoniflorin (200 mg/kg/day) reduced body weight gain, plasma triglyceride and cholesterol in control HFC-fed rats. Similar results observed in the present study, triglyceride as well as cholesterol levels were significantly reduced in the liver of albiflorin-treated HFC-fed mice (Section 4.3.2).

Furthermore, another group showed after 8-day administration of paeoniflorin (200 mg/kg body weight), it showed significant anti-hyperglycaemic effect at 30 mins during oral glucose tolerance test in neonatal streptozotocin-induced diabetic rats [231]. However, in the present study, 4-week administration of albiflorin in HFC-fed mice did not show improvement in glucose intolerance indicated by glucose tolerance test (Section 4.3.6). As a recent study in our laboratory showed that inclusion of cholesterol lessens insulin resistance, a further study was conducted in mice fed with a high-fat (HF) diet alone, a more established insulin resistant model [152, 235]. The results showed that albiflorin attenuated glucose intolerance in HF-fed mice (Section 4.3.7). The treatment of albiflorin reduced the fasting blood glucose suggesting liver might be the major target of albiflorin, because fasting blood glucose is mainly maintained by the liver. However, to further confirm the effects of albiflorin on insulin
Chapter Six – Future Directions

resistance, studies are required with more sensitive and reliable technique, such as hyperinsulinaemic-euglycaemic clamp with glucose tracers [66, 250].

In terms of inflammation, several groups have investigated the anti-inflammatory effects of paoniflorin. One study was conducted in immunological liver injury mice induced by tail vein injection of bacillus Calmette-Guérin (BCG) and lipopolysaccharide (LPS). Their data showed that 12-day treatment of paoniflorin (100 mg/kg) significantly decreased plasma ALT activities, attenuated the area and extent of necrosis and reduced the immigration of inflammatory cells, and reduced gene expression of TNFα in mice liver after BCG and LPS injection [227]. Another study showed that paoniflorin (12.5 µg/ml ~ 312.5 µg/ml) reduced gene expression of TNFα in synoviocytes isolated from rats with collagen-induced arthritis (CIA) [221]. Another study presented that 20-day administration of paoniflorin (10 mg/kg) can protect against LPS-induced rat liver inflammation [222]. Similarly, results observed in the present study clearly showed that albiflorin administration reduced the elevated plasma AST levels in HFC-fed mice. Moreover, albiflorin indicated its potential effect on anti-inflammation as it showed significantly effect on the reduction of TNFα gene expression in the liver (Section 4.3.5).

As albiflorin has showed its hepatic protective property as well as anti-inflammatory effect, it may be more suitable for its effects on anti-diabetes with liver complications. The detailed effects of albiflorin on hyperglycaemia and inflammation deserve further investigations as described in Section 6.3.
6.1.3 Sustained Anti-hyperglycaemic Effects of Oleanolic Acid in Type 2 Diabetic Mice

As hyperglycaemia is a major cause of the organ damages in type 2 diabetes, it is essential to control the hyperglycaemia effectively to prevent its serious consequences [252]. However, sustained control of hyperglycaemia in type 2 diabetes is still an unmet task by the current oral medications due to the gradual loss of efficacy and/or various adverse effects as discussed in Section 1.4.

Our recent study demonstrated that oleanolic acid (OA) is able to reduce hyperglycaemia and such effect is maintained even 4 weeks after the cessation of administration with liver as a major target site [154]. Moreover, the screening results showed OA exerted significant lipid-lowering efficacy in 3T3-L1 cells (Figure 34). The second in vivo study presented in this thesis (Chapter 5) further investigated the molecular mechanisms underlying the sustained anti-hyperglycaemic effects (metabolic memory) of OA in type 2 diabetic mice model induced by high-fat (HF) feeding with low doses of streptozotocin (STZ) injections.

The results showed that along with the attenuation of increased triglyceride accumulation in the liver, the administration of OA triggered a marked increase in the phosphorylation and acetylation of FoxO1 in type 2 diabetic mice. More importantly, these post-translational regulations were memorised, leading to the sustained inhibition of G6Pase gene expression, hence reducing hepatic glucose production (sustained correction of hyperglycaemia) well beyond the cessation of OA treatment with liver as a major responsible site. This mechanism
is supported by a subsequent study showing the suppression of the increased hepatic glucose production in HF-fed mice from the gluconeogenesis with pyruvate as the substrate (Figure 55). The persistent acetylation of FoxO1 is likely to be fostered by the action of histone acetyl-transferase 1 (HAT1) and histone deacetylases (HDACs) independent of AMPK.

Several mechanisms have been reported to be involved in the anti-diabetic effects of triterpenoids [255]. The anti-hyperglycaemic effects of OA observed in the present study is consistent with a recent study in db/db mice showing reduced hyperglycaemia and hepatic G6Pase expression in response to OA treatment [253]. Studies from several groups showed that OA has beneficial effects on the insulin signalling pathway by targeting AMPK [259] and GSK3β [297] which bring into line with our results. However, the findings of acetylation of FoxO1 from our present study demonstrate, for the first time, a plausible mechanism of the metabolic memory for the therapeutic effect of a pentacyclic triterpenoid on hepatic glucose metabolism and glycaemic control in a model of type 2 diabetes in mice.

6.2 SUMMARY & CONCLUSIONS

Based on the results obtained from this thesis, a number of conclusions can be drawn as follows:

- The newly-established phenotypic assay to determine triglyceride accumulation in cells is an effective and reliable high-throughput screening tool capable of identifying new drugs with anti-obese and anti-diabetic properties by reducing lipid accumulation via different mechanisms (increased fatty acid oxidation or decreased lipid synthesis).
• Albiflorin reduces the hyperlipidaemia caused by high-fat cholesterol. Albiflorin reduces fasting glucose level without reverse hyperglycaemia suggesting liver as its possible target site to reduce hepatic glucose production. Moreover, albiflorin could become a potential compound for the treatment of inflammatory conditions. Albiflorin attenuates hyperglycaemic in high-fat mice also indicating its therapeutic potential in type 2 diabetes.

• Oleanolic acid normalises the hyperglycaemia in type 2 diabetic mice, and its effects last beyond the treatment period due to inhibition of hepatic glucose production. The anti-hyperglycaemic effect observed in post-oleanolic acid treatment may result from the inactivation of the gluconeogenic regulator of FoxO1 via persistent acetylation and phosphorylation. Oleanolic acid may have a potential to become long-lasting therapeutic for type 2 diabetes.

Taken together, in vivo data further support the use of the newly-established high-throughput assay for the identification of new anti-diabetic and anti-obese compounds. Overall, the results shown in this thesis support my hypothesis which is compounds derived from Traditional Chinese Medicine with lipid-lowering efficacy may become potential candidates to improve insulin sensitivity and treatment of type 2 diabetes.

6.3 FUTURE DIRECTIONS

This thesis has presented a number of studies and several aspects arising from this work require further investigations.
6.3.1 Future Directions for Newly-developed Screening Assay

More than 200 compounds from our unique compound library have been screened by using the cell-based endpoint screening assay, which is the first line screening guiding us to select more suitable candidates. In addition, the present study provides a proof of concept for the use of this approach which could be extended to other cell types (such as HepG2 hepatocytes or L6 myocytes) to examine the effect on cell-specific lipid metabolism. Apart from the novel screening assay method, our lab has been using GLUT4 traslocation assay [298] to screened BBR dirivitaeves [142], ingredients from bitter melon and triterpenoids [165] with anti-diabetic or anti-obese proportites. Several other laboratories have also used GLUT4 traslocation assay to screen for anti-diabetic compounds [299, 300].

Based on all the available results for each compound obtaining from us or others, it would be useful to generate a database for our compound library, which will guide us to select suitable compounds for further investigations. Moreover, it is necessary to extend the application of our novel screening method to other traditional medicine (e.g. Traditional Indian Medicine). This will help us to explore more targeted compounds with anti-obese, anti-diabetic or anti-inflammatory properties.

6.3.2 Future Directions for Albiflorin Study

The results showed in Chapter 4, firstly, in order to better characterise the effect of albiflorin on insulin resistance, a hyperinsulinaemic-euglycaemic clamp with continuous infusion of glucose tracer experiment is needed [66, 250, 301]. Hyperinsulinaemic-euglycaemic clamp is
regarded as the gold standard technique to assess insulin action on glucose metabolism \textit{in vivo} [302]. This technique allows the assessment of \textit{in vivo} insulin action at an elevated level of plasma insulin within the physiological range by a constant infusion of insulin. By matching the hyperinsulinaemic levels among groups, the confounding factor of different degree of insulin stimulation (as occurs in a glucose tolerance test) will be avoided. As the blood glucose level is also clamped at an equal and normal level, the glucose turnover can be accurately determined by the combined use of glucose tracers (e.g. 14-glucose and 3H-2-deoxy-glucose) based on the Steal's equation to examine insulin action on endogenous glucose production (mainly from the liver) and glucose disposal (mainly in the muscle) [301]. The glucose tracer (e.g. 3H-2-deoxy-glucose) trapped in individual tissue such as skeletal muscle and adipose tissue can be measured to determine insulin action at the tissue level [303]. As this technique is labour-intensive and low output, it is usually used for highly promising candidates. The results from this thesis provide a strong rationale for the use of this technique for albiflorin.

Secondly, a burning question that could not be addressed was: How to explain the contradiction of triglyceride, AST and morphology data? It has been shown that albiflorin reduced the elevated hepatic triglyceride and plasma AST levels in the high-fat cholesterol mice. However, albiflorin did not present improvement on the liver morphology observed by H & E staining. Other staining methods, such as oil red O staining or Masson’s trichrome staining, is necessary for further confirmation of the histological changes among each group.
In addition, to confirm the effects of albiflorin on the liver function, a typical animal model of non-alcoholic steatohepatitis (NASH) is needed, such as choline-deficient animal model.

The third interesting question is the mechanism by addressing albiflorin may have potential effects on anti-inflammatory conditions. Albiflorin reduced the plasma AST level and gene expression of TNFα in HFC-fed mice, and the other steps of inflammation pathway would be important to investigate. In this thesis, we used insulin resistance mice model induced by high-fat cholesterol feeding. However, to investigate the anti-inflammation effect of albiflorin, a typical inflammatory mice model is needed, such as lipopolysaccharide (LPS) injection induced inflammation animal model.

### 6.3.3 Future Directions for Oleanolic Acid Study

Chapter 5 demonstrated the sustained improvement in hyperglycaemia after the treatment of oleanolic acid (OA). Several aspects have been arisen from this study and demanded further investigations. First, an intriguing question is whether AMPK activation is required to initiate the sustained anti-diabetic property of OA. The AMPK knockout mice or cell-based model can be conducted to examine the role of AMPK on the sustained anti-hyperglycaemia effects of OA. A comparative study with other activators (e.g. metformin) or inhibitors (e.g. compound C) of AMPK would be potentially informative.

Second, another two questions to be addressed are how OA promotes acetylation of FoxO1 and whether the acetylation site is specific to FoxO1. Intervention studies by using Class IIa HDACs inhibitors in cell-based experiments could confirm whether OA promotes acetylation
of FoxO1 via Class IIa HDACs or any specific HDAC. An immunoprecipitation experiment could be performed to answer the latter question. Third, although OA presented effects on the acetylation of FoxO1, this acetylation is not likely the first target of OA. To identify the primary target, chemical proteomics using Mass Spectroscopic Proteomic Analysis approach will be helpful [304].

Additionally, a functional assay is needed to determine the sustained effect of OA on reducing hepatic glucose production. For example, pyruvate tolerance test could be performed after the removal of OA administration to confirm whether reduction of glucose production is sustained. Another functional assay can be performed to further confirm hepatic glucose production by using tracer for gluconeogenic substrates, such as glycerol and lactate [305, 306]. Finally, in this thesis, it is important to investigate the sustained anti-hyperglycaemic effect of OA in other animal models, such as db/db model, which could extend the application of OA in different diabetic animal model for the treatment of type 2 diabetes.

### 6.3.4 Advantages, Limitations and Future Solutions

One of biggest bottleneck in a new drug development from the conventional approach (bench to bedside) is the unpredicted adverse effects in humans. The approach adopted in this thesis started with natural products which have already been exposed to humans for various other conditions. This approach for bedside (known usage) - bench - bedside (new application) (also described as drug repurposing) has an advantage to overcome the drug development bottlenecks [307] because of the exiting knowledge of the adverse effects in humans.
However, cautions still need to be exercised because the tolerable adverse effects for the current clinical conditions may not necessarily be the same for the potential new therapeutic applications for metabolism disease. This is an obvious limitation at this stage.

In this study, albiflorin and OA have been investigated in the treatment of insulin resistance and type 2 diabetes, respectively. Albiflorin has been approved to use clinically in Traditional Chinese Medicine for its beneficial pharmacological actions with little adverse effects [220]. OA has been used in humans for its potential therapeutic application for cancer [207] which condition may not be necessarily more serious than insulin resistance or type 2 diabetes. In addition, the studies in mice did not show any signs of physical or behavioural abnormalities of albiflorin and OA for the intended metabolic effects. New chronic toxicity studies in relation to the therapeutic effects on diabetes in animals need to be performed. The data presented in this thesis also provide strong reasons to conduct further research for this particular purpose. Ultimately, specific clinical trials are required to determine whether or not the identified new therapeutics from this thesis (particularly albiflorin and OA) would be suitable as a treatment for insulin resistance or type 2 diabetes and for which types of patient populations. To this end, it is imperative to conduct rigorous studies for these intended new applications in the well-controlled laboratory conditions to guide such future clinical trials.

In the sense of drug development, the findings from this thesis have provided the proof of principle for targeting the natural products in traditional Chinese medicine for the discovery of potential new anti-diabetic drugs.
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PUBLICATION
Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds

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A B S T R A C T

Reducing lipid accumulation in insulin target tissues is critical for the treatment of type 2 diabetes. This study aimed to develop a biochemical assay in cells for high throughput (HTP) screening of anti-diabetic drugs by reducing lipid accumulation via different mechanisms. We designed a new method to extract triglyceride (TG) with KOH to allow biochemical quantification of TGs for HTP screening in 3T3-L1 cells. This new method was validated for its biochemical properties with identical results of TG obtained with or without KOH (r2 = 0.9978, p < 0.001) and a fourfold improvement in TG extraction recovery rate (88–95%, p < 0.001) as compared to the conventional chloroform/methanol extraction (12–18%). The ability of this phenotype screening to capture potential anti-diabetic drugs was verified by pharmacological agents well known to alter lipid accumulation by different mechanisms including AMPK activators, fatty acid synthesis inhibitors, PPARγ activator and several lipogenic substrates. To further demonstrate the application of this screening tool for discovery of new anti-diabetic drugs, we screened >200 new candidates selected from Chinese medicine and identified 49 compounds from different classes which reduced TG content by >50% at 1 μM or >75% at 10 μM. Finally, we tested two selected leads (albiflornin and oxamtramine) in vivo and confirmed their efficacy in reducing visceral adiposity, glucose intolerance and hepatic steatosis in high fat-fed or high fructose-fed mice. Our results indicate that screening for the efficacy on lipid accumulation in cells by biochemical quantification of TGs with KOH extraction is an effective tool for the identification of new anti-diabetic compounds.

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1. Introduction

The prevalence of type 2 diabetes (T2D) is increasing at an alarming rate worldwide largely due to the increased prevalence of obesity. It has been estimated that there are around 200 million people suffering from T2D, and this figure is expected to increase to over 320 million by 2030 [1]. It has been established that insulin resistance (a reduced responsiveness of tissues, i.e. muscle and liver, to insulin) is a major factor linking obesity (particularly central adiposity) and T2D. At the pre-diabetic (insulin resistance alone) stage, blood glucose is maintained relatively normal via a compensatory increase in insulin secretion by the pancreas. However, prolonged insulin resistance may eventually result in beta-cell failure and the circulating insulin levels become insufficient to control blood glucose, leading to overt hyperglycaemia and the development of diabetes-associated complications.

Triglycerides (TGs) are commonly used as indicators of intracellular accumulation of lipids. Although TGs do not directly cause insulin resistance by themselves alone, they constitute the major storage of lipid intermediates that can interrupt insulin action by multiple mechanisms. For example, the intracellular forms of fatty acids (FAs) and long-chain fatty acyl-CoAs can inhibit hexokinase and glycogen synthase [2,3]; diacylglycerols can block insulin signalling pathways by promoting protein kinase C-dependent serine/threonine phosphorylation of IRS-1 [4]; furthermore, ceramide can dephosphorylate Akt and thus inhibiting its activity [5]. The toxicity of these lipid metabolites can also
trigger other mechanisms including pro-inflammatory cytokines to exacerbate insulin resistance at a later stage [6]. Therefore, reducing ectopic lipid accumulation has been shown to reverse insulin resistance and obesity.

The cellular level of TG is determined by de novo lipogenesis (mainly by FA synthesis) and FA oxidation (mainly in mitochondria). One of the most attractive mechanisms to promote a reduction on intracellular lipid levels is the activation of AMP-activated protein kinase (AMPK). This protein phosphorylates and inhibits the activity of acetyl-CoA carboxylase (ACC). ACC mediates the first step in the de novo synthesis of fatty acids and simultaneously, its product malonyl-CoA blocks the entry of FAs in the mitochondria for oxidation by inhibiting the carnitine palmitoyltransferase shuttle system. Thus, AMPK has been an attractive target for the discovery of drugs with potential efficacy for T2D and obesity [7]. The other mechanism to reduce cellular TG level is the inhibition of FA synthesis, particularly in adipose tissue and liver, because these tissues exhibit elevated de novo lipogenesis capacity that can exacerbate lipid accumulation. Indeed, a number of compounds acting at key steps of the lipid pathway have been shown to reduce obese mice's hepatic steatosis and/or insulin resistance, such as specific inhibitors of fatty acid synthase C75 and cerulinin [8], ACC inhibitor 5-(tetradecylxoy)-2-furanacryloylic acid (TOFA) [9] or sterol regulatory element-binding protein-1c (SREBP-1c) [10].

Here, we established a HTP phenotypic screening assay with a cellular TG level as a measurement capable of capturing the lipid-lowering efficacy from actions at different sites in lipid metabolic pathways. This newly developed screening assay was made possible by using KOH based on its ability to lyse cells and hydrolyse TGs [11] to overcome the obstacles encountered with the conventional organic solvent. Following the validation by a series biochemical and pharmacological tests, we used this HTP assay to screen more than 200 candidates selected from different classes of compounds from traditional Chinese medicine (TCM). Our results identified over 40 potential anti-diabetic compounds and among them we confirmed the efficacy of newly identified compounds alibofirin in high-fat-fed mice and oxymatrine in high fructose-fed mice. These findings provide strong support for the application of this screening assay for the discovery of novel anti-obese and anti-diabetic drugs.

2. Materials and methods

2.1. Regents, chemicals and test compounds

Insulin, dexamethasone, DMSO, biotin, 3-isobutylxanthine (IBMX), AICAR, berberine (BBR), oleic acid (OA), arctigenin (ATG), betulin, C75, cerulenin, 5-(tetradecylxoy)-2-furanacryloylic acid (TOFA), rosiglitazone (RSG), fructose, genipin (>98.0%), bile acids including cholic acid, deoxycholic acid and chenodeoxycholic acid, as well as chemicals and solvents used in various buffers were purchased from Sigma–Aldrich (Melbourne, Australia). Tissue culture reagents were obtained from Invitrogen (Melbourne, Australia). The compounds tested were kindly supplied by or Dr. LiHong Hu and Dr. Yang Ye of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China) and Dr Jun Xu of the School of Pharmaceutical Sciences, Sun Yat-sen University (Guangzhou, China). Specifically, alibofirin (98.0%) was obtained from Baoding Yunxin Trade (Hebei, China) and oxymatrine (98.0%) from Hangzhou Tianlong Biotechnology (Hangzhou, China). Tiliroside (>98.0%) was obtained from Meryer Chemical Technology (Shanghai, China). The derivatives of alibofirin, oxymatrine, berberine, genipin and tiliroside were developed at Shanghai Institute of Materia Medica by modifying their chemical structures. Triterenoids were obtained from the purification of the extracts of bitter melon as we recently described [12]. FXR ligands and other bile acid analogues were developed in Sun Yat-sen University. Other test compounds were obtained from the chemical library of Sun Yat-sen University after computer-based virtual screening against the targets for lipid metabolism [13]. To improve the likelihood of success and minimize the numbers for screening, we selected the candidates with a wide range of chemical diversity by one of the following virtual screening processes [12–14]. The first virtual screening was based on careful evaluation of documented (or reported) effects implicated for diabetes or related conditions as used in our recent identification of BBR and its derivatives from Golden Seal [14] and cucurbitane triterpenoids from Bitter Melon [12]. The second process involved computer modelling possible binding of test compounds to cellular targets, such as the farnesoid X receptor (FXR) (a regulator of lipid metabolism [15]), in the software available to us by the similar approach as described for the liver X receptor [13].

2.2. Cell culture

3T3-L1 mouse embryonic fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagles’s medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin-glutamine (PSG) at 37 °C in 5% CO2. The 3T3-L1 pre-adipocytes were seeded into 48- or 96-well micro-plates and allowed to reach full confluence. To induce pre-adipocyte differentiation, 2-day post-confluent cells were incubated in differentiation medium (DMEM containing 10% FBS, 1% PSG, 2 μg/ml insulin, 100 ng/ml dexamethasone, 500 μM BMX and 10 ng/ml biotin) for 3 days before switching to post-differentiation medium (DMEM with 10% FBS, 1% PSG and 2 μg/ml insulin) for additional 3 days. To assess their effects on lipid accumulation, the test compounds were dissolved in DMSO and supplemented at the concentrations indicated in the figure legends throughout the course of the differentiation. Test compounds were made as stock solutions and stored at –20 °C in aliquots. They were added on day 0 in differentiation medium (for 3 days) and replaced with another aliquot in post-differentiation medium on day 3 (for another 3 days). 3T3-L1 cells cultured in DMEM supplemented with 0.1% DMSO were used as a vehicle control for all experiments. Cells were monitored and photographed using the Nikon TS100F inverted microscope and camera (Nikon Instruments Inc., USA).

2.3. Design for a novel technique of TG extraction for biochemical assay

At the end of the 3-day post-differentiation medium incubation, the culture medium was removed and the cells were washed twice with ice-cold PBS buffer (0.2 M NaCl, 10 mM Na2HPO4, 3 mM KCl, 2 mM KH2PO4, pH 7.4), before subjected to drying in a 37 °C incubator (Racket Instruments, Australia). Cells were lysed and TGs were extracted by the addition of KOH (final concentration 50 mM) followed by incubation at 60 °C for 10 min and centrifuged at 4000 rpm for 5 min. For the conventional extraction method [16], cells were scraped immediately following the addition of the chloroform/methanol (2:1) solution. The cell lysate was mixed with 0.6% NaCl solution and centrifuged at 2000 rpm for 10 min. The bottom organic layer containing the TG was collected and dried under nitrogen gas at 45 °C. TG was reconstituted in ethanol for the measurement using a commercial Peridochrom TG GPO-PAP kit (Roche Diagnostics, Castle Hill, New South Wales, Australia) following the manufacturer's instructions in a spectrophotometer. To evaluate the influence of KOH on the enzymatic assay, the TG kit was tested in the presence of 50 mM KOH (used
for TG extraction) at various concentrations of intralipid (Sigma-Aldrich, Melbourne, Australia) as a TG donor [3,4]. The recovery rate of TG extraction from 3T3-L1 cells was assessed by adding known amounts of TGs from the intralipid before the extraction either by KOH or the C/M mixture.

2.4. Oil red O (ORO) staining

Oil red O (ORO) staining was used in combination with light microscopy to assess pre-adipocyte differentiation as previously described [17]. Briefly, the treated differentiated 3T3-L1 cells were washed twice with ice-cold PBS buffer and fixed in 10% neutral formalin overnight before staining with a 0.35% (w/v) ORO solution in isopropanol for 10 min. Excess stains were then removed by rinsing the cells under water and then dried before microscopic examination. For quantitative analysis, ORO was eluted with isopropanol and the absorbance was measured at 485 nm in a spectrophotometer.

2.5. Determination of lactate dehydrogenase (LDH) release

After cells were treated with compounds for 6 days, the medium and lysate were collected to measure the activity of lactate dehydrogenase (LDH) released into the culture medium from cells. The medium was collected and centrifuged at 1000 rpm at 4 °C for 10 min to remove detached cells. To measure the intracellular LDH activity, 3T3-L1 cells were lysed by two freeze-thaw cycles (chilling the plate at −80 °C for approximately 30 min followed by thawing at 37 °C for 15 min) after wash with ice-cold PBS buffer. The cell lysate was then centrifuged at 1000 rpm for 4 min, and the supernatant was stored at 4 °C until ready to use. The enzyme activity in the whole cell lysate and medium was determined by the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Melbourne, Australia) according to the manufacturer's protocol. Briefly, 50 μl of the diluted medium (1:5) or cell lysate (1:10) were transferred onto a plate followed by addition of the reconstituted substrate mixture. The absorbance of the samples was read at 490 nm using the Polarstar Optima microplate reader (BMG Labtech, Germany). LDH released into the medium was expressed as a percentage of the total LDH activity (ratio of LDH in lysate and in medium) as described.

2.6. Animal studies

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. Animals were randomly assigned to receive a high fat (60%) diet (HFD) ad libitum to generate insulin resistance [12,14] or a high fructose (35% fructose and 35% starch) diet (HFrU) to generate hepatic steatosis [18]. Animals were fed for 8 weeks with or without albutin (AF, 100 mg/(kg day) in HFD), for the final 2 weeks. In a separate study, HFrU-fed mice received oxymatrine (OM, 100 mg/(kg day) in diet, 1 week) after 3-day HFrU-feeding (unless indicated otherwise). Body weight, food intake and fasting blood glucose level were monitored on weekly basis. After two weeks of AF treatment in HFD mice, glucose tolerance test (glucose load 2 g/kg BW, i.p.) was performed after 6-h fasting. Glucose level of blood collected from the tail vein was measured at designed time points using the Accu-Chek glucometer (Roche Diagnostics, Castle Hill, New South Wales, Australia). The effect of OM on glucose tolerance in HFrU-fed mice was assessed after 2 weeks of treatment at a glucose load of 3 g/kg (i.p.) based on our previous studies [18] All animal experiments were approved by the Animal Ethics Committee of RMIT University (Approval No #1012) following the guidelines issued by the National Health and Medical Research Council of Australia.

2.7. Statistical analyses

Data were presented as means ± SE. Unpaired student test was performed for comparison of relevant groups. Pearson’s two-sided correlation was used for correlation calculation. Differences at p < 0.05 were considered to be statistically significant.

3. Results

3.1. Validation of the novel TG extraction method for HTP biochemical assay

The conventional method for the extraction of TGs from tissues with organic solvents requires a series of steps including the separation of organic and aqueous phases [16]. Thus we designed a one-step method to extract TG with the use of KOH based on its ability to lyse cells and hydrolyse TGs [11] (Fig. 1). We first tested whether or not KOH may influence the biochemical assay. Fig. 2A shows that at the concentration (50 mM) designed for TG extraction KOH did not have any significant influence on the biochemical assay for the quantification of TG. The obtained results in the presence of KOH were almost identical to those obtained in the absence of KOH as indicated by a high degree of linear correlation (Y = 1.1416X, r² = 0.9978, p < 0.001). We next examined if KOH could extract a similar amount of TGs from cells, by adding 1.0 or 2.5 μg TG (50-100% of TG from 3T3-L1 adipocytes) to a cell culture plate. As shown in Fig. 2B, the recovery rates of TGs extracted with KOH at these two concentrations of added TG were 87.8 ± 4.6% and 95.1 ± 0.5%, respectively. These recovery rates were approximately four times of those extracted by C/M (12.1 ± 8.9 and 18.0 ± 8.4%, respectively, p < 0.001). Furthermore, the obtained values of TGs extracted with KOH were quantitative in proportion to amount added (0.88 ± 0.05 μg from 1.0 μg and 2.4 ± 0.01 μg from 2.5 μg) (Fig. 2C).

![Diagram](image-url)

Fig. 1. Design of KOH extraction for biochemical assay of TG content in 3T3-L1 cells for high throughput (HTP) drug screening. (A) ORO staining for the quantification of cell triglyceride (TG) content. Multiple procedures involved in the ORO method which usually required two days. (B) Biochemical determination of TG using the conventional extraction with chloroform/methanol (C/M). Multiple steps involved including separation, drying and reconstitution which usually requires one full day. (C) Designed method for TG extraction and cell lysis with KOH. This new method intended to avoid multiple steps, shorten the time to ~2 h and allow miniaturization of the biochemical assay of TG for HTP drug screening.
3.2. Validation of the detectability of TG altered by known pharmacologic agents

In order to verify the ability of this new assay as an end-point measurement to capture the effect on lipid accumulation induced by different upstream mechanisms, we tested a number of pharmacological agents well known to promote FA oxidation, inhibit or enhance lipogenesis in our newly developed assay system. As expected, the AMPK activators AICAR [19], BBR [14], oleoanolic acid (OA) [12], arctigenin (ATG) [20] all reduced the TG levels significantly (p < 0.01) at 1 and/or 10 μM (Fig. 3A). Detailed examination revealed a dose-depend efficacy for these agents.
(Fig. S1). Similar results were observed after the incubation with C75 and cerulenin (specific FAS inhibitors [9]), betulin (specific SREBP-1c inhibitor [10]), and tetracyclolxyloxyacetic acid (TTOA, specific ACC inhibitor [9]) (all \( p < 0.05 \), Fig. 3A). In contrast, incubation with 20 mM fructose (Fru, lipogenic substrate [21]) or 1–10 \( \mu \)M rosiglitazone (RSG, specific PPAR\( \gamma \) agonist [22]) increased TG accumulation by more than 150% (\( p < 0.05 \) for both).

To exclude a possibility of the decrease in TG content resulting from cytotoxicity, we determined the LDH activity in the cultured media as an indicator of cell damage. Fig. 3B shows that apart from the higher concentration (10 \( \mu \)M) of BBR and betulin which moderately elevated the LDH activity to the similar levels of Fru and RSG, there was no significant increase in LDH activity for other test agents at the concentrations which reduced TG content. Consistent with the results of LDH, the cells displayed a healthy morphology in response to these treatments (Fig. S2).

3.3. Comparison with results obtained from ORO staining

As ORO staining is widely used to screen the changes in lipid content in cultured cells [17], we compared the values of intracellular lipid content obtained from new method with the semi-quantitative ORO method. As shown in Fig. 4A, the ORO staining method confirmed all the responses we detected using our new screening assay. The obtained values of TG were all significantly reduced by lipogenesis inhibitors BBR, betulin, C75, cerulenin and TTOA (\( > 40\% \)) but increased by Fru and RSG (\( > 130\% \)) under the experimental conditions (all \( p < 0.01 \)). The mean values of results obtained from these two assays were significantly correlated (\( Y = 1.0996X, r^2 = 0.9032, p < 0.001 \)) (Fig. 4B).

3.4. Screening of test compounds for their effects on TG accumulation in 3T3-L1 adipocytes

Following the biochemical and pharmacological validations as described above, we applied this new screening protocol in 48-well plates (as illustrated in Fig. 5A) in an attempt to identify potential lipid-lowering drugs by targeting the compounds developed from traditional Chinese medicine (TCM). Among the 207 candidates selected for the screening, we detected 49 hits (compounds with efficacy in reducing TG accumulation by \( > 50\% \) at 1 \( \mu \)M or \( > 75\% \) at 10 \( \mu \)M) (Fig. 5B). These identified hits included several classes of compounds with different core structures, particularly derivatives of BBR (Fig. 6A), triterpenoids (Fig. 6B), isoroside (Fig. 6C), oxytomatine (Fig. 6D), albiflorin (Fig. 6E) and ligands for farnesoid X receptor (FXR, Fig. 6F).

3.5. Demonstration of therapeutic effects in vivo of leads selected from the identified hits

We next tested if our new screening system may guide the identification of new leads with anti-diabetic and/or anti-steatotic efficacy in vivo by confirming the therapeutic effect of the selected candidates in a well-recognized model of insulin resistance and obesity (HFD), and hepatic steatosis (HFrU), respectively. Based on the screening results (illustrated in Fig. 5), we elected to examine albiflorin (AF, an analogue of paoniflorin). AF has been suggested to be a key ingredient of Paonia radix which has been shown to exert acute effect in reducing blood glucose in diabetic mice [23]. As shown in Table 1, HFD mice with oral administration of AF at 100 mg/(kg day) (based on the reported i.p. dose of 30 mg/kg used

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**Fig. 5.** Application of the new HTP screening for identification of anti-diabetic compounds from TCM. (A) Schematic diagram for the drug discovery process using the newly developed HTP screening in 96-well plates for the identification of anti-diabetic leads by targeting TCM. It also illustrates the consideration of additional information for the selection of promising compounds (leads) for further studies in vivo. (B) Overview of the numbers of compounds identified (namely hits) from different classes. A hit is defined as a compound with the efficacy in lowering TG for \( > 50\% \) at 1 \( \mu \)M or \( > 75\% \) at 10 \( \mu \)M. Results were obtained from three independent experiments.
in mice [23] for 2 weeks had significantly lesser gain of body weight (by 45%, p < 0.01) and visceral adiposity (indicated by a 68% reduction in epididymal fat mass, p < 0.01) compared with control HFD mice. These effects occurred in the absence of any influence on caloric intake (p > 0.05). Consistent with the efficacy on obesity, AF treatment significantly improved glucose tolerance, as indicated by 43% reduction (p < 0.01) in the area under the curve (iAUC) for blood glucose during the ipGTT. To further test the application of new screening system, we examined the effect of oxymatrine (OM) because this alkaloid (rich in *Sophora flavescens*) has been used in humans as a safe oral drug in treatment of hepatic fibrosis [24]. As shown in Table 2, oral administration of OM for one week was able to...
to reduce body weight gain (p < 0.05) and eliminating hepatic steatosis (p < 0.05) in HFrEF fed mice, a model of fatty liver due induced by de novo lipogenesis [18]. After 2 weeks of administration of OM, glucose tolerance was significantly improved (AUC: 1050 ± 92 vs 1260 ± 60 mmol h -1). p < 0.05, n = 8/group).

4. Discussion

As excess accumulation of lipids in cells is a hallmark of obesity and insulin resistance [25], elimination of elevated cellular lipid levels is an effective approach for the treatment of these pathological conditions [2–4]. Based on this concept, we established a biochemical assay of TGs in cells as an HTP screening tool by designing a new lipid extraction method in order to identify compounds with potential efficacy for obesity and associated metabolic diseases. With this newly developed screening tool, we screened 207 compounds selected from two TCM banks based on extensive evaluation of TCM database [12,14] and/or computer modelling [13,26]. Among them, 49 showed an efficacy in reducing TG by >50% at 1 μM or >75% at 10 μM, which we regarded as identified hits. Based on their drug-like properties and sufficient quantity available to us, we demonstrated the in vivo anti-diabetic and anti-steatotic effects of two novel leads (AF and OM) identified from these hits. Our results provide the proof of concept for the use of this HTP biochemical screening tool for the discovery of novel anti-diabetic drugs.

Up till now, the ORO staining has been commonly used in adipocytes to screen the efficacy on lipid content [17], particular for drugs promoting cell differentiation such as PPARy agonists [22]. However, this method involves multiple processes which are time consuming and can be labour intensive when used at a large scale. Furthermore, there is concern about the specificity and the inaccuracy of the results obtained from the ORO staining [17]. Therefore, it would be advantageous to develop an HTP screening for the cell TG levels based on a biochemical assay of TG widely used for in plasma and tissue samples. To overcome the technical obstacles in applying the biochemical assay for HTP screening, we designed a method of TG extraction using KOH based on its ability to lyse cells and hydrolyze TGs [11]. This new method provides advantages over the organic extraction that requires multiple steps, allowing the biochemical assay performed in a micro-plate for HTP drug screening. Importantly, KOH extraction produced an almost full recovery of TG (88–95%), which is far more superior to the 12–18% of recovery obtained by the C/M method. Our new procedure also substantially improves the efficiency of whole TG assay (approximately 2 h) compared to the ORO staining and the C/M extraction method, which usually require much longer time.

Cellular TG level can be regulated by either the lipogenic and FA oxidative pathways. The HTP assay established with the new extract method was able to capture the expected effects of well-defined pharmacological agents in lipid metabolism. Among them, AICAR, BBR, bitter melon triterpenoid and ATG have been demonstrated to activate AMPK in previous studies including ours [12,14,19,20]. Activated AMPK can reduce cellular lipid accumulation by promoting FA oxidation [27] and inhibiting lipogenesis [28]. Decreased TG was also evident, as predicted, in the presence of lipogenic inhibitors acting at the site of SREBP-1c (betulin, [10]), ACC (TOFA [9]) or FAS (C75 and cerulenin [9]). Furthermore, our results also showed that our screening system was sensitive in detecting increased TG accumulation in response to lipogenic substrates (indicated by fructose [21]) and PPARy agonists (indicated by RSG [22]). All these results verify the suitability of this HTP screening to capture potential anti-diabetic drugs which alter lipid metabolism by acting at different cellular sites.

Recently, we identified two anti-diabetic compounds such as BBR and cucurbitane triterpenoids from the plants used in TCM for the treatment of diabetes and related conditions [12,14]. Compared with a random screening of natural products, TCM has been more fruitful resource as it enables us to perform careful prior analysis [12,14], as well as computation [13] to reduce the large numbers of compounds to a handful of highly selected candidates for biological screening. Using this approach, we selected 207 candidates representing a wide range of chemical diversity (for new discovery) or different structures of the same class of compounds (e.g. BBR derivatives) for the screening of novel anti-diabetic drugs using the method established in this study. Apart from obtaining detailed information for the new derivatives from BBR and TP which would allow for detailed structure–activity analysis, our results also confirmed a number of compounds which have been suggested to affect lipid metabolism including tilisolride [29], oxymatrine [30] and FXR ligands [13]. Collectively, these data demonstrate that the screening of the efficacy on cellular lipid accumulation is an effective tool to identify novel compounds with anti-diabetic properties.

Based on the screening data along with the analysis for their drug-like properties, we selected AF and OM for further studies in a model of insulin resistant induced by chronic high fat feeding and hepatic steatosis induced by high fructose feeding, respectively. AF is an active ingredient found in herbal remedies (e.g. Paeria radix) with a good oral absorption property and pharmacokinetics in vivo [31]. AF is widely used in TCM for its antibiotic, anti-inflammatory and anti-allergic properties with little adverse effects [32,33]. In addition, AF has been reported to lower blood glucose in hyperglycaemic mouse models without increasing pancreatic insulin secretion [34]. We found that AF was able to attenuate the increased body weight gain, visceral adiposity and glucose intolerance induced by chronic high fat feeding without significant effects on food intake, indicating its therapeutic potential for treatment of obesity and diabetes. We further tested the potential

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*Fig. 7. Illustration of the screening on lipid accumulation for identification of anti-diabetic and anti-obese drugs. As excess lipid accumulation is a major cause of insulin resistance in T2D and obesity, drugs that eliminate excess lipid accumulation (in liver and muscle) can improve glucose homeostasis by reversing lipid-induced insulin resistance. Similarly, reducing lipid accumulation in fat tissue can alleviate obesity. The present study has established a HTP assay in cells to determine TG accumulation as a phenotypic screening tool for the identification of new anti-diabetic and anti-obese drugs. This screening assay is capable of capturing drugs that act at various cellular targets in the upstream pathways of lipid metabolism which either increase FA oxidation (energy expenditure) or inhibit de novo lipogenesis.*