Validation and Mechanistic Studies of Gluconeogenesis Regulators Identified from Zebrafish Chemical Genetic Screens

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
University of Toronto

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2017

Abstract

Phosphoenolpyruvate carboxykinase (PEPCK) is the rate limiting enzyme in hepatic gluconeogenesis, which produces glucose from pyruvate to maintain circulating glucose level according to the body’s needs. Cytosolic PEPCK (pck1) is widely studied for its crucial role in hepatic gluconeogenesis. High levels of pck1 are associated with the development of type 2 diabetes; conversely, improved insulin sensitivity is observed in mice with decreased pck1 level. Ten potential lead compounds from previous screening of 727 FDA-approved compounds were examined using pck1 zebrafish reporter lines, Tg(pck1:luc2) and Tg(pck1:Venus). Compounds were tested in larval zebrafish and ranked based on their ability to regulate pck1 expression and glucose levels. Subsequently, a diabetic mouse model was used to further validate the potential anti-hyperglycemic effects of the selected compound (Esomeprazole, Eso). Eso treatment improved glucose homeostasis in diabetic mice as determined by pyruvate tolerance tests. Here we describe a possible mechanism of action of Eso, which involves regulation of hepatic gluconeogenesis through insulin-dependent down-regulation of pck1.
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Table of Contents

Acknowledgments........................................................................................................................................iii

Table of Contents.........................................................................................................................................iv

List of Abbreviations .....................................................................................................................................ix

List of Tables ................................................................................................................................................xvi

List of Figures ................................................................................................................................................xvii

List of Appendices .........................................................................................................................................xix

Chapter 1 .....................................................................................................................................................1

1 Introduction ...............................................................................................................................................1

1.1 Diabetes mellitus...................................................................................................................................1

1.1.1 Metabolic syndrome and diabetes mellitus .....................................................................................1

1.1.2 Complications of diabetes mellitus .................................................................................................2

1.1.3 Etiology and pathophysiology of type 2 diabetes mellitus ............................................................2

1.1.4 History of type 2 diabetes medications ............................................................................................7

1.2 Drug development ................................................................................................................................13

1.2.1 Drug discovery ................................................................................................................................13

1.2.2 Drug repurposing and its advantages ...............................................................................................15

1.3 Zebrafish as an in vivo model organism .............................................................................................16

1.3.1 Zebrafish background .....................................................................................................................16

1.3.2 Zebrafish embryos for high throughput screening (HTS) ................................................................16

1.3.3 Zebrafish as a model for human diseases .........................................................................................17

1.3.4 Glucose homeostasis in zebrafish and its suitability as a model for glucose metabolism .................18

1.3.5 Pancreas and liver development in zebrafish ..................................................................................20
3.3 Examination and ranking of the ten lead compounds ................................................. 37
   3.3.1 Luciferase assay ........................................................................................................ 37
   3.3.2 Fluorescence reading ............................................................................................... 37
   3.3.3 Glucose assay .......................................................................................................... 38
   3.3.4 Glucose assay and luciferase assay for dose response curves ............................. 38
   3.3.5 Fluorescence imaging ............................................................................................. 39
3.4 RNA extraction .............................................................................................................. 39
   3.4.1 Zebrafish ................................................................................................................ 39
   3.4.2 Mouse ..................................................................................................................... 40
3.5 RT-qPCR ....................................................................................................................... 40
   3.5.1 Zebrafish and mouse .............................................................................................. 40
3.6 Protein extraction ......................................................................................................... 41
3.7 Western blot ................................................................................................................ 41
3.8 Intraperitoneal pyruvate tolerance test ........................................................................ 41
3.9 Serum gastrin and insulin measurement ...................................................................... 41
3.10 Statistical analysis ...................................................................................................... 42
Chapter 4 .......................................................................................................................... 43
4 Results .............................................................................................................................. 43
   4.1 Examination and ranking of the ten lead compounds ............................................. 43
      4.1.1 Luminescence and fluorescence readings identified significantly reduced pck1 expression levels by compounds A, Epi, Fluo, Eso .................................................. 43
      4.1.2 Real-time PCR (qPCR) analysis identified significantly reduced pck1 mRNA levels by compounds Tol, Eso, C, A, and Dau ......................................................... 45
      4.1.3 Whole body free glucose levels were significantly reduced by compounds Epi, Eso, A and I ........................................................................................................ 46
      4.1.4 Compounds A, Epi, Eso, and Tol showed pronounced pck1 and glucose lowering effects .......................................................... 46
4.2 Comparison studies for the top three lead compounds: Epi, Eso, and Tol

4.2.1 Fluorescent liver images of the top three lead compounds: Epi, Eso, and Tol

4.2.2 Compound Epi showed a dose-dependent reduction of pck1 expression while compounds Epi and Eso decreased glucose levels in a dose-dependent manner.

4.3 Validation of esomeprazole (Eso) in mouse models

4.3.1 Eso treatment in normal mice shows euglycemia but reduced pck1 levels, and increased serum gastrin and insulin levels.

4.3.2 Eso treatment in diabetic mice shows improved glycemic control with reduced pck1 and nuclear FoxO-1 levels, and increased serum gastrin and insulin levels.

Chapter 5

5 Discussion

5.1 Zebrafish studies identified esomeprazole (Eso) as effective at lowering glucose levels through regulating pck1.

5.1.1 Ten lead compounds were narrowed down to three: Epi, Eso, and Tol.

5.1.2 Results from fluorescence screening correspond to the fluorescence liver imaging.

5.1.3 Dose response assessment of the top three lead compounds.

5.2 Mouse studies validated the potential anti-diabetic effects of esomeprazole (Eso).

5.2.1 Esomeprazole enhances glycemic control under hyperglycemic condition but does not cause hypoglycemia under normal condition.

5.2.2 Esomeprazole promotes insulin secretion through increasing gastrin levels.

5.2.3 Eso treatment in normal mice suggests involvement of other factors in maintaining glucose homeostasis.

5.2.4 Eso treatment in diabetic mice enhances glucose control through regulating pck1.

5.2.5 Eso treatment at 3mg/kg BW in diabetic mice suggests insulin-independent pathways in maintaining glucose homeostasis.

5.3 Four lead compounds

5.3.1 Amlexanox (A - #1)
5.3.2 Epirubicin (Epi - #2) ........................................................................................................68
5.3.3 Esomeprazole (Eso - #3) ................................................................................................69
5.3.4 Tolterodine (Tol - #4) ....................................................................................................70
5.4 Limitations .........................................................................................................................71
  5.4.1 Zebrafish studies ..........................................................................................................71
  5.4.2 Mouse studies .............................................................................................................71

Chapter 6 ..................................................................................................................................73
6 Conclusion ...............................................................................................................................73

Chapter 7 ..................................................................................................................................75
7 Future directions ...................................................................................................................75

Bibliography .............................................................................................................................77

Appendices ................................................................................................................................85
  Levofloxacin (C - #5) ............................................................................................................87
  Dicloxacillin (L - #6) ..........................................................................................................87
  Fluorocytosine (Fluo - #6) ..................................................................................................88
  Daunorubicin (Dau - #8) ....................................................................................................88
  Naproxen (I - #9) ...............................................................................................................88
  Zolpidem (Zol - #10) .........................................................................................................88
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCC8</td>
<td>ATP-binding cassette, sub-family C, member 8</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-coenzyme A carboxykinase</td>
</tr>
<tr>
<td>ACS</td>
<td>acetyl CoA-synthase</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, excretion</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor 2</td>
</tr>
<tr>
<td>ATZ</td>
<td>atrazine</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>C/EBPβ</td>
<td>CCAAT enhancer-binding protein β</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CKK-B</td>
<td>cholecystokinin B</td>
</tr>
<tr>
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<td>carbon dioxide</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRTC2</td>
<td>CREB regulated transcription coactivator 2</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
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<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>DPP-4</td>
<td>dipeptidyl peptidase 4</td>
</tr>
<tr>
<td>ECL</td>
<td>enterochromaffin-like</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EWAS</td>
<td>environmental-wide association study</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport protein</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FoxO-1</td>
<td>forkhead box protein O1</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose-dependent insulinoitropic polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide 1</td>
</tr>
<tr>
<td>GLP-1 RA</td>
<td>glucagon-like peptide-1 receptor agonist</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HFD</td>
<td>high fat diet</td>
</tr>
<tr>
<td>HBF-3β</td>
<td>hepatocyte nuclear factor 3-beta</td>
</tr>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screening</td>
</tr>
<tr>
<td>ICM</td>
<td>intermediate cell mass</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
</tr>
<tr>
<td>IKKε</td>
<td>inhibitor of nuclear factor kappa-B kinase subunit epsilon</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational New Drug</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>iPTT</td>
<td>intraperitoneal pyruvate tolerance test</td>
</tr>
<tr>
<td>IRE-1α</td>
<td>inositol requiring enzyme-1α</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrates-1</td>
</tr>
<tr>
<td>Iso</td>
<td>isoprenaline</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP sensitive potassium</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 11</td>
</tr>
<tr>
<td>KCNQ1</td>
<td>potassium voltage-gated channel, KQT-like subfamily, member 1</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>K⁺-channel subunit</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>KvLQT1</td>
<td>voltage-gated K⁺ channel</td>
</tr>
<tr>
<td>Luc</td>
<td>luciferase</td>
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LPL  lipoprotein lipase
MET  metformin
mTOR mammalian target of rapamycin
NAFLD non-alcoholic fatty liver disease
NDA New Drug Application
Neg negative control
NF-1 nuclear factor-1
NFκB nuclear factor kappa light chain enhancer of activated b cells
NIH National Institutes of Health
NMR nuclear magnetic resonance
NSIS non-sulfonylurea insulin secretagogue
NO$_2$ nitrogen dioxide
NOD non-obese diabetic
OAA oxaloacetate
PAI-1 plasminogen activator inhibitor-1
PBI posterior blood island
PBS phosphate-buffered saline
PCK1 cytosolic phosphoenolpyruvate carboxykinase (or PEPCK-C)
PD pharmacodynamics
PEP phosphoenolpyruvate
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PHHI</td>
<td>persistent hyperinsulinemic hypoglycemia of infancy</td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetics</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (or Akt)</td>
</tr>
<tr>
<td>PM</td>
<td>particulate matter</td>
</tr>
<tr>
<td>PP</td>
<td>pancreatic polypeptide</td>
</tr>
<tr>
<td>PP4C</td>
<td>protein phosphatase 4 catalytic subunit</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator activated receptor γ (or PPARG)</td>
</tr>
<tr>
<td>PPG</td>
<td>postprandial glucose</td>
</tr>
<tr>
<td>PPI</td>
<td>proton pump inhibitor</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PTU</td>
<td>phenyl 2-thiourea</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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</table>
RXR  retinoid X receptor
SIK  salt-inducible kinase
SGLT2 sodium glucose transporter 2
SMEK suppressor of MEK null
SNP single nucleotide polymorphism
SPF specific pathogen free
SREBP-1 sterol-regulatory-element-binding-protein-1
STZ streptozotocin
SU sulfonyleurea
SUR sulfonyleurea receptor subunit
T1DM type 1 diabetes mellitus (or T1D)
T2DM type 2 diabetes mellitus (or T2D)
TCA tricarboxylic acid
TCF7L2 transcription factor 7-like 2
TBK1 TANK-binding kinase 1
Tg transgenic
TGF-α transforming growth factor-α
TNF-α tumor necrosis factor-α
TU Tuebingen
TZD thiazolidinedione
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>5-HMT</td>
<td>5-hydroxymethyl tolterodine</td>
</tr>
</tbody>
</table>
List of Tables

Table 4.1 Ranking of each compound for each experiment .......................................................... 47
List of Figures

Figure 1.1: Mechanism of action of insulin secretagogues in pancreatic β-cells ...................... 9

Figure 1.2: Target sites of current anti-hyperglycemic medications for T2DM......................... 12

Figure 1.3: Drug discovery process from basic research to FDA filing................................. 15

Figure 1.4: PEPCK as a cataplerotic enzyme involved in metabolic pathways from the TCA cycle to various biosynthetic and oxidative processes.......................................................... 26

Figure 1.5: Transcriptional regulation of hepatic gluconeogenesis under (A) fasting and (B) feeding states........................................................................................................................................ 28

Figure 4.1: Relative luciferase activity of the Tg(pck1:Luc2) zebrafish larvae at 6dpf ............ 44

Figure 4.2: Relative fluorescence intensity of the Tg(pck1:Venus) zebrafish larvae at 6dpf...... 44

Figure 4.3: Relative pck1 mRNA expression from WT larvae at 6dpf .................................. 45

Figure 4.4: Whole body free glucose levels of WT zebrafish larvae at 6dpf ......................... 46

Figure 4.5: Fluorescence intensity in Tg(pck1:Venus) zebrafish larvae at 6dpf .................... 49

Figure 4.6: Pck1 and glucose levels examined in control groups............................................ 51

Figure 4.7: Luciferase assays to plot dose response curves for the four compounds pursued in comparison studies............................................................ 52

Figure 4.8: Glucose assays to plot dose response curves for the four compounds pursued in comparison studies............................................................ 52

Figure 4.9: iPTT results in normal C57BL/6 mice ......................................................... 54

Figure 4.10: Relative pck1 mRNA expression in normal C57BL/6 mice ............................. 55

Figure 4.11: Pck1 protein expression in normal C57BL/6 mice ........................................... 55
Figure 4.12: Cytosolic and nuclear FoxO-1 protein expression in normal C57BL/6 mice .......... 56

Figure 4.13: Serum gastrin and insulin levels in normal C57BL/6 mice................................. 57

Figure 4.14: Body weight and blood glucose levels in mice after starting HFD feeding and receiving five consecutive STZ ip injections................................................................. 58

Figure 4.15: iPTT results in diabetic C57BL/6 mice................................................................. 59

Figure 4.16: Relative pck1 mRNA expression in diabetic C57BL/6 mice................................. 60

Figure 4.17: Pck1 protein expression in diabetic C57BL/6 mice ............................................. 60

Figure 4.18: Cytosolic and nuclear FoxO-1 protein expression in diabetic C57BL/6 mice..... 61

Figure 4.19: Serum gastrin and insulin levels in diabetic C57BL/6 mice................................. 62

Figure 7.1: The possible mechanisms of proton pump inhibitors (PPIs) on the improvement of glycemic control............................................................................................................. 76
List of Appendices

Appendix 1: Work flow of zebrafish studies................................................................. 85

Appendix 2: Work flow of (A) normal and (B) diabetic mouse studies................... 86

Appendix 3: Compounds #5-10.................................................................................. 87
Chapter 1

1 Introduction

1.1 Diabetes mellitus

1.1.1 Metabolic syndrome and diabetes mellitus

The metabolic syndrome is defined as a cluster of health conditions that increase a person’s risk of diabetes and cardiovascular disease. Approximately 20-25% of the world’s adult population is estimated to have the metabolic syndrome (IDF, 2006). It increases the risk of heart attack or stroke by threefold and is associated with a fivefold greater risk of developing type 2 diabetes (Bhowmik et al. 2015 and Stern et al. 2004). Strikingly, according to International Diabetes Federation (IDF), approximately 3.2 million people around the world die from complications associated with diabetes each year (IDF, 2006).

Diabetes mellitus (DM) is a growing worldwide epidemic that requires global attention and action. In 2012, diabetes was the direct cause of 1.5 million deaths (WHO 2016) and it is projected that diabetes will be the 7th leading cause of death in 2030 (Mathers and Loncar 2006).

It is a complex metabolic disorder that is characterized by impaired glucose metabolism and hyperglycemia caused by defects in insulin secretion from pancreatic β-cells, insulin action, or both. DM is a chronic disease that requires lifelong medical care combined with multifactorial risk-reduction strategies beyond glycemic control to maintain a good quality of life and reduce the incidence of complications associated with DM (ADA 2013). According to World Health Organization (WHO), the four main types of chronic diseases are cardiovascular diseases, cancers, chronic respiratory diseases, and diabetes. Chronic diseases significantly influence the morbidity and mortality of the worldwide population.

Most cases of DM can be divided into two broad etiopathogenic categories: Type 1 (T1DM) and Type 2 (T2DM).

Type 1 diabetes mellitus (T1DM) is an autoimmune disease, in which a person’s own immune system attacks and destroys insulin-producing pancreatic β-cells, leading to decreased insulin
levels, low glucose uptake, and thus hyperglycemia. Individuals with T1DM require daily administration of insulin for survival (Zimmet et al. 2001). Unfortunately, T1DM is not currently preventable (WHO 2016).

In contrast, the pathophysiology of type 2 diabetes mellitus (T2DM) is defined by increased insulin resistance, impaired insulin secretion and decreased β-cell function, which eventually lead to β-cell failure (CDA 2011). T2DM is the most common form of diabetes, accounting for 90% of all diabetes cases (WHO 2016). The prevalence of T2DM, which was once known as a disease of the West and an adult-onset-disease, has dramatically increased even in the populations where the incidence of T2DM was rare. The rapid increase is likely attributable to rapid urbanization, westernization, nutrition transition and sedentary lifestyles (Hu 2011).

Symptoms of all types of diabetes are similar, which include polyuria (excessive excretion of urine), polydipsia (thirst), polyphagia (constant hunger), vision changes and fatigue. However, in type 2 diabetes, those symptoms are often less marked, which causes delay in diagnosis and treatment (WHO 2016).

1.1.2 Complications of diabetes mellitus

Individuals with diabetes have increased risk of developing physical and psychological complications. The chronic hyperglycemia due to poorly controlled DM can lead to various complications such as heart attack, stroke, kidney failure, blindness, foot ulcers, non-traumatic limb amputations, and depression. Complications can be life-threatening as life expectancy may be shortened by as much as 15 years for people with T1DM and 5-10 years for those with T2DM (CDA 2011). According to International Diabetes Federation (IDF), when individuals with diabetes maintain their blood glucose levels, blood pressure, and cholesterol close to normal values, it can help delay or prevent complications associated with diabetes. Hence it is crucial for those living with diabetes to tightly control and maintain their well-being.

1.1.3 Etiology and pathophysiology of type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a multifactorial disease caused by genetic factors related to insulin resistance and impaired insulin secretion, as well as environmental factors including obesity, overeating, lack of exercise, stress, and aging (Kaku, 2010).
The significantly higher concordance rate of T2DM between monozygotic twins compared to dizygotic twins supports the involvement of genetic factors in the development of T2DM (Doria et al. 2008). Moreover, there is a 3.5-fold or 6-fold higher risk for T2DM in offspring with a single diabetic parent or two diabetic parents, respectively, compared to offspring without parental diabetes, further supporting the existence of genetic determinants for T2DM (Meigs et al. 2000). Previous studies have indicated that approximately 30-70% of type 2 diabetes risk can be attributed to genetic factors (Poulsen et al. 1999). Nevertheless, despite many efforts, the range of risk genes for the development of T2DM is not yet fully elucidated.

Present knowledge of genetic determinants for type 2 diabetes (T2D) include the transcription factor 7-like 2 gene (TCF7L2), potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11), and peroxisome proliferator-activated receptor-γ (PPARG/PPARγ), which are the three-well-known T2D susceptibility genes (Thorsby et al. 2009).

The TCF7L2 gene, which shows the strongest association with T2D to date (Grant et al. 2006), encodes a transcription factor in the Wnt-signaling pathway that promotes transcription of several genes, including proglucagon, in the intestine (Hertel et al. 2013). Previous studies by Lyssenko et al. have shown that increased expression of TCF7L2 in the pancreatic islets leads to impaired glucose-stimulated insulin secretion in T2D (Lyssenko et al. 2007).

Another T2D susceptible gene is KCNJ11, which encodes the potassium channels in the pancreatic beta cells that trigger the release of insulin based on a concentration gradient of electrolytes, potassium, sodium, and calcium, across the membranes. A membrane potential is created by ion channels through regulating the passage of ions. The ATP sensitive potassium (KATP) channels are responsible for insulin release from the pancreatic β-cells by regulating the membrane potential.

An increase in glucose levels results in ATP increment which closes the KATP channels in the beta cell membrane, depolarizing the cell by blocking the passage of potassium ions out of the cell. Depolarization results in the opening of calcium channels, allowing the entry of calcium into the cell, which triggers the release of insulin via exocytosis. Hence, drugs that close the KATP channels in the pancreatic beta cells to stimulate insulin secretion are used as one of the type 2 diabetes therapeutics. Such drugs include sulfonylureas (Dean and McEntyre 2004).
This KAPT channel consists of two subunits: a K^+ -channel subunit (Kir6.2) and a sulfonylurea receptor subunit (SUR), which belongs to the family of ATP-binding cassette (ABC) transporter proteins. Each potassium channel has four Kir6.2 subunits and four SUR subunits (Inagaki et al. 2003). The KCNJ11 gene encodes for the Kir6.2 subunit, and the ATP-binding cassette, sub-family C, member 8 (ABCC8) gene encodes for the SUR subunit. Mutations in either gene can lead to decreased KATP channel activity, resulting in increased insulin secretion and low blood glucose levels. This is a rare disorder known as persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Thomas et al. 1995). On the other hand, mice with targeted overactivity of the KATP channel were found to have severe diabetes (Koster et al. 2000) while activating mutations of KCNJ11 results in permanent neonatal diabetes in humans (Gloyn et al. 2004).

There are three common single nucleotide polymorphisms (SNPs) found within the KCNJ11 gene: E23K, L270V, and I337V in Caucasians (Hani et al. 1998), which cause non-synonymous amino acid changes in the mature protein. Among these three, E23K and I337V have shown to be associated with impaired insulin response and increased risk of T2DM (Dean and McEntyre 2004).

Lastly, peroxisome proliferator-activated receptors (PPAR) are both hormone receptors and transcription factors that are classified into three types: α, γ and δ. Hence, PPARs have two binding sites, one site for ligands and another site for DNA (Dean and McEntyre 2004). Ligands that activate PPARs include fatty acids, hormones, and specific diabetic drugs such as fibrates (PPARα) and thiazolidinediones (PPARγ) (Maltarollo et al. 2017). Among these three subtypes of PPARs, PPARγ is abundantly expressed in adipose tissue and plays a crucial role in adipocyte differentiation (Dean and McEntyre 2004). PPARγ as a transcription factor forms a complex with another transcription factor known as retinoid X receptor (RXR). When the PPARγ-RXR complex is activated, it binds to the promoter region of specific genes to activate their transcription. Identified PPARγ target genes include lipoprotein lipase (LPL), fatty acid transport protein (FATP), and acetyl CoA-synthase (ACS), which play a role in the metabolism of fatty acids (Arner 2003). Thiazolidinediones (TZDs), which are the ligand for PPARγ, are drugs for T2DM that activate the PPARγ-RXR complex in adipocytes to improve insulin sensitivity and lower blood glucose levels. Genetic variants of the PPARγ gene were some of the first to be identified as contributing risk factors for developing T2DM (Altshuler et al. 2000). A single nucleotide polymorphism (SNP) in the PPARγ gene coding region at position 12, with an alanine
substitution for proline (Pro12Ala), has been associated with increased risk for T2DM (Stumvoll and Haring 2002).

Interestingly, a high prevalence of T2DM was found in the Pima Indian population of Arizona. Screening this population for \textit{PPAR}\gamma2, an isoform of \textit{PPAR}\gamma, identified two SNPs in the coding region and seven SNPs in the promoter region. Among these SNPs, a novel functional variant in the \textit{PPAR}\gamma2 promoter region, C-2821T, was associated with metabolic predictors of T2DM and obesity (Muller \textit{et al.} 2003).

In addition to the genetic variants stated above, a recent genome wide association study (GWAS) in Asian subjects identified the potassium voltage-gated channel, KQT-like subfamily, member1 (\textit{KCNQ1}) gene; this genetic variant was later confirmed in European subjects to be a type 2 diabetes susceptibility gene (Hertel \textit{et al.} 2013). \textit{KCNQ1} encodes the pore-forming subunit of a voltage-gated \textit{K}$^+$ channel (KvLQT1) which plays an important role in repolarizing the cardiac action potential, and also facilitates water and salt transport in epithelial tissues (Sun \textit{et al.} 2012). Mutations in the \textit{KCNQ1} gene result in the long QT syndrome and deafness (Splawski \textit{et al.} 2000). Furthermore, \textit{KCNQ1} is also expressed in the pancreatic islets and inhibition of the \textit{KCNQ1} channel stimulates insulin secretion (Ulrich \textit{et al.} 2005), suggesting its potential role in regulation of insulin secretion. rs2237892 and rs2237895 are two important \textit{KCNQ1} polymorphisms associated with increased T2D risk (Sun \textit{et al.} 2012).

Apart from the genetic factors for increased risk of T2D, environmental factors also have a significant impact on increased T2D risk. The high prevalence of T2D in Pima Indians, compared to other races, supports not only the existence of genetic determinants but also environmental and/or behavior factors for T2D susceptibility. Murea \textit{et al.} analyzed the progression to diabetes in high-risk individuals with impaired glucose tolerance, and found that lifestyle intervention can prevent one case of T2D for every 6.9 enrolled persons while intervention using metformin medication prevents one case per 13.9 persons. Lifestyle intervention in this study refers to low-fat diet, exercise, and behavior modification. Further studies in individuals with high risk of T2D have shown that either metformin or lifestyle intervention is effective in decreasing the risk of T2D at any level of genetic risk (Hivert \textit{et al.} 2011). In addition, lifestyle interventions in overweight adult participants with impaired glucose tolerance resulted in 58\% reduction in the incidence of diabetes. However, approximately 10\%
of the participants progressed to T2D within 3 years despite rigorous lifestyle modification, which may be explained by the presence of non-modifiable genetic risk factors (Tuomilehto et al. 2001).

In addition to sedentary lifestyles and high-fat diets, epidemiologic studies have identified a number of new environmental factors that raise the risk of T2D including stress, cultural and socioeconomic variables, chronic low-grade infection, and environmental pollutants (Rajagopalan and Brook 2012). For example, chronic exposure to organic land pollutants such as pesticides and herbicides was shown to disturb glucose metabolism and insulin resistance. A strong dose-dependent relationship was found between serum concentrations of organic pesticides and prevalence of diabetes (Lee et al. 2006). Moreover, chronic exposure to herbicide, atrazine (ATZ), has been shown to cause mitochondrial toxicity and induce obesity and insulin resistance in rats (Lim et al. 2009). The association between prolonged herbicide/pesticide exposure and insulin resistance was found to be stronger and more detrimental in overweight individuals and rats fed a high-fat diet (Lee et al. 2007 and Lim et al. 2009, respectively). Furthermore, epidemiologic studies have also found a close link between air pollutants such as traffic-related pollutants (particulate matter [PM] and nitrogen dioxide [NO₂]) and prevalence of T2D. One plausible mechanism for how air pollutants contribute to T2D was described in a mouse model of diet-induced obesity, wherein PM exposure led to adipose inflammation and insulin resistance (Sun et al. 2009).

As only few methods are currently available to investigate potential linkages between environmental factors and risk of T2D, Patel et al. have conducted a pilot environmental-wide association study (EWAS), in which epidemiological data are comprehensively and systematically interpreted in an equivalent manner to GWAS. From this pilot study, they have found two novel environmental factors associated with higher risk of T2D: γ-tocopherol (vitamin E), and heptachlor epoxide (a pesticide) (Patel et al. 2010). The identification of γ-tocopherol as a risk factor for T2D was unexpected, as it was previously found to be a preventive agent against colon cancer (Campbell et al. 2003). Further studies on this vitamin should be conducted for its potential adverse metabolic effects.
1.1.4 History of type 2 diabetes medications

The prevalence of T2DM is continuously rising globally in parallel with being overweight and being obese (Mokdad et al. 2000). Previously the incidence of T2DM in children was very rare; however, over the past 30 years the prevalence of T2DM has approached epidemic proportions, affecting people of all ages (Mazzola 2012). As a consequence, there have been a growing number of oral medications and insulin analogs for the treatment of T2DM over the past years (Sheehan 2003).

Type 2 diabetes mellitus has been considered as a less severe disease relative to type 1 diabetes mellitus despite a much greater prevalence of T2DM. Due to its greater prevalence, the vast majority of microvascular complications occur in T2DM patients. In addition, there has been an increase in the risk of cardiovascular complications, which are mainly responsible for the high morbidity and mortality in people with diabetes (Stirban and Tschoepe 2008). As a result, the awareness of physicians and scientists of the importance for managing overall cardiovascular health through controlling cholesterol levels and blood pressure not just hemoglobin A$_{1C}$ (HbA$_{1C}$) has been increased (Sheehan 2003).

Prior to 1994, the only option for patients with T2DM was to select which type of sulfonylurea to take as an oral agent. Subsequently, different classes of new agents with unique mechanisms of action have been released for monotherapy or combination regimens (Sheehan 2003).

Current therapeutics for T2DM can be categorized into five main types: (1) drugs increasing insulin secretion (insulin secretagogues, e.g. sulfonylureas), (2) insulin sensitizer (increasing insulin action, e.g. thioazolidinediones), (3) drugs reducing hepatic glucose production (e.g. biguanides), (4) drugs inhibiting glucose absorption in the gut (decreasing insulin need, e.g. $\alpha$-glucosidase inhibitors), and (5) drugs mimicking the incretin hormones (e.g. GLP-1 receptor agonists or DPP-4 inhibitors) (King 2012).

The first oral agents released for the treatment of T2DM in the mid-1950s were sulfonylureas, which are insulin secretagogues. They increase insulin secretion through binding to a regulatory protein, commonly known as the sulfonylurea (SU) receptor, on pancreatic $\beta$-cells. This binding eventually leads to closure of ATP-dependent potassium (KATP) channels. Closure of these
channels results in membrane depolarization and influx of calcium ions through voltage-dependent channels, causing insulin release through exocytosis (Figure 1.1) (Sheehan 2003).

There are two types of insulin secretagogues: sulfonylurea (SU) and non-sulfonylurea (NSIS). Examples of SU insulin secretagogues include glyburide, glipizide, chloropamide, and tolbutamide. The common side effects of the SUs are hypoglycemia and weight gain, in which the degrees of symptoms vary among the agents. Glyburide has a 2-fold higher risk of hypoglycemia due to its longer duration of binding to the SU receptor than other SUs (Shorr et al. 1996 and McCall 2001). There is some evidence that weight gain tends to be greatest with glyburide while glipizide and glimepiride are more weight neutral (Cefalu et al. 1998). There were some concerns on the use of SUs for their increased risk of myocardial events and progressive death of β-cells. However, the United Kingdom Prospective Diabetes Study (UKPDS) has diminished these concerns; they did not observe any adverse effect on cardiovascular outcomes and the rate of β-cell functional decline was parallel to other classes of anti-diabetic drugs (UKPDS Group 1998 and 1995). Overall, despite their potential side effects, sulfonylurea class drugs are considered as a potent, safe and cost-effective management option for patients with T2DM (Sheehan 2003).

Examples of non-sulfonylurea insulin secretagogues (NSISs) include repaglinide, which belongs to the meglitinide family, and nateglinide, which is a derivative of phenylalanine. The major difference of these agents from the SUs is that they have less risk of hypoglycemia due to their rapidity and shorter duration of stimulation of insulin secretion. Patients taking the NSISs therefore need to take the agent at each meal. Nateglinide is less effective compared to repaglinide as it is exclusively metabolized and excreted by hepatic mechanisms. Hence, nateglinide can be used in patients with advanced renal insufficiency (Sheehan 2003). Importantly, due to the faster onset of action of NSISs, NSISs are thought to better control postprandial glucose (PPG), which has shown to be associated with the risk of cardiovascular disease (CVD) (Hanefeld et al. 1996). However, few studies have been published in support of the ability of NSISs to better manage PPG, while others failed to show a significant difference between SUs and NSISs in terms of PPG control (Sheehan 2003).
Different from insulin secretagogues, insulin sensitizers work by improving cellular sensitivity to insulin (Kirpichnikov et al. 2002). Thiazolidinediones (TZDs) are insulin sensitizers and there are two TZDs that are currently available in Canada; rosiglitazone and pioglitazone (Cheng and Fantus 2005). Prior to the approval of these two drugs, troglitazone was introduced in 1997 but removed due to liver toxicity (Sheehan 2003). Limiting factors for the use of rosiglitazone and pioglitazone are their potential adverse effects such as edema and weight gain. Furthermore, the use of these two TZDs is contraindicated in patients with hepatic dysfunction. TZDs bind to peroxisome proliferator-activated receptor gamma (PPARγ), which is a nuclear receptor that is highly expressed in adipocytes. PPARγ plays a role in the regulation of the gene expression involved in carbohydrate and lipid metabolism. TZDs are thought to enhance insulin sensitivity through reducing lipolysis, free fatty acids (FFAs), tumor necrosis factor-α (TNF-α) and leptin, while increasing adiponectin (Cheng and Fantus 2005). The decrease in the number of FFAs improves pancreatic β-cell function through decreasing lipotoxicity, which is a process of β-cell death by increased FFAs (Bell and Ovalle 2001). Both agents, rosiglitazone and pioglitazone, are approved to be used as monotherapy as well as in combination with metformin, SUs, NSISs or α-glucosidase inhibitors (Cheng and Fantus 2005). There is evidence that TZDs have beneficial effects beyond glycemic control including reduced urinary albumin excretion (Bakris et al. 2003), increased levels of high-density lipoprotein (HDL) cholesterol, reduced triglyceride levels (Herz et al. 2003), lower blood pressure, and reduced levels of plasminogen activator inhibitor-1 (PAI-1) (Inzucchi 2002). In contrast, the major side effects of TZDs are weight gain, edema, anemia, and congestive heart failure (Nesto et al. 2003).
Next, metformin is the only biguanide class of drug that is available in most of the world (Mazzola 2012). Other biguanide class of anti-hyperglycemic drugs was removed from the international market in the 1970s due to their high risk of lactic acidosis (Bailey 1996). The mechanism of action of metformin is still not entirely clear, but its main effect is decreasing hepatic gluconeogenesis, thereby improving fasting hyperglycemia (Hundal et al. 2000). It also increases glucose uptake by skeletal muscles by improving insulin sensitivity (Kirpichnikov et al. 2002). Zhou et al. have discovered that metformin activates hepatic and muscle adenosine monophosphate-activated protein kinase (AMPK), which is activated by a cellular signal, adenosine monophosphate, for increased energy requirements (Zhou et al. 2001). The activation of AMPK phosphorylates and inhibits acetyl-coenzyme A carboxykinase (ACC), the rate-limiting enzyme in lipogenesis. Moreover, activation of hepatic AMPK reduces the expression of sterol-regulatory-element-binding-protein-1 (SREBP-1), a transcription factor involved in the pathogenesis of insulin resistance, dyslipidemia and diabetes (Cheng and Fantus 2005). There is evidence that metformin reduces a variety of factors related to increased cardiovascular risk (Palumbo 1998) and significantly reduces the rate of myocardial infarction (UKPDS group 1998). Metformin is frequently the first-line option for most patients with T2DM as it does not induce weight gain, is associated with fewer hypoglycemic attacks than SUs or insulin therapy (UKPDS Group 1995), and tends to have a lower risk of diabetes-related endpoint or death such as hyper- or hypoglycemia, fatal or non-fatal myocardial infarction, angina, heart failure, stroke, renal failure amputation, vitreous hemorrhage, retinopathy, and blindness (UKPDS Group 1998). The possible side effects of metformin include gastrointestinal intolerance such as nausea, abdominal pain and diarrhea. Approximately 30% of patients taking metformin experience these symptoms (Garber et al. 1997). The use of metformin should be avoided in patients with liver disease and with conditions that may predispose them to acidosis such as congestive heart failure and/or pulmonary disease (UKPDS group 1998). Nevertheless, very few patients need to withdraw metformin due to adverse effects, as the risk of lactic acidosis with metformin is minimal.

Another class of anti-hyperglycemic drugs is α-glucosidase inhibitor. The only available α-glucosidase inhibitor in Canada is acarbose while the use of miglitol, another α-glucosidase inhibitor, is approved in the United States. These drugs lower postprandial glucose (PPG) levels by competitively inhibiting the binding of oligosaccharides to the α-glucosidase enzyme in the
small intestine, delaying the absorption of carbohydrate. Similar to metformin, these drugs do not cause weight gain and hypoglycemia. However, as α-glucosidase inhibitors only control PPG levels, they are less potent than other agents (Sheehan 2003).

In addition to the aforementioned diabetes medications, there has been an explosion of diabetes therapeutics released since the mid-1990s including amylin agonists, glucagon-like peptide-1 receptor agonists (GLP-1 RA), dipeptidyl peptidase-4 (DPP-4) inhibitors, incretin mimetics, and sodium glucose transporter 2 (SGLT2) inhibitors (White JR 2014). Also, significant strides have been made to develop newer insulin therapies including inhaled insulin, which was first approved in the United States in 2006, and ultra-long-acting basal insulins in 2015 (Cahn et al. 2015).

Amylin is the endogenous neuroendocrine hormone that is co-secreted with insulin by the pancreatic β-cells in equimolar amounts. Pramlintide is the only currently available amylin analog in the market. It induces weight loss, delays gastric emptying, and leads to reduction in both PPG and glucagon levels, while the main side effect is nausea (White JR 2014).

Due to the short half-life of native incretins, which are gut hormones released from enteroendocrine cells upon increased glucose levels shortly after eating to stimulate insulin secretion while inhibiting glucagon release (Baggio et al. 2007), there have been many studies on the development of longer-acting incretin mimetics. The effect of incretin at secreting insulin is responsible for approximately 50-70% of the total insulin secretion after oral glucose intake. The two main incretins that play roles in the maintenance of glycemic control are glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). As mentioned above, these incretins have short half-life as they are readily hydrolyzed by the dipeptidyl peptidase 4 (DPP-4) enzyme inhibitors (Nauck and Meier 2016). Therefore, one avenue of therapeutic development is to increase the half-life of incretins. Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) are the best-known incretin mimetics that decrease blood glucose levels through enhancing glucose-dependent insulin secretion. The first GLP-1 RA released on the market was exenatide followed by liraglutide (White JR 2014).

As noted above, DPP-4 inhibitors hydrolyze the incretins. Hence, there has been an increase in the number of studies targeting the incretin system including DPP-4 inhibitors, which would
prolong the half-life of circulating endogenous incretins. The first drug of this class that became available is sitagliptin followed by saxagliptin and linagliptin (White JR 2014). These agents may be used as single therapy or combined with metformin, sulfonylurea, or TZD. They are weight neutral and do not tend to cause hypoglycemia (Chaudhury et al. 2017). Possible adverse effects of DPP-4 inhibitors include pancreatitis (White JR 2014).

Lastly, sodium-glucose cotransporter 2 inhibitors (SGLT-2) are a new class of anti-diabetic medications. The first SGLT-2 inhibitor, canagliflozin, was approved by FDA in 2013. These compounds function through antagonizing high-capacity and low-affinity glucose transporters that are primarily found in the kidney. SGLT-2 is responsible for approximately 90% of glucose reabsorption in the kidney. Hence antagonizing SGLT-2 leads to excretion of excess glucose in the urine, causing a net loss of glucose and a reduction in hyperglycemia through an insulin-independent mechanism (White JR 2014). Therefore, these agents may be effective in advanced stages of T2DM where pancreatic β-cell reserves are permanently lost (Chaudhury et al. 2017).

To sum up, figure 1.2 shows the target organs of currently available medications for T2DM.

Figure 1.2: Target sites of current anti-hyperglycemic medications for type 2 diabetes mellitus (T2DM) (Jenssen and Hartmann 2015).
Despite the anti-hyperglycemic efficacy of current anti-diabetic therapeutics, patients taking anti-diabetic medications may have undesirable side effects such as hypoglycemia, GI problems, weight gain, β-cell loss, vitamin B12 deficiency, and pancreatitis (Philis-Tsimikas 2009). These limitations justify further studies to develop new classes of anti-hyperglycemic agents to supplement existing therapies. Compared to traditional agents, which focus on pancreatic β-cell failure or insulin resistance, newer agents tend to target other defects such as incretin deficiency or resistance. As well, common comorbidities of diabetes including dyslipidemia, hypertension, obesity, and hypercoagulability have become an additional therapeutic focus (Mazzola 2012).

1.2 Drug development

1.2.1 Drug discovery

Drug discovery is a process of finding a promising molecule that is suitable for clinical applications for treatment of diseases. The discovery of new drugs is a long, complex, costly, and rigorous process. On average, it takes at least 10 years and $2.6 billion to develop a potential new medicine (PhRMA 2015). Unfortunately, many drugs fail to reach the marketplace due to lack of efficacy or toxicity (Silber 2010).

Often, the initial research occurs in academia to establish a hypothesis that the intervention, either through inhibition or activation of a protein or pathway, will result in a therapeutic effect in a disease state. As shown in figure 1.3, there are five major stages in a drug discovery process: basic research, lead discovery, preclinical development, clinical development, and FDA filing (Hughes et al. 2011).

Selection of a target is achieved during the basic research phase. Targets can be proteins, genes and RNAs, but the resulting drug candidates need to be efficacious, safe, and meet clinical and commercial needs. Following the target selection and validation process, compound screening assays are developed to identify so-called “hit” molecules, which are defined as compounds that exhibit the desired activity by affecting the target. The assays to identify hit molecules include high throughput screening (HTS), focused screening, and physiological screening (Hughes et al. 2011). High throughput screening involves screening of a drug library using complex laboratory automation without prior knowledge of the compounds for the desired activity at the target, while focused screening selects compounds that are deemed likely to have desired activity based
on knowledge of the target (Boppana et al. 2009). The activity of compounds can be detected with quantitative outputs such as absorbance, fluorescence, or chemiluminescence. In addition, the physiological screening method is a tissue-based approach that highlights the desired response within an in vivo context, in contrast to measuring only one specific molecular component (Hughes et al. 2011).

Prior to progression into lead discovery phase, further validation studies may be required to increase confidence in the relationship between target and disease, thereby justifying further drug discovery efforts. The hits obtained from screening methods may be grouped based on their structural similarity, to develop a broad spectrum of chemical classes. Subsequently, hit compounds are ranked through validation studies such as dose-response curves, which allow comparison of the potency of candidate compounds. Lead compounds from the series of hits can be selected through validation studies using in vivo models, known as the hit-to-lead phase (Hughes et al. 2011).

Further studies of the lead compounds in animal models are performed to determine pharmacokinetics (PK) and pharmacodynamics (PD) including the absorption, distribution, metabolism and excretion (ADME). The selected initial lead compounds undergo the lead optimization phase in order to retain the favorable properties of the lead compounds while improving target specificity, selectivity, PK, PD, and toxicological properties (Baxter and Lockey 2007).

The outcome of the lead discovery phase is the selection of candidate compounds that will progress into the pre-clinical phase which typically involves both in vivo and in vitro studies to extensively examine the candidate compound, especially for toxicity, before clinical studies. If successful in the pre-clinical phase, an Investigational New Drug (IND) application is submitted prior to Phase I-III clinical trials. If the candidate compound appears to be safe and promising for its intended purpose, a New Drug Application (NDA) can be submitted to the Food and Drug Administration (FDA). Upon approval by FDA after extensive review, the compound can continue to progress into phase IV trials. Ultimately, following the clinical development phase, the selected target compound will become available in the market (Hughes et al. 2011).
1.2.2 Drug repurposing and its advantages

Drug repurposing or repositioning, which is the application of drugs that were already approved for other uses to new indications (Sleigh and Barton 2010), has received great attention as new drug discovery from bench to bedside is costly, time-consuming, and risky (Ashburn and Thor 2004). Since drug repurposing examines potential efficacy of previously approved drugs (but for new indications), detailed information on ADME, toxicity, and clinical efficacy may already be available, thereby reducing the time, effort, and cost for the transition of a promising molecule from the bench into an approved drug for new uses (Chong and Sullivan Jr 2007).

Among many repurposed drugs, the two best known repurposed drugs are sildenafil and minoxidil. The original purpose of sildenafil was for the treatment of angina. However, it was discovered later that sildenafil is in fact an effective therapeutic for erectile dysfunction, thereby rebranded as Viagra (Ghofrani et al. 2006). Its repositioning as a therapeutic for erectile dysfunction generated more than $2 billion worldwide in 2012 (Beachy et al. 2014).

In addition, minoxidil was originally developed as a treatment for ulcers, however; it was later proved to act as a vasodilator. For this reason, minoxidil was approved by the FDA as a treatment for high blood pressure in 1979. Upon approval by the FDA, a new study was conducted to examine minoxidil as a therapeutic for hypertension, in which a potential role of minoxidil to promote hair growth was determined. In 1988, minoxidil was finally approved to be used for the treatment of hair loss by the FDA (Conrad 2007).
1.3 Zebrafish as an *in vivo* model organism

1.3.1 Zebrafish background

Zebrafish, *Danio rerio*, has become one of the most widely used animal models in developmental research due to a number of features that make them an attractive experimental vertebrate including genetic and physiological similarities to mammals (Miscevic et al. 2012). Recently, the whole zebrafish genome has been sequenced and showed that approximately 70% of zebrafish genes have human orthologues (Howe et al. 2013). In addition to physiological and biological similarities to humans, zebrafish is deemed to be a robust *in vivo* drug and toxicological screening model organism due to its high fecundity, low cost, transparent embryo, rapid *ex utero* development, ease of manipulation and maintenance, and suitability for high throughput screening (HTS) (Miscevic et al. 2012). The availability of techniques for transgenic zebrafish construction, targeted mutation, and nuclear transfer make the zebrafish model even more attractive and useful in research.

1.3.2 Zebrafish embryos for high throughput screening (HTS)

The small size and optically transparent zebrafish embryos make them an attractive and powerful screening vertebrate model organism particularly suitable for high throughput screening (HTS) in drug discovery as well as toxicological screening (Elo et al. 2007).

Traditional drug discovery involves screening of a large chemical library to identify compounds that affect a specific target molecule, then measuring the target’s activity. Hence, target selection is a key decision to make in this approach of drug discovery. However, it is possible that the target has been intensively studied in a particular area but poorly studied in other cell types or tissues. Furthermore, animal models for preclinical phases can be expensive and/or highly inbred. As a result, it is common for marketed drugs to have been tested in less than 1,000 animals. Therefore, it is not surprising that many drugs suffer from on-target and off-target toxicity or unanticipated effects (Kithcart and MacRae 2017).

For example, amiodarone, the most effective anti-arrhythmic therapeutic to date, was originally discovered as an anti-anginal drug but was later found to be effective for arrhythmia (Rutitzky et al. 1982). It is now understood that complex effects on multiple targets are important to the final profile of a drug, underscoring the value of *in vivo* systems that include all the relevant targets in
their native context. Such a model will allow researchers to investigate possible toxic/adverse/unanticipated effects in parallel with ongoing examination for efficacy, which would increase the success rate of a drug reaching the market (Kithcart and MacRae 2017).

The larval zebrafish is a useful vertebrate model organism that it is amenable to both genetic and chemical screening. Genetic manipulation or gene knockouts in zebrafish have become easier since the whole genome has been sequenced. Techniques for genetic manipulation or gene knockouts include morpholino antisense oligonucleotides, zinc finger nuclease, and CRISPR/Cas9 (Meng et al. 2008).

The use of larval zebrafish in genetic or chemical screening is increasing due to the following features. A pair of zebrafish can produce hundreds of offspring and the maintenance of zebrafish embryos is simple and affordable compared to other vertebrate models; they can be sustained in large numbers in multi-well plates without food for a few days after fertilization as they take the nutrients from their embryonic yolk sac (Truong et al. 2011). The complex physiology in zebrafish is established within few days post fertilization (dpf), which can expedite screening. Organogenesis in zebrafish begins by 24 hours post fertilization (hpf). Insulin-positive pancreatic β-cells are first detectable at 15 hpf (Kinkel and Prince 2009) and the pancreas is fully developed by 3 dpf (Tehrani and Lin 2011). By 5 dpf, all major organs including the liver are established (Chu and Sadler 2009). Another advantage of using zebrafish in high throughput chemical and genetic screens is the permeability of zebrafish embryos to small molecules, expediting drug biodistribution studies (Kithcart and MacRae 2017).

High throughput screening in drug discovery is often accompanied by automated equipment such as COPAS, which can sort and dispense transgenic zebrafish larvae into microtiter plates, automated liquid handling robotics, and an automated plate reader to measure signal or desired outcome from treated transgenic zebrafish larvae (White et al. 2016). These advanced automated systems facilitate drug screening by increasing the throughput.

1.3.3 Zebrafish as a model for human diseases

Being genetically and physiologically similar to mammals, zebrafish is an excellent model to study pathological conditions in vertebrates. After its complete genome sequencing in 2013, zebrafish has increasingly been used as a model organism to study human diseases (Howe et al.
As optically transparent and rapidly developing vertebrates, zebrafish embryos have been particularly useful in overcoming the difficulty in studying embryogenesis in utero in mammalian models (Chu and Sadler 2009).

Although mammalian models including mice are commonly used due to anatomical and physiological similarities with humans, difficulty in producing large numbers of progeny, longer in utero embryonic development, and maintenance expenses restrict the ability to conduct high throughput chemical or genetic analyses (Wilkins and Pack 2013).

The conservation of genes between zebrafish and humans, and the ability to perform genetic manipulations, have driven efforts to establish transgenic zebrafish models to study variety human diseases in vivo (Seth et al. 2013) such as congenital and hereditary disease, carcinogenesis, infection, inflammation, immunological disease, metabolic disease, and psychological abnormality (Lieschke and Currie 2007). Recently, a zebrafish model has been used to study platelet formation, which is crucial for maintaining haemostasis (Gieger et al. 2011). Zebrafish models are also helping to understand human cardiac regeneration, since zebrafish maintains the ability to regenerate heart tissue throughout its lifetime (Bournele and Beis 2016). In addition, there are a number of established zebrafish models to study metabolic diseases including obesity, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and atherosclerosis (Seth et al. 2013).

1.3.4 Glucose homeostasis in zebrafish and its suitability as a model for glucose metabolism

The regulation of glucose homeostasis in zebrafish closely resembles that in mammals (Jurczyk et al. 2011). Zebrafish also possess key organs for metabolism similar to humans, from the appetite circuits to the pancreas and insulin-sensitive tissues, liver, muscle, and white adipose tissue (WAT) (Seth et al. 2013).

It was found that the zebrafish pancreas and liver, which play important roles in the regulation of glucose homeostasis, are morphologically and physiologically similar to humans and other mammals (Tehrani and Lin 2011). Therefore, the observations made in zebrafish are applicable to human as well as other mammalian models. The optically transparent zebrafish embryos
provide an advantage in monitoring organ morphogenesis without requiring sacrifice and necropsy (Kinkel and Prince 2009).

As with mammals, the zebrafish pancreas is composed of exocrine and endocrine glands. The endocrine portion of the pancreas, the islets, consists of alpha (α), beta (β), delta (δ), epsilon (ε), and PP (or gamma, γ) cells, which secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide (PP), respectively. Insulin-producing β-cells occupy the central portion of the islets and play an important role in glucose homeostasis with glucagon-producing α-cells. The exocrine portion, comprising acinar and duct cells, is responsible for the production of digestive enzymes such as amylase and trypsin, which are secreted into a ductal system where these enzymes are transported to the digestive tract. In addition to morphological similarity, important genes for islet development in mammals have been found to play similar roles in zebrafish (Elo et al. 2007).

The liver plays an essential role for metabolism, detoxification and homeostasis. Hepatocytes are the major components of the liver and are responsible for most of the liver’s functions, including metabolism of endogenous and exogenous substances, storage of glycogen, amino acids, fat and iron, and secretion of bile acids (Field et al. 2003). Genetic analyses have found that the genes and pathways involved in liver development are largely conserved between mammals and zebrafish (Chu and Sadler 2009).

Recent studies by Andersson et al. have used transgenic zebrafish with a fluorescent reporter for pancreatic β-cells for high throughput screening of drugs that promote β-cell regeneration. The hits identified from zebrafish studies were subsequently validated in mouse models, further confirming similar biology between zebrafish and mammals. Although it is not feasible to measure blood glucose levels in zebrafish larvae due to their small size, measuring the levels of free glucose from embryo extracts is used as an alternative method. Andersson et al. demonstrated an approximately three-fold increase in free glucose levels following targeted β-cell ablation (Andersson et al. 2012). Also, Gut et al. have observed a decrease in free glucose levels in drug treated larvae relative to their control group, further supporting that this method can be used for quantifying glucose homeostasis in zebrafish larvae (Gut et al. 2013). In addition, Rovira et al. have found small molecules capable of inducing β-cell neogenesis through chemical screening using zebrafish (Rovira et al. 2011). Collectively, the studies described
above highlight the suitability and advantages of zebrafish as a model organism in studies related to glucose metabolism, and its amenability to high throughput screening.

1.3.5 Pancreas and liver development in zebrafish

As in mammalian pancreas, the zebrafish pancreas develops from the two buds from the posterior foregut endoderm (Field et al. 2003). By 24 hpf, the dorsal bud emerges to exclusively give rise to endocrine cells, where in mammals, the dorsal bud gives rise to both endocrine and exocrine cells. By 24 hpf, a single primary islet consisting of α, β, δ, and ε cells is already present. Then the ventral bud emerges at 32 hpf to form exocrine cells as well as endocrine cells. The ventral bud-derived β-cells contribute to both the primary islet and the secondary islet, including γ-cells that secrete pancreatic polypeptide (PP) (Li et al. 2009 and Wang et al. 2011). Intriguingly, the ventral bud-derived β-cells secrete more insulin compared to the dorsal bud-derived β-cells (Hesselson et al. 2009). At 50 hpf, the dorsal and ventral buds fuse and a mature pancreas with the primary islet surrounded by exocrine cells is observable. Following a gut rotation, the pancreas is located on the right side of the embryo (Field et al. 2003).

The zebrafish liver is formed from the ventral foregut endoderm, which gives rise to the hepatoblast. At 6 hpf, the ventral part of endoderm cells likely differentiate to the liver bud whereas the endoderm cells located in the dorsal side give rise to the pancreas (Tao and Peng 2009). Field et al. have divided the liver morphogenesis into two phases, budding and growth. Budding phase is further divided into three phases. The budding stage I occurs at 24 hpf to form an endoderm rod via aggregation of endoderm cells caudal to the pharyngeal region. By 28 hpf, the thickening of the endoderm rod occurs indicating the beginning of liver morphogenesis. Then the budding stage II starts from 28 hpf, where gut-looping occurs through increasing in size and bending of the anterior thickening region to the left side of the middle line. By 50 hpf, the budding phase is complete as the liver becomes connected to the intestine. As the growth stage begins, the size, shape and placement of the liver dramatically change due to rapid cell proliferation. At this stage, hepatoblasts differentiate into hepatocytes and bile duct cells. As the second liver lobe forms by 96 hpf, liver morphogenesis is complete and the liver is fully functional (Tao and Peng 2009).

Unlike in mammals, embryonic hematopoiesis in zebrafish does not take place in the liver but occurs first in the intermediate cell mass (ICM) followed by posterior blood island (PBI) and
kidney sequentially. Therefore, defects or mutations affecting the liver development in zebrafish do not result in lethality from anemia as in mammals, allowing researchers to investigate liver formation in the presence of mutations affecting blood or liver development, which would lead to fatal anemia in mammals (Thisse and Zon 2002). Indeed, this difference has allowed rapid advances in understanding liver development and disease in zebrafish, and thereby in mammals (Tao and Peng 2009).

1.4 Mouse as a model organism

1.4.1 Use of mouse models in drug discovery

Drug discovery and development involve a variety of experimental models ranging from cell cultures to animal models, healthy human subjects, and patients to intensively examine the efficacy and safety of a drug before it becomes available in the market (Zhang et al. 2012). Although zebrafish is increasingly used in drug discovery and development due to its number of advantageous features, including small size, high fecundity, rapid ex utero development, ease of maintenance and manipulation, and most importantly, suitability for high throughput screening (HTS) (Miscevic et al. 2012), being an aquatic organism leads to important divergences between zebrafish and mammals. For example, because of the permeability of zebrafish to small molecules, drug delivery can be achieved transdermally in zebrafish (Kithcart and MacRae 2017) whereas drugs are administered to mammalian models through a variety of routes such as oral administration or intraperitoneal, subcutaneous and intravenous injections (Turner et al. 2011). Although it is possible to perform intraperitoneal (ip) injection in zebrafish, it is technically more challenging compared to ip injection in larger species such as mice. It is important to note that the difference in drug delivery method may result in divergent effects (Seth et al. 2013).

In addition, blood sampling in zebrafish is only possible in adult fish; however, it requires sacrificing of fish, which increases the number of animals for studies and limits the analysis of disease progression by tracking the changes in metabolite or hormone levels via repeated measurements. As well, radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs) for zebrafish are limited in availability (Seth et al. 2013).

Preclinical studies often involve at least two animal species to further validate the desired effects of candidate druggable compounds (Brewer 2007). Mouse models, due to highly conserved
biological pathways, are the foremost mammalian model organism to study human disease by elucidating the underlying physiological mechanisms and examining the efficacy of candidate drugs (Weidner et al. 2016). Mice and humans show Mendelian and polygenic similarities in common inherited diseases including diabetes, atherosclerosis, heart disease, cancer, glaucoma, anaemia, hypertension, obesity, osteoporosis, asthma, bleeding disorders, and neurological disorders. Consequently, many efforts have been made to study human diseases as well as normal biological processes using mice as a model organism over the past 100 years (Peters et al. 2007). Mouse disease models are generated through genetic means such as transgenic knockout and knockin, and conditional gene mutation, as well as by radiation and chemical induction (Perlman 2015 and Simmons 2008). Hence, despite the limitations of using mice to recapitulate and study human systems, they are still the most widely used animal model in biomedical research (Peters et al. 2007).

1.4.2 Mouse as a model in diabetes research

The major feature of type 1 diabetes (T1D) is the destruction of pancreatic β-cells via autoimmune processes, which results in hyperglycemia due to the lack of insulin secretion. There are currently many available mouse models of T1D including non-obese diabetic (NOD) mice, which develop spontaneous diabetes when kept in specific pathogen-free (SPF) conditions; chemically induced diabetic mice which involve either streptozotocin (STZ) or alloxan; genetically induced AKITA diabetic mice, and virally induced diabetic mice (King 2012).

Meanwhile, since type 2 diabetes (T2D) is characterized by insulin resistance and impaired insulin secretion as well as loss of β-cell function that ultimately leads to β-cell failure, mouse models of T2D often include insulin resistance and/or β-cell failure. Since there is a close link between T2D and obesity in human, many mouse models of T2D are obese such as Lep$^{ob/ob}$, Lepr$^{db/db}$, KK, and high fat diet (HFD) induced diabetic mice. The Lep$^{ob/ob}$ mice are characterized by deficiency in leptin, which is a hormone secreted from adipocytes that plays a role in inhibiting food intake by signaling the hypothalamus (Cammisotto and Bukowiecki 2002). On the other hand, the Lepr$^{db/db}$ mice have an autosomal recessive mutation in the leptin receptor (Chen et al. 1996). Both models are hyperphagic, obese, hyperinsulinaemic, and hyperglycemic. KK mice are also obesity-induced diabetic mice yet appear to be mildly obese (King 2012). Similar to genetically induced obese diabetic mice, HFD-induced diabetic mice develop obesity,
hyperinsulinaemia, and altered glucose homeostasis (Winzell and Ahren 2004). It is important to consider the strain of mice when inducing T2D through HFD feeding as certain strains of mice are resistant to diet-induced metabolic changes (King 2012). Another method to induce T2D involves STZ or alloxan injection. Since pancreatic β-cell destruction is also an important manifestation in T2D (Donath et al. 2005), low dose STZ or alloxan is also used to induce T2D in mouse models (Srinivasan and Ramarao 2007). STZ is an antibiotic that specifically targets pancreatic β-cells due to its glucose moiety, and exerts cytotoxic effects by its highly reactive methyl nitrosourea moiety which results in partial pancreatic β-cell destruction (Wu and Yan, 2015). Mice are fasted prior to injection to increase the susceptibility of mice to STZ, as glucose competes with STZ due to their structural similarity. Meanwhile, alloxan gets reduced to dialuric acid and is subsequently re-oxidized back to alloxan, leading to the formation of free radicals, which cause β-cell destruction. Like STZ, alloxan is rapidly taken up by the pancreatic β-cells due to its structural similarity to glucose. Genetically induced non-obese mouse models of T2D include hIAPP mice, which are characterized by amyloid deposition in pancreatic islets, leading to the destruction of β-cells (King 2012).

1.5 Energy metabolism

1.5.1 Liver glucose metabolism

The essential organ responsible for energy metabolism and homeostasis is the liver, where its metabolic activity is closely associated with the action of insulin and other metabolic hormones. Food is digested in the gastrointestinal (GI) tract and glucose, amino acids and fatty acids enter the bloodstream where they are transported to the liver through the portal vein. In the liver, glucose is metabolized into pyruvate through a process called glycolysis in the cytoplasm. Pyruvate is completely oxidized in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria. From this process, ATP is generated (Rui 2014).

Under fed state, pancreatic β-cells secrete insulin, which plays an important role in the regulation of fuel metabolism along with its counter-regulatory hormone, glucagon. Insulin stimulates the storage of fuels through glycogenesis, which is the synthesis of glycogen in both the liver and muscle, and de novo lipogenesis which is the synthesis of fatty acids (Berg et al. 2002). In hepatocytes, fatty acids are converted into triglyceride, which is either stored in hepatocytes as
lipid droplets or incorporated into very low density lipoprotein (VLDL) along with cholesterol and apolipoproteins, and then enters the bloodstream (Rui 2014).

During fasted state, glucagon and catecholamines, the counter-regulatory hormones of insulin, are secreted by pancreatic α-cells and adrenal medulla, respectively, to stimulate glucose release while inhibiting glycolysis, glycogenesis, and lipogenesis. Glucose is released from the liver through two different pathways: glycogenolysis, which is the breakdown of glycogen, and gluconeogenesis, which is \textit{de novo} glucose synthesis from pyruvate, TCA cycle intermediates, and fatty acids. The breakdown of lipid, lipolysis, is also activated during fasting state to contribute in increasing glucose levels in blood stream. For short-term fasted state, glycogenolysis is the main pathway to replenish glucose, while gluconeogenesis predominates during prolonged fasting (Rui 2014)

1.5.2 Phosphoenolpyruvate carboxykinase (PEPCK) and gluconeogenesis

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that catalyzes the first committed rate-limiting step in hepatic gluconeogenesis; it converts oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and carbon dioxide (CO\textsubscript{2}) (Matte \textit{et al}. 1997). The expression of PEPCK is induced by glucagon, glucocorticoids, and cyclic adenosine monophosphate (cAMP), and dominantly inhibited by glucose-induced insulin (Quinn and Yeagley 2005).

The four major pathways in which PEPCK plays an important role are 1) gluconeogenesis, 2) glyceroneogenesis, 3) serine synthesis, and 4) conversion of the carbon skeletons of amino acids, including glutamine and glutamate, to TCA cycle intermediates, which then enter the TCA cycle for subsequent oxidation as shown in figure 1.4 (Yang \textit{et al}. 2009).

The most widely studied pathway for the enzyme PEPCK is gluconeogenesis, which is a reverse metabolic pathway of glycolysis. It is essential for the maintenance of circulating glucose levels during starvation (Chandramouli \textit{et al}. 1997). This process occurs mainly in the liver to ensure a continuous supply of glucose (Burgess \textit{et al}. 2007). In healthy individuals, gluconeogenesis is under exquisite control to match its rate with the body’s glucose requirement. However, this regulation appears to be dysregulated in individuals with diabetes (Boden \textit{et al}. 2001; Wajngot \textit{et al}. 2001). The mechanism of gluconeogenesis regulation still remains incompletely understood.
PEPCK has cytosolic and mitochondrial isozymes. Cytosolic PEPCK (abbreviated as pck1 or PEPCK-C), encoded by the Pck1 gene in humans as well as in rodents, is responsible for the vast majority of the activity in the liver and has been extensively studied for its essential role in hepatic gluconeogenesis regulation and diabetes (She et al. 2000 and Beale et al. 2007).

Overexpression of pck1 in mice contributes to insulin resistance and hyperglycemia (Sun et al. 2002 and Valera et al. 1994), while reduced pck1 levels through gene silencing in the liver of diabetic mice leads to improved glycemia and insulin sensitivity (Gómez-Valadés et al. 2008). Furthermore, studies by Cao et al. have found a close link between polymorphism in the pck1 promoter and the development of T2DM (Cao et al. 2004). Collectively, pck1 plays an important role in glucose homeostasis, and due to its close association with T2DM, pck1 has been identified as a potential therapeutic target (Beale et al. 2007).

Studies by She et al. and Hakimi et al. have shown severe hypoglycemia and lethality in mice lacking PEPCK enzyme within 3 days of birth. However, both studies have revealed that lethality was due not only to the lack of gluconeogenesis, which has been thought to be the main function of PEPCK enzyme, but also the lack of integration of hepatic metabolic pathways as PEPCK is responsible for various cataplerotic processes. Cataplerosis is responsible for maintaining TCA cycle flux by removing TCA cycle anions (She et al. 2000 and Hakimi et al. 2005). Gluconeogenesis and glyceroneogenesis are cataplerotic processes that recycle TCA cycle intermediates for their biosynthesis of glucose and glycerol-3-phosphate, respectively (Hakimi et al. 2005).

Strikingly, She et al. have observed euglycemia in fasted mice with a liver-specific knockout of PEPECK as well as in fasted mice with 50, 90, and 95% PEPCK reduction even though hepatic gluconeogenesis, hepatic lipid metabolism, and glycogen synthesis in both the liver and muscle were impaired. These studies indicated the existence of compensatory mechanisms for maintaining normal fasting blood glucose levels in mice with markedly diminished or absence of hepatic PEPCK (She et al. 2003).

Taken together, the above studies have highlighted the potential role of the enzyme PEPCK in maintaining the balance in hepatic energy metabolism by acting as a cataplerotic enzyme in various pathways, including gluconeogenesis and glyceroneogenesis.
Figure 1.4: PEPCK as a cataplerotic enzyme involved in metabolic pathways from the TCA cycle to various biosynthetic and oxidative processes; (a) gluconeogenesis, (b) glyceroneogenesis, (c) serine synthesis, and (d) recycling and oxidation of the carbon skeletons of amino acids back into the TCA cycle (Yang et al. 2009).

1.5.3 Expression of PEPCK

There are two isoforms of PEPCK enzyme that are highly conserved throughout phyla: cytosolic PEPCK (pck1 or PEPCK-C) and mitochondrial PEPCK (pck2 or PEPCK-M). They are expressed in various organs but predominantly expressed in the liver and kidney. The cytosolic PEPCK is encoded by the Pck1 gene located on human chromosome 20 and contains 622 amino acids while the mitochondrial PEPCK is encoded by the Pck2 gene located on human chromosome 14 and contains 640 amino acids. These two isozymes share 68% identity and 82% similarity in their sequences (Beale et al. 2007).

Less information is known about the mitochondrial (pck2) isoform of PEPCK, possibly because in rats and mice, which are the most commonly used model organism so far, pck2 accounts for only around 5% of PEPCK enzymatic activity. In addition, cytosolic PEPCK (pck1) in rodents is tightly regulated by various hormonal stimuli, including insulin and glucagon, while mitochondrial PEPCK (pck2) is constitutively expressed in rodents, birds, and humans (Tilghman et al. 1976).

The abundance of pck1 and pck2 varies with species. In rats, mice and zebrafish, the enzymatic activity of PEPCK is exclusively due to the cytosolic isozyme whereas it is the opposite in
rabbits and birds. In humans, however, both isoforms are equally abundant (Tilghman et al. 1976). It is thought that the mitochondrial isozyme is involved in gluconeogenesis as there is no need to transport oxaloacetate as malate through a malate shuttle for cytosolic isozyme. This is because phosphoenolpyruvate produced by mitochondrial isozyme can be easily transported to the cytosol, where gluconeogenesis occurs, via mitochondrial dicarboxylate and tricarboxylate transporters (Berg et al. 2002).

The varying abundance of these two isozymes in species, and nearly equal enzymatic activity in humans, underlines the importance of understanding the role of the mitochondrial isozyme, which would allow further understanding of the role of PEPCK in human physiology (Beale et al. 2007).

1.5.4 Regulation of PEPCK (pck1)

The widely accepted pathway in which insulin regulates expression of the *pck1* gene is via the phosphoinositide-3 kinase (PI-3K) pathway. The PI-3K has been found to be a vital element in the inhibition of hepatic *pck1* gene transcription by insulin. The pathway involves the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which activates protein kinase B (Akt/PKB). Activation of Akt/PKB in hepatoma cells suppresses the glucocorticoid and cyclic adenosine monophosphat (cAMP) induction of *pck1* gene transcription (Chakravarty et al. 2005). Schmoll et al. have shown decreased levels of PEPCK mRNA in hepatoma cells with overexpression of the gene for Akt/PKB. This suggests that transcription factors such as forkhead box protein O1 (FoxO-1), hepatocyte nuclear factor 3-beta (HNF-3β), and cAMP response element-binding protein (CREB), which are downstream targets of Akt/PKB, are involved in insulin-mediated suppression of gluconeogenic gene expression including PEPCK.

FoxO-1 is an important transcription factor that facilitates PEPCK gene expression. As shown in figure 1.5, upon insulin action under fed state, Akt/PKB activates salt-inducible kinase (SIK), which leads to phosphorylation of CREB regulated transcription coactivator 2 (CRTC2), restricting the nuclear localization of CRTC2 and thus inhibition of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) transcription (Oh et al. 2013). As well, FoxO-1 becomes phosphorylated by Akt/PKB and is retained in the cytoplasm, preventing its
induction of PEPCK gene expression. This also prevents the binding of PGC-1α, a transcriptional co-factor which is positively regulated by FoxO-1 (Gross et al. 2008).

Under fasting condition, cAMP levels are increased by the action of glucagon and glucocorticoids, and cAMP-dependent protein kinase A (PKA) becomes phosphorylated and activated. PKA then phosphorylates and activates CREB, a transcription factor that stimulates PEPCK gene expression through the coactivator PGC-1α, a major regulator of PEPCK transcription (Yoon et al. 2001). The transcription of PGC-1α is dependent on CREB; thus, transcription of PGC-1α is indirectly affected by insulin, which reduces cAMP levels and leads to inactivation of CREB (Gross et al. 2008). In addition, PKA dephosphorylates CRTC2 by inactivating SIK and activating suppressor of MEK null (SMEK)/protein phosphatase 4 catalytic subunit (PP4C), resulting in nuclear localization of CRTC2 and increased association with CREB to promote transcription of PGC-1α, eventually leading to increased expression of gluconeogenic genes including PEPCK as shown in figure 1.5 (Oh et al. 2013).

![Figure 1.5: Transcriptional regulation of hepatic gluconeogenesis under (A) fasting and (B) feeding states (Oh et al. 2013).](image)

1.5.5 Inhibitors of PEPCK (pck1)

Currently, there are several molecules that competitively mimic the binding of either OAA (oxaloacetate, substrate of pck1 reaction), or PEP (phosphoenolpyruvate, product of pck1 reaction).
reaction) such as, oxalate and phosphonoformate, which are known to mimic the binding of OAA, and phosphoglycolate and 3-phosphonopropionate, which are known to mimic the binding of PEP. Another competitive inhibitor, sulfooacetate, is known to mimic the bindings of both PEP and OAA in a hybrid fashion (Stiffin et al. 2008). In contrast, 3-mercaptopicolinic acid is an inhibitor of gluconeogenesis through non-competitively inhibiting pck1, suggesting that it binds to a site other than the product (PEP) or substrate (OAA) binding site (Katiyar et al. 2015). Finding highly selective pck1 inhibitors is significant as pck1 plays an important role in glucose metabolism. Pck1 inhibitors that act through different binding sites could be of interest due to the potential synergistic effects of different pck1 inhibitors on inhibiting gluconeogenesis.

1.5.6 Transgenic zebrafish pck1 reporter lines

Aspects of glucose metabolism are highly similar between zebrafish and mammals; for example, the first rate-limiting enzyme in gluconeogenesis, PEPCK, is transcriptionally regulated by glucoregulatory hormones including insulin and glucagon in both species. Changes in the level of PEPCK expression can be readily examined by real-time PCR analysis of whole larval RNA (Elo et al. 2007).

PEPCK expression is also increased by glucocorticoids, all-trans retinoic acid, and thyroid hormone (Stafford et al. 2001 and Waltner-Law et al. 2003). Studies by Elo et al. have shown that treatment of larval zebrafish with cyclic adenosine monophosphate (cAMP)/dexamethasone and all-trans retinoic acid enhanced PEPCK levels; conversely, significantly decreased PEPCK expression levels were observed in larval zebrafish treated with the anti-diabetic drugs, glipizide, metformin, and rosiglitazone. The glucoregulatory hormone, glucagon, is secreted from pancreatic α-cells and increases cAMP which binds to the promoter of PEPCK, activating its transcription (Elo et al. 2007). Dexamethasone is a glucocorticoid drug that also enhances expression of PEPCK in zebrafish (Gut et al. 2013). These data support the use of zebrafish as a model for glucose metabolism and changes in PEPCK expression.

Recently, transgenic bioluminescence and fluorescence reporter lines, Tg(pck1:Luc2) and Tg(pck1:Venus), were generated by Gut et al. Both luciferase and Venus fluorophore are under the control of the zebrafish pck1 promoter (they were cloned downstream of a 2.8kb zebrafish pck1 promoter fragment), thus the intensity of reporter signal is indicative of pck1 expression levels. Gut et al. were successful in screening 2400 bioactive compounds for their ability to
modulate $pck1$ expression by utilizing the $Tg(pck1:Luc2)$ line, which allowed them to select two novel compounds capable of lowering glucose levels that were subsequently validated in diabetic mice (Gut et al. 2013). Thus, transgenic zebrafish $pck1$ reporter lines successfully identified compounds with potential anti-diabetic effects in more than one species.
Chapter 2

2 Rationale, hypothesis, and objectives

2.1 Rationale

The prevalence of T2DM is rapidly increasing worldwide in both developed and developing countries and thus it is a growing global burden (Van Dieren et al. 2010). Currently available therapeutics for diabetic patients include sulfonylureas, biguanides, alpha-glucosidase inhibitors, glucagon like peptide-1 receptor agonists (GLP-1 RA), dipeptidyl peptidase-4 (DPP-4) inhibitors, and sodium-glucose cotransporter 2 inhibitors (SGLT-2). Despite their effectiveness, they are associated with various side effects and are contraindicated for pre-existing health conditions including liver and kidney diseases. Therefore, novel therapeutics targeting different sites and organs may widen the choice of medications and supplement existing medications.

The enzyme pck1 has been extensively studied as a determinant of hepatic gluconeogenesis as it conducts the first rate-limiting step in gluconeogenesis. This role has been underscored by various studies showing that overexpression of pck1 in mice leads to insulin resistance and hyperglycemia (Sun et al. 2002 and Valera et al. 1994); conversely, reduction of pck1 expression in diabetic mice results in improved glycemia and insulin sensitivity (Gómez-Valadés et al. 2008).

In the drug discovery and development process, in vivo models are more beneficial than in vitro models because they allow researchers to examine the effect of a drug at the target site as well as the drug’s pharmacokinetic/pharmacodynamic profile. This way, potential unanticipated or adverse effects as well as toxicity can be investigated at the preclinical stage, thereby improving the efficiency and success rate of drugs that progress beyond preclinical studies (Silber 2010).

The use of zebrafish as an in vivo model organism in high throughput screening for drug discovery is increasing due to its advantageous features including small size, rapid and ex utero development, ease of manipulation and maintenance, and genetic and physiological similarities to humans (Miscevic et al. 2012).
Gut and his colleagues have generated two transgenic zebrafish pck1 reporter lines, \textit{Tg(Pck1:Luc2)} and \textit{Tg(Pck1:Venus)} and successfully screened 2400 bioactive compounds to identify novel pck1 modulators with potential therapeutic effects on glucose levels. They found several drugs that influence gluconeogenesis in humans, validating their strategy, as well as two novel compounds which were further validated in obese mice.

Findings from high throughput screenings are often validated using a second \textit{in vivo} model. Mouse models are the most commonly used mammalian model organism to study human systems due to anatomical and physiological conservation, enabling the examination and validation of candidate drugs (Weidner \textit{et al.} 2016 and Peters \textit{et al.} 2007).

One of the common strategies to induce type 2 diabetes in mice is high fat diet feeding and streptozotocin injection (HFD/STZ). Multiple low doses of STZ are used to induce type 2 diabetes by recapitulating pancreatic β-cell destruction (Donath \textit{et al.} 2005). The HFD feeding prior to STZ injections mimics pre-diabetic conditions such as increased body weight and glucose levels.

Collectively, drug repositioning using transgenic zebrafish pck1 reporters as a primary model organism to identify candidate compounds with potential anti-diabetic effects may reduce the time, cost and duration in drug discovery and development. Additionally, subsequent validation studies in mouse models will contribute to enhance success rate of a drug candidate’s success in reaching the market.

2.2 Hypothesis

As described above, pck1 is a valuable therapeutic target for anti-diabetic drug discovery. In this study, we hypothesize that the compounds identified from the larval zebrafish screening that down regulate \textit{pck1} gene expression could potentially be developed as anti-diabetic drugs by targeting the molecular pathways leading to activation or inhibition of transcription factors or co-factors within the PEPCK promoter region.

2.3 Objectives

The three main objectives of this study include:
1) To evaluate and rank the efficacy of the ten lead compounds, which were identified from the previous screening of a drug library consisting of 727 FDA-approved drugs, using two transgenic zebrafish pck1 reporter lines, \textit{Tg(pck1:Luc2)} and \textit{Tg(pck1:Venus)}, based on their potential anti-diabetic effects. Ranking of the compounds will be based on their ability to down-regulate \textit{pck1} expression and lower glucose levels. \textit{Pck1} expression levels can be measured in two ways; 1) quantitation of luminescence and fluorescence intensities, which both are indicative of \textit{pck1} expression levels, using \textit{Tg(pck1:Luc2)} and \textit{Tg(pck1:Venus)}, respectively, and 2) measuring \textit{pck1} mRNA levels through qPCR analysis. Free glucose levels from whole larval extracts will also be considered in ranking the ten lead compounds.

2) To select top three lead compounds and perform further validation studies, examine whether there is a dose-dependent response on \textit{pck1} and glucose lowering (IC\textsubscript{50}), and take individual fluorescent liver images.

3) Using mouse (healthy and diabetic) as our next model organism, validate the efficacy of the selected top lead compound(s) identified from the zebrafish studies by intraperitoneal pyruvate tolerance test (iPTT), measurement of \textit{pck1} and FoxO-1 mRNA and/or protein levels (qPCR and Western blot analyses), and serum insulin and gastrin levels (ELISA).
Chapter 3  
Materials and Methods  

3 Materials and Methods  

3.1 Animal models: Zebrafish  

3.1.1 Zebrafish husbandry  

Adult zebrafish were maintained at 25°C and pH 6.8-7.4 in a 14:10 light:dark cycle, following the ZebTEC aquarium housing system (Techniplast Inc, USA). Fish were fed with TetraMin Tropical Flakes (Big Al’s Canada, Toronto, Ontario) supplemented with live brine shrimp. Embryos and larvae were raised in 28 °C incubator with normal oxygen concentrations (20%) in embryo water (E2, 13.7mM NaCl, 5.4mM KCl, 0.25mM Na₂HPO₄, 0.44mM KH₂PO₄, 1.3mM CaCl₂, 1.0mM MgSO₄, 4.2mM NaHCO₃, pH 7.2 with NaOH) and kept in 5cm petri dishes until treatment (4 dpf). The fish facilities are located at Li Ka Shing Knowledge Institute, St. Michael’s Hospital, Toronto, Ontario. 

Studies involving zebrafish as a model organism utilized two transgenic zebrafish pck1 reporter lines, Tg(pck1:Luc2) and Tg(pck1:Venus), which were provided by Dr. Philipp Gut (Stainier Lab, University of California, San Francisco), wild type Tuebingen (TU), which was provided by the Zebrafish International Resource Center, and spotted Leo. 

3.1.2 Compound preparation for zebrafish studies  

Nine lead compounds (amlexanox (A), levofloxacin (C), naproxen sodium (I), dicloxacillin sodium salt monohydrate (L), esomeprazole magnesium hydrate (Eso), 5-fluorocytosine (Fluo), tolterodine L-tartrate (Tol), epirubicin hydrochloride (Epi) and daunorubicin hydrochloride (Dau)), as well as metformin hydrochloride (positive control, Met), were purchased from Sigma Aldrich and dissolved in 100% DMSO with a stock concentration of 100mM. The tenth lead compound, zolpidem (Zol), was dissolved in methanol (zolpidem tartrate solution, 1mg/mL, Sigma Aldrich). Both stock and working solutions were kept at -20°C, and working solutions were kept to less than ten-freeze-thaw cycles or less than one month. Undissolved compounds were kept either at 4 or -20°C according to the manufacturer’s instructions.
Working concentrations for all compounds were either 10mM or 1mM, and in the case of metformin was diluted to 100μM final concentration as per the following dilution examples: In a 96-well plate, in which each well contained 1 larva in 100μL 0.5% DMSO embryo water, 1μL of 10mM metformin was added to each well for a final concentration of 100μM. In a 12-well plate, in which each well contained 10-15 larvae in 1.5mL 0.5% DMSO embryo water, 15μL of 10mM metformin was added to each well for a final concentration of 100μM.

3.1.3 Treatment in zebrafish

The Tg(pck1:Luc2) or Tg(pck1:Venus) was out-crossed with WT (Leo or TU). As both transgenic lines express mCherry fluorophore in the eyes, screening and sorting of transgenic embryos were done at 3dpf. At 4 dpf, larval zebrafish in embryo water were transferred to embryo water containing 0.5% DMSO and subsequently transferred to either 96-well (1 larva/well) or 12-well plate (10-15 larvae/well) to be treated. Treated larvae were kept at 28˚C for 48 hours. Following a 48-hour incubation, treated larvae were measured for luminescence or fluorescence in a 96-well plate, or harvested for qRT-PCR, glucose measurement, or luminescence measurement using whole larval extracts.

3.2 Animal models: Mouse

3.2.1 Mouse husbandry

For mouse studies, 10-week-old and 4-week-old C57BL/6 male mice were ordered from the Jackson Labs for two different conditions; normal and diabetic groups. Mice were kept in specific pathogen free (SPF) facilities, five animals per cage at 21˚C with 12:12 light:dark cycle. Normal mice groups were fed with a normal diet (2918 Tekland Global 18% Protein Irradiated Rodent Diet) while diabetic mice groups were fed with a high fat diet starting from 5-week-old age (D12492: 60% kcal as fat; Research diets, USA) ad libitum.

3.2.2 Compound preparation for mouse studies

For mouse studies, all the compounds (esomeprazole magnesium hydrate (Eso), metformin hydrochloride (Met), sodium pyruvate, streptozotocin (STZ)) were purchased from Sigma Aldrich. Due to insolubility of esomeprazole magnesium hydrate in water, a stock solution (100mg/mL) was made with 100% DMSO and subsequently diluted in autoclaved double
distilled water (ddH$_2$O) to 0.3 and 1.5mg/mL. The rest of the compounds were dissolved in autoclaved ddH$_2$O. The stock concentration of Met control was also 100mg/mL. For Met and Eso treatments, which were done in five days, working solutions were made on the first day of the treatment and aliquoted into five tubes and kept at -20°C. Sodium pyruvate was made on the day of intraperitoneal pyruvate tolerance test (iPTT) with a working concentration of 0.2mg/mL. Streptozotocin powder was weighed and transferred to a 1.5mL Eppendorf tube wrapped with aluminum foil, and kept at -20°C. Just prior to STZ injection, appropriate volume of filtered 0.1M Na-Citrate buffer (pH 4.5) was added to make a working concentration of 5mg/mL. All injections for mouse studies were done intraperitoneally and all solutions for intraperitoneal injections (ip) were filtered through a 0.22μm syringe filter and transferred to a new 15mL falcon tube or 1.5mL Eppendorf tube.

3.2.3 Inducing type 2 diabetes (T2D) in mice

T2D was induced in mice following the protocol by Vickers et al. (2011). Mice were fed with a high fat diet (HFD) for 3 weeks. Following a 3-week HFD feeding, five consecutive low doses of STZ (50mg/kg bodyweight (BW)) injections were given intraperitoneally to induce pancreatic β-cell destruction. The dosing volume was 10μL/g BW. Mice were monitored for 4 weeks to ensure they reached and maintained diabetes, denoted by blood glucose levels above 20mmol/L. After the confirmation of sufficient diabetes, mice were treated with the selected lead compound (esomeprazole magnesium hydrate, Eso) through intraperitoneal injection (ip).

3.2.4 Treatment in normal and diabetic mice

Both normal and diabetic mice received daily ip injection of the selected lead compound, Eso, at two difference doses, 3 and 15mg/kg BW, for five days. Metformin (50mg/kg BW) was used as a positive control to compare its efficacy with our lead compound and the corresponding negative control group received saline (0.9% sodium chloride) at 10μL/g BW. Treatment was administered in two separate rounds with one week of rest in between. The dosing volume was 10μL/g BW. On the last day of each 5-day treatment, mice were subjected to intraperitoneal pyruvate tolerance test (iPTT).
3.3 Examination and ranking of the ten lead compounds

3.3.1 Luciferase assay

The homozygous Tg(pck1:Luc2) was out-crossed with WT Leo or TU. Transgenic larvae were transdermally treated with one of the ten lead compounds at two different concentrations (5 and 10μM, or 1 and 2μM) or metformin (100μM) at 4 dpf in 96-well plates (96-well Microlite™ White Microtiter™ plates, Thermo Scientific). The condition for negative control group was 0.5% DMSO embryo water without treatment. At 6 dpf, treated larvae as well as negative control were subjected to luciferase assay using Steadylite Plus™ (Perkin Elmer) reagents according to the manufacturer’s instructions. The Steadylite plus reagent was added at a ratio of 1:4 (reagent:embryo water containing treated larva). Each well contained treated larva in 100μL of embryo water, thus 25μL of the reagent was directly added to lyse the larva. Then the plate was incubated for 1 hour at room temperature (RT) in the dark followed by luminescence reading in the plate reader SpectraMax M5e (Molecular Devices) using the software SoftMax Pro. Each plate was read twice and the average values were used for quantification analysis and to compare the efficacy of the compounds at down-regulating pck1 expression levels. The protocol was adapted from Gut et al. (2013).

3.3.2 Fluorescence reading

The results from luciferase assay were validated using another transgenic pck1 reporter line, Tg(pck1:Venus). Heterozygous Tg(pck1:Venus) was out-crossed with WT Leo or TU. 600μL of 20X 1-phenyl 2-thiourea (PTU) was added to petri dishes containing approximately 20mL of embryo water at 1 dpf to inhibit melanogenesis so that embryos remain transparent for imaging. Transgenic embryos were screened and sorted for experiments at 3 dpf. At 4 dpf, transgenic larvae were treated with one of the ten lead compounds at two different concentrations (5 and 10μM, or 1 and 2μM) or metformin (100μM) in 96-well, black wall, clear bottom plates (Thermo Scientific). The condition for negative control group was 0.5% DMSO embryo water without treatment. At 6 dpf, 20μL of 100ppm clove oil was added to each well to anesthetize the larva, and 80μL of water was removed from each well, leaving approximately 40μL of water remaining to bring the larva to the bottom of the well. Just prior to reading, plates were spun down to position the larva at the bottom center. Then, fluorescence intensity from each well was measured using a plate reader (ImageXpress Ultra, Molecular Devices). The fluorescence
intensity values were used to compare and validate the efficacy of the compounds at down-regulating pck1 expression levels.

3.3.3 Glucose assay

WT larvae were treated with one of the ten lead compounds at 2μM (Tol, Epi, and Dau), 10μM (the rest of the seven compounds), or metformin (100μM) at 4 dpf in 96-well plates (1 larva/well). The condition for negative control was 0.5% DMSO embryo water without treatment. Treated larvae as well as negative controls were collected in 1.5mL Eppendorf tubes following a 48-hour incubation. Water was removed and samples were stored at -80°C until ready to use. Samples were then thawed and reconstituted in 50μL phosphate-buffered saline (PBS). Each larva was homogenized using a hand-held homogenizer. 50μL of sample was used for glucose assay using a glucose colorimetric/fluorometric assay kit (BioVision Inc.)

3.3.4 Glucose assay and luciferase assay for dose response curves

The protocol for glucose and luciferase assays using the same larval extracts to plot dose response curves was adapted from Gut et al. (2013). Homozygous Tg(pck1:Luc2) was out-crossed with WT Leo or TU and transgenic larvae were treated with one of the three lead compounds (Eso, Epi, or Tol), amlexanox (A), metformin (Met), or isoprenaline (Iso) in 12-well plates (10 larvae/well). The negative control group of larvae were incubated in 0.5% DMSO embryo water without treatment. Met and Iso were treated at 100μM and 20μM, respectively. Five concentrations were used to plot a dose response curve for A, Eso, and Epi, in which 2, 4, 8, 16, 32uM were used for A, 1.25, 2.5, 5, 10, 20uM for Eso, 0.5, 1, 2, 4, 8uM for Epi, while nine concentrations were used for Tol, 2, 4, 8, 16, 31, 62, 125, 250, 500nM. Following a 48-hour incubation, treated larvae as well as negative control were collected in 1.5mL Eppendorf tubes and the water was removed. Collected larvae were stored at -80°C until ready to use.

Samples were thawed and reconstituted in 200μL phosphate-buffered saline (PBS). The larvae in each tube were homogenized using a hand-held homogenizer. 15μL of sample was used for glucose assay using a glucose colorimetric/fluorometric assay kit (BioVision Inc.) whereas 20μL of sample was used for luciferase assay using Steadylite Plus™ (Perkin Elmer) reagents according to the manufacturer’s instructions. For glucose assay, 96-well black wall, clear bottom plates (Thermo Scientific) were used for fluorescence quantitation while 96-well Microlite™
White Microtiter™ plates (Thermo Scientific) were used for luciferase quantitation. For both assays, the plate reader SpectraMax M5e (Molecular Devices) with the software SoftMax Pro was used.

3.3.5 Fluorescence imaging

Heterozygous Tg(pck1:Venus) was out-crossed with WT Leo or TU. Again, for transparent embryos, 600μL of 20X 1-phenyl 2-thiourea (PTU) was added to petri dishes containing approximately 20mL of embryo water at 1 dpf. Transgenic larvae were screened and sorted out at 3 dpf. At 4dpf, transgenic larvae were treated with one of the three lead compounds (Eso, Epi, or Tol), amlexanox (A), metformin (Met), or isoprenaline (Iso) in 12-well plates (10-15 larvae/well). Negative control group of larvae were in 0.5% DMSO embryo water without treatment. Met and Iso were treated at 100μM and 20μM, respectively. 5 and 20μM doses were used for A and Eso, 2 and 8μM for Epi, and 0.25 and 1μM for Tol. Fluorescence images of Tg(pck1:Venus) were acquired using an inverted microscope (Leica Microsystems). Fluorescence images were taken at 40X magnification with constant exposure time, which was identified according to the fluorescence intensity of the metformin treated larvae. Fluorescence intensity was measured using the ImageJ software (version 1.48, NIH).

3.4 RNA extraction

3.4.1 Zebrafish

WT larvae were treated with one of the ten lead compounds at 2μM (Tol, Epi, and Dau) or 10μM (for the rest of the seven compounds), or metformin (100μM) at 4 dpf in 12-well plates (15 larvae/well). Negative control group was in 0.5% DMSO embryo water without treatment. Treated larvae as well as negative controls were collected in 1.5mL Eppendorf tubes following a 48-hour incubation. Water was removed and samples were stored at -80°C until ready to use.

The larval zebrafish samples were suspended in 300μL of Trizol® (Invitrogen). RNA extraction was done following the modified Trizol RNA extraction protocol accompanied by RNeasy® Mini Kit (Qiagen) by Untergasser (2008).
3.4.2 Mouse

The liver tissues were collected, immediately frozen in liquid nitrogen, and stored at -80°C until ready to use. Liver tissue (approximately 30-50mg) was homogenized in 1mL of Trizol® (Invitrogen) in Eppendorf tubes. Then 0.2mL of chloroform (Sigma Aldrich) was added to each tube followed by a vigorous shaking for approximately 15 seconds. Tubes were spun down at 12,000 rpm for 15 minutes at 4°C. The supernatant from each tube was immediately collected in a new 1.5mL Eppendorf tube and 0.5mL of isopropanol was subsequently added. Tubes were incubated for 10 minutes at RT and centrifuged at 12,000 rpm (revolutions per minute) for 10 minutes at 4°C. The RNA pellet was washed in 1mL of 75% ethanol twice. Then the RNA pellet was air dried and dissolved in 40μL of ddH2O. The concentration of RNA was measured using the NanoDrop (Thermo Scientific). The purity of each sample was confirmed by 260/280 ratio.

3.5 RT-qPCR

3.5.1 Zebrafish and mouse

cDNA for qPCR experiments was generated using the QuantiTect Reverse Transcription Kit (Qiagen) and relative mRNA expression levels were determined using the Power SYBR® Green Master Mix (Life Technologies) according to manufacturers’ instructions. The reaction volume for qPCR experiments was 10μL per well in 384-well plates (Thermo Scientific). Relative mRNA expression levels were determined using the ΔΔCt method. Values were normalized to reference genes: EF1α (Elongation factor 1α) for zebrafish and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) for mouse.

The primer sequences used for real time PCR experiments for zebrafish studies are as followed:
PEPCK Fwd 5’-GAGTGGGACAAAGCCATGAA-3’, PEPCK Rev 5’-AGCTCCACCCCTATCTTGGA-3’; EF1α Fwd 5’-GATGCACCACGAGTCTCTGA-3’, EF1α Rev 5’-TGATGACCTGAGCGTTGAAG-3’

The primer sequences used for real time PCR experiments for mouse studies are as followed:
PEPCK Fwd 5’-AGCCTTTGGTCAACAACCTGG-3’, PEPCK Rev 5’- TGCCCTCGGGGTAGTTATG-3’; GAPDH Fwd 5’-AACCTTTGGCATTTGGAAGG-3’, GAPDH Rev 5’-ACACATTGGGCTAGGAACA-3’.
3.6 Protein extraction

Cytoplasmic and nuclear protein extracts from mouse liver tissues (approximately 20mg) were obtained using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce Biotechnology) according to the manufacturer’s instructions.

3.7 Western blot

Protein concentrations for murine cytoplasmic and nuclear extracts were determined using the BSA protein assay kit (Thermo Scientific) following the manufacturer’s instructions. 40µg of cytoplasmic and nuclear extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat milk-TBST (10mM Tris, 150mM NaCl, 0.5% Tween 20, pH 8.0) for 1 hour at RT. Following a 1 hour-blocking, membranes were briefly washed in TBST for 5 minutes and incubated in primary antibodies directed against pck1 (Abcam), β-actin (Cell Signaling), FoxO-1 (Abcam), and histone H3 (Cell Signaling) overnight at 4°C. On the following day, membranes were washed 5 times in TBST (5 minutes for each wash) and incubated in secondary horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology) for 1 hour at RT. After 5 TBST washes (5 minutes for each wash), immunoblots were exposed to Clarity™ and Clarity Max™ Western ECL Blotting Substrates (BioRad) for 3 minutes prior to chemiluminescence detection (Chemidoc, BioRad).

3.8 Intraperitoneal pyruvate tolerance test

Treated mice were subjected to the intraperitoneal pyruvate tolerance test (iPTT). Prior to iPTT, mice were fasted for 16 hours (treatment day 4). Mice were given the last treatment (5th treatment) 30 minutes prior to iPTT. Then, fasting blood glucose levels were measured using a glucometer and 2g/kg BW sodium pyruvate was given intraperitoneally. Subsequently, blood glucose levels at 15, 30, 60, and 120 minutes post pyruvate injection were measured using a glucometer.

3.9 Serum gastrin and insulin measurement

Mouse blood was collected through cardiac puncture. Blood samples in 1.5mL Eppendorf tubes were incubated at RT for 30 minutes and then centrifuged at 12000rpm for 10 minutes at 4°C.
Serum supernatant was immediately collected into a new 1.5mL Eppendorf tube. Serum samples were stored at -80°C until ready to use. Serum gastrin and insulin levels were measured using the mouse gastrin (Sigma Aldrich) and insulin (Millipore) ELISA kits following the manufacturer’s instructions.

### 3.10 Statistical analysis

All statistical analyses were performed with Microsoft Excel 2016, Graphpad Prism 5, and SigmaPlot 13.0 software. The statistical significance for each experiment was calculated using the two-tailed student’s t-tests with thresholds of p<0.05 (*) and p<0.01 (**). For the dose-response curves, non-linear regression analysis was used to determine either IC$_{50}$ or EC$_{50}$. All error bars represent ± SEM (standard error mean).
Chapter 4

4 Results

4.1 Examination and ranking of the ten lead compounds

4.1.1 Luminescence and fluorescence readings identified significantly reduced pck1 expression levels by compounds A, Epi, Fluo, Eso.

Each of the ten lead compounds was tested 5-7 times at two different concentrations (1μM and 2μM for compounds Tol, Epi, and Dau, due to toxicity; 5μM and 10μM for the rest of the compounds) using the luciferase pck1 reporter Tg(pck1:Luc2) (Figure 4.1) to quantify the expression levels of the pck1 gene. The negative control was 0.5% DMSO embryo water without treatment while the positive control for the entire study was metformin (Met, 100μM), which is a known anti-diabetic drug that works by reducing hepatic glucose production and enhancing insulin sensitivity (Hundal et al. 2000 and Kirpichnikov et al. 2002). Meanwhile, independent quantification of pck1 expression was done using the fluorescence pck1 reporter line Tg(pck1:Venus) under the same experimental condition described above in parallel with luciferase assays to measure the potential effects of the ten lead compounds as gluconeogenesis regulators (Figure 4.2). The two aforementioned transgenic reporter lines contain either luciferase or Venus fluorophore, respectively, under the control of the same pck1 promoter.

Compound A showed the most significant reduction in pck1 expression followed by compounds Epi and Fluo in luciferase assay (Figure 4.1 and Table 4.1). Similarly, although there is a discrepancy in the level of reduction, compounds A and Epi showed the most significant reduction in pck1 expression in fluorescence intensity reading followed by compounds Eso, and L. (Figure 4.2 and Table 4.1).

The discrepancy between these two readings may be attributed to a different sensitivity of the experiment and the fact that luciferase assay was performed in larval lysate whereas fluorescence reading was done in live larvae which may potentially introduce more variables.
Figure 4.1: Relative luciferase activity of the $Tg(pck1:Luc2)$ zebrafish larvae at 6dpf. Each compound had a low and high dose. Compounds Tol, Epi, and Dau were given at 1uM and 2uM due to toxicity while the remaining compounds were given at 5uM and 10uM. All values are normalized to negative control values, and are reported as a ratio. Data are expressed as mean ratios ± s.e.m. *p<0.05, **p<0.01 versus negative control. Numbers depict n (number of wells measured).

Figure 4.2: Relative fluorescence intensity of the $Tg(pck1:Venus)$ zebrafish larvae at 6dpf. Each compound had a low and high dose. Compounds Tol, Epi, and Dau were treated at 1uM and 2uM due to toxicity while the remaining compounds were treated at 5uM and 10uM. All values are normalized to negative control values, and are reported as a ratio. Data are expressed as mean ratios ± s.e.m. *p<0.05, **p<0.01 versus negative control. Numbers depict n (number of wells measured).
4.1.2 Real-time PCR (qPCR) analysis identified significantly reduced pck1 mRNA levels by compounds Tol, Eso, C, A, and Dau.

Subsequently, relative pck1 mRNA expression level was measured 3-4 times by qRT-PCR to further confirm the effect of the ten lead compounds (Figure 4.3). Fifteen healthy WT larvae were pooled for each treatment in 12-well plates. Pck1 gene expression results were normalized to the housekeeping gene EF1α. Interestingly, there was a robust reduction in pck1 mRNA expression in Tol treated WT larvae followed by compounds Eso, C, A, and Dau (Figure 4.3 and Table 4.1). Again, metformin (Met) was the positive control. The eight lead compounds (excluding compounds Fluo and Zol) showed stronger pck1 mRNA reduction than Met treatment.

![Figure 4.3: Relative pck1 mRNA expression from 15 WT larvae (n=4 for Neg, Met and Tol and n=3 for the rest of the compounds) at 6dpf treated with one of the compounds; compounds Tol, Epi, and Dau were treated at 2μM while rest of the compounds were treated at 10μM. All values are normalized with regulatory gene EF1α. Data are expressed as means ± s.e.m. *p<0.05, **p<0.01 versus negative control.](image-url)
4.1.3 Whole body free glucose levels were significantly reduced by compounds Epi, Eso, A and I.

Next, whole-larva free glucose level was measured in three independent assays to investigate the potential glucose lowering effect of the ten lead compounds (Figure 4.4). The treatment was performed in 96-well plates, in which each well contained one WT larva. Using metformin treatment as our threshold, there were three compounds that showed stronger glucose lowering effects than metformin, which are as follows; Epi, Eso, and A (Figure 4.4 and Table 4.1).

![Figure 4.4: Whole body free glucose levels of WT zebrafish larvae at 6dpf treated with one of ten compounds; compounds Tol, Epi, and Dau were treated at 2uM while the remaining compounds were treated at 10uM. Numbers depict n, sample number; each n represents larval lysate from 1 WT zebrafish larva at 6dpf. Data are expressed as means ± s.e.m. *p<0.05, **p<0.01 versus negative control.](image)

4.1.4 Compounds A, Epi, Eso, and Tol showed pronounced pck1 and glucose lowering effects.

The ten lead compounds were ranked according to their efficacy and consistency at lowering pck1 expression and glucose levels to prioritize them for further examination and validation studies. Each compound was ranked for each experiment. Because luciferase assay and fluorescence intensity reading were performed at two different concentrations, compounds were ranked separately for low and high doses, and the two ranking numbers for each compound were averaged. Each compound was ranked within each experiment, then the ranking numbers were aggregated to provide a final Average Ranking (Table 4.1). The four compounds that consistently
showed pronounced pck1 and glucose lowering effects throughout the four experiments are compounds 1) A (amlexanox), 2) Epi (epirubicin), 3) Eso (esomeprazole), and 4) Tol (Tolterodine).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Luciferase assay</th>
<th>Fluorescence intensity</th>
<th>qRT-PCR</th>
<th>Glucose assay</th>
<th>Average ranking</th>
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<tr>
<td>Amlexanox (A)</td>
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<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Levofloxacin (C)</td>
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<td>3</td>
<td>7</td>
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<tr>
<td>Naproxen (I)</td>
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<td>7</td>
<td>4</td>
<td>7.5</td>
<td>9</td>
</tr>
<tr>
<td>Dicloxacillin (L)</td>
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<td>7</td>
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<td>5.75</td>
<td>6</td>
</tr>
<tr>
<td>Esomeprazole (Eso)</td>
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<tr>
<td>Zolpidem (Zol)</td>
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<tr>
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<td>4</td>
<td>8</td>
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</tbody>
</table>

Table 4.1 Ranking of each compound for each experiment

4.2 Comparison studies for the top three lead compounds: Epi, Eso, and Tol.

4.2.1 Fluorescent liver images of the top three lead compounds: Epi, Eso, and Tol.

The three compounds selected for comparison studies are compounds Epi, Eso, and Tol. Although amlexanox (A) was ranked to be No. 1, it was excluded for further studies as its potential anti-diabetic effects and its possible mechanism of action have already been published (Reilly et al. 2015). However, we used amlexanox to compare its pck1 and glucose lowering effects with our top three lead compounds.

The Tg(pck1:Venus) line was used to take individual liver images via fluorescence microscopy to further confirm their effects on pck1 expression (Figure 4.5).

The fluorescent liver images were acquired in parallel with dose response studies. Hence, the middle and highest concentrations used for each compound’s dose response studies were selected for each of the compound for fluorescence imaging: 5 and 20uM for compounds A (amlexanox)
and Eso (esomeprazole), 2 and 8uM for compound Epi (epirubicin), and 0.25 and 1uM for compound Tol (tolterodine). However, compounds A and Tol did not show complete sigmoidal curves for glucose assay; they both showed only one part of the plateau, therefore; concentrations were adjusted to identify complete sigmoidal curves.

Consistent with the previous readings, compounds A and Epi showed a lower pck1 expression at higher dose while compounds Eso and Tol showed a higher pck1 expression at higher dose (Figure 4.5). Compound A showed the most significant reduction in pck1 expression followed by compounds Eso (5uM) and Epi (2 and 8uM).
Figure 4.5: Fluorescence intensity in Tg(pck1:Venus) zebrafish larvae at 6dpf. (A) Images were captured via fluorescence microscope. Each larva was positioned on its lateral side to fully visualize the fluorescent liver. Each compound was tested at two concentrations. (B) Fluorescence intensity was measured using ImageJ. Numbers depict n (number of larvae measured). All values are normalized to negative control group and are expressed as mean % response ± s.e.m. **p<0.01 versus negative control.
4.2.2 Compound Epi showed a dose-dependent reduction of pck1 expression while compounds Epi and Eso decreased glucose levels in a dose-dependent manner.

Each of the three compounds Epi (epirubicin), Eso (esomeprazole), and Tol (tolterodine) was assessed by luciferase and glucose assays to determine whether they reduce pck1 and glucose levels in a dose-dependent manner. IC₅₀ value, which is the concentration of a compound at 50% inhibitory response (Beck et al. 2012), was used to compare efficacy among the top three lead compounds.

Five concentrations were used to plot a dose response curve; 2, 4, 8, 16, 32µM were used for amlexanox (A); 1.25, 2.5, 5, 10, 20µM for esomeprazole (Eso); and 0.5, 1, 2, 4, 8µM for epirubicin (Epi); while nine concentrations were used for tolterodine (Tol), 2, 4, 8, 16, 31, 62, 125, 250, 500nM. Doses were selected with consideration of the previous concentrations used to rank the ten lead compounds. Ten healthy Tg(pck1:Luc2) larvae were pooled for each concentration of the compound.

The protocols for glucose and luciferase assays were adapted from Gut et al., and uses homogenized larval lysate to test both pck1 expression and glucose levels. This method allows us to better monitor and examine changes in the pck1 expression and glucose levels, and reduces variability because the measurements from both assays are from the same pool of larvae. Control groups include 0.5% DMSO, metformin (Met, 100µM), and isoprenaline (Iso, 20µM) (Figure 4.6). Isoprenaline, which increases both pck1 expression and glucose levels (Gut et al. 2013), was added to our control group to verify an increase (by Iso) and a decrease (by Met) in pck1 and glucose levels.

Using luciferase assays to measure the ability of each compound to decrease pck1 expression levels, compound A lowered pck1 expression in a dose-dependent manner with an IC₅₀ of 5.33µM (Figure 4.7A). Compound Epi also showed a dose-dependent reduction of pck1 expression with a superior IC₅₀ of 2.51µM (Figure 4.7C). On the other hand, compound Eso did not show dose dependency on pck1 expression level (Figure 4.7B); there was a decrease in pck1 expression from 1.25 to 5µM but an increase from 5 to 20µM. Compound Tol showed a linear reduction of pck1 expression (Figure 4.7D). The IC₅₀ from glucose assays was found to be 5.22 and 1.28µM for compounds Eso (Figure 4.8B) and Epi (Figure 4.8C), respectively while the
EC₅₀, which is the concentration of a compound at 50% inducing response (Beck et al. 2012), for compounds A (Figure 4.8A) and Tol (Figure 4.8D) was found to be 11 µM and 16 nM, respectively. Our data suggest that both compounds A and Tol increase glucose levels in a dose-dependent manner regardless of reduced pck1 expression (Figure 4.7A and D), indicating the involvement of other factors responsible for the increase in glucose levels.

Collectively, among the top three compounds, compound Epi (epirubicin) was most potent at lowering pck1 and glucose levels (Figures 4.7C and 4.8C) followed by compound Eso (esomeprazole). Although compound Epi showed the most significant reduction in pck1 and glucose levels in a dose-dependent manner, due to its current use (anthracycline) and higher toxicity than compound Eso, our study focused in on compound Eso for mouse models.

![Figure 4.6](image)

Figure 4.6: (A) pck1 and (B) glucose levels examined in control groups, Neg (0.5% DMSO), Met100 (Metformin 100µM), and Iso20 (Isoprenaline 20µM), using Tg(pck1:Luc2) from 5 independent experiments (10 larvae/well). Luminescence (pck1 level) and fluorescence (glucose level) values are normalized to negative control group (0.5% DMSO) and data are expressed as mean % response ± s.e.m. **p<0.01 versus negative control.
Figure 4.7: Luciferase assays to plot dose response curves for the four compounds pursued in comparison studies using Tg(pck1:Luc2) larvae at 6dpf. Each point represents results from 5 independent luciferase assays (10 larvae/well). Concentrations are expressed as log10(concentration). Luminescence values are normalized to negative control and are expressed as mean % response ± s.e.m. Compounds A (amlexanox) and Epi (epirubicin) reduce pck1 expression in a dose-dependent manner with the IC50 of 5.33 and 2.51μM, respectively.

Figure 4.8: Glucose assays to plot dose response curves for the four compounds pursued in comparison studies using Tg(pck1:Luc2) larvae at 6dpf. Each point represents results from 5 independent glucose assays (10 larvae/well). Concentrations are expressed as log10(concentration). Values are normalized to negative control and are expressed as mean % response ± s.e.m. Compounds Eso (esomeprazole), and Epi (epirubicin)
reduce glucose levels in a dose-dependent manner with the IC$_{50}$ of 5.22 and 1.28μM, respectively while compounds A (amlexanox) and Tol (tolterodine) increase glucose levels in a dose-dependent manner with the EC$_{50}$ of 11μM and 16 nM, respectively.

4.3 Validation of esomeprazole (Eso) in mouse models.

4.3.1 Eso treatment in normal mice shows euglycemia but reduced pck1 levels, and increased serum gastrin and insulin levels.

For treatment in normal mice, 10-week-old C57LB/6 male mice were ordered and given a week for the accommodation to the new environment. Following the accommodation period, mice were subjected to intraperitoneal pyruvate tolerance test (iPTT), which reflects the glycemic excursion in response to intraperitoneally injected pyruvate (Hughey et al. 2014) to obtain the baseline parameters. Then mice were separated into four treatment groups: saline (10μL/g BW), metformin (Met, 50mg/kg BW) and esomeprazole (Eso, 3 and 15mg/kg BW). Metformin suppresses gluconeogenesis in the liver and enhances insulin sensitivity, and was used as our positive control (Hundal et al. 2000 and Kirpichnikov et al. 2002).

On the last day of treatment (treatment day 5), mice were subjected to iPTT 30 minutes post last treatment injection. Hence, the glucose levels at t=0 minute reflects the fasting glucose levels 30 minutes post treatment. After pyruvate was given intraperitoneally, blood glucose levels were measured at 15, 30, 60, and 120 minutes post injection.

Neither Met nor Eso treatments showed a significant difference in glucose levels compared to saline group in normal mice (Figures 4.9A-C). Differences in glucose AUC did not reach statistical significance among groups (Figure 4.9D).
Figure 4.9: iPTT results of normal C57BL/6 mice treated with A) metformin at 50mg/kg BW or compound Eso at B) 3mg/kg BW and C) 15mg/kg BW for 5 days. Time 0 min indicates the blood glucose levels 30 min post 5th treatment. D) AUC calculations show no enhancement in glycemic control by Met and Eso treatments. Each group consisted of 5 mice, with the experiment replicated twice. **p<0.01 versus control, error bars represent ± s.e.m.

Next, pck1 mRNA level was examined to validate the pck1 lowering effect of Eso observed in zebrafish studies. In normal mice, the level of pck1 mRNA expression was not statistically reduced by Eso treatment at 3 and 15mg/kg BW, or by Met treatment. The reference gene GAPDH was used for normalization.
Figure 4.10: Relative pck1 mRNA expression of normal C57BL/6 mice treated with metformin (50mg/kg BW) or compound Eso at two doses (3 and 15mg/kg BW). Values are normalized with regulatory gene GAPDH. *p<0.05, **p<0.01 versus negative control, error bars represent ± s.e.m.

Western blot was performed to examine and compare the expression level of pck1 protein in compound Eso treated mice against saline received mice. As shown in figure 4.11, there was a significant decrease in pck1 protein levels in both Met and Eso treated mice, the higher dose of compound Eso (15mg/kg BW) being the most significant at lowering pck1 levels (approximately 48% reduction).

Figure 4.11: Pck1 protein expression of normal C57BL/6 mice treated with metformin (50mg/kg BW) or compound Eso at two doses (3 and 15mg/kg BW). Quantifications of western blots were done with ImageJ. Data are expressed as means ± s.e.m. *p<0.05, **p<0.01 versus negative control.
To further explore the possible pathway in which compound Eso takes place in regulating glucose metabolism, FoxO-1, an important transcription factor for the expression of genes involved in gluconeogenesis, was subsequently examined (Figure 4.12). Despite significantly decreased pck1 levels in Met and Eso treated normal mice (Figure 4.11), there was a trend toward increased nuclear FoxO-1 levels in all treatment groups (approximately 25-40% increase) but the difference did not reach statistical significance (Figure 4.12B).

Figure 4.12: (A) cytosolic and (B) nuclear FoxO-1 protein expression of normal C57BL/6 mice treated with metformin (50mg/kg BW) or compound Eso at two doses (3 and 15mg/kg BW). Quantifications of western blots were done with ImageJ. Data are expressed as means ± s.e.m.

Lastly, serum gastrin and insulin levels were measured using mouse gastrin and insulin ELISA kits to examine whether or not Eso treatment has an effect on insulin secretion through increasing gastrin levels as previously reported by Rooman et al. There was an approximately 2.3-, 6.1-, and 8.4-fold greater serum gastrin levels in Met, Eso 3, and Eso 15 treatments, respectively, compared to saline (Figure 4.13A).

Serum insulin levels were significantly increased by 2.8 times in Eso treatment at higher dose (15mg/kg BW), but were not significantly increased at the lower dose (3mg/kg BW). The higher the dose of Eso treatment, the higher the gastrin and insulin levels (Figure 4.13B).

On the other hand, Met treatment resulted in significantly decreased serum insulin levels (approximately 6 times less than the negative control group). This may be explained by the
increased insulin sensitivity following metformin treatment, thereby requiring less insulin to maintain glucose homeostasis.

Taken together, we demonstrate that Eso treatment, a proton pump inhibitor, increases serum gastrin levels, which promotes insulin secretion from pancreatic β-cells, corresponding to the current characteristics of proton pump inhibitors (PPIs).

![Graph](image)

Figure 4.13: Serum (A) gastrin and (B) insulin levels of normal C57BL/6 mice treated with metformin (50mg/kg BW) or compound Eso at two doses (3 and 15mg/kg BW). Data are expressed as means ± s.e.m. *p<0.05, **p<0.01 versus negative control.

4.3.2 Eso treatment in diabetic mice shows improved glycemic control with reduced pck1 and nuclear FoxO-1 levels, and increased serum gastrin and insulin levels.

For diabetic mouse studies, 4-week-old C57LB/6 male mice were given a week for accommodation to the new environment. Following the accommodation period, mice were on HFD feeding and received five consecutive STZ intraperitoneal (ip) injections to induce type 2 diabetes. HFD feeding primes a pre-diabetic state in mice, including increased body weight and glucose level. Blood glucose levels pre- and post-HFD feeding did not show a profound change, however; HFD feeding resulted in weight gain of up to 6g in 3 weeks (Figure 4.14A) whereas chow diet fed mice gained up to 3g throughout the entire study (Data not shown).

Followed by a 3 week-HFD feeding, mice received five consecutive low dose STZ injections (50mg/kg BW) intraperitoneally. Increased glucose levels (above 20mmol/L) were confirmed through monitoring the mice for 4 weeks after receiving the five STZ injections (Figure 4.14B).
After we compared the iPTT results of the same group of normal mice pre- and post-treatment, we decided not to obtain baseline parameters as post-treatment glucose levels of all four groups of mice were higher than pre-treatment glucose levels (Data not shown). It is thought that mice were under stressful environment due to consecutive ip injections and glucose measurements. Hence, we decided to omit this step and only compare post-treatment glucose levels among the four treatment groups.

![Graph](image)

**Figure 4.14:** (A) Body weight and (B) blood glucose levels of mice after starting HFD feeding and receiving five consecutive STZ ip injections.

Each group of mice received daily ip injection of saline (10μL/g BW), Met (50mg/kg BW) or Eso (3 or 15mg/kg BW) for 5 days and iPTT was performed on the last day of the treatment. Intriguingly both groups of mice that received Eso at low and high doses (3 and 15mg/kg BW) showed significantly enhanced glycemic control compared to saline-treated mice (Figures 4.15B-C), indicating that Eso treatment suppresses hepatic glucose production. Likewise, Met treated mice (positive control) showed significantly enhanced glucose control as expected (Figure 4.15A). Changes in food uptake and body weight prior to and during the treatment were unremarkable (Data not shown), indicating that the improvement in glucose control in Met and Eso treated diabetic mice is not due to the differences in food uptake among groups. The fact that Eso treatment did not lead to significant weight change at either dose is notable, since weight gain is one of the common side effects of current anti-diabetic medications including sulfonylureas and thiazolidinediones classes of drugs (Sheehan 2003).
Figure 4.15: iPTT results of diabetic C57BL/6 mice treated with A) metformin at 50mg/kg BW or compound Eso at B) 3mg/kg BW and C) 15mg/kg BW for 5 days. The first and second rounds of treatments were given on the 5th and 7th week post STZ injections, respectively. Time 0 min indicates the blood glucose levels 30 min post 5th treatment D) AUC calculations show significantly enhanced glycemic control in all drug treated groups. Each group consisted of 5 mice, with the experiment replicated twice. *p<0.05, **p<0.01 versus negative control, error bars represent ± s.e.m.

Next, pck1 mRNA was quantified to investigate the role of Eso treatment in pck1 regulation under hyperglycemic condition. Both Met and Eso treatments led to statistically decreased pck1 mRNA levels compared to saline treatment (Figure 4.16), reflecting less hepatic gluconeogenesis, corresponding to the iPTT results (Figures 4.15B-C). The pck1 mRNA expression levels were decreased by 40%, 39%, and 46% by Met, Eso 3 and Eso 15 treatments, respectively.
Figure 4.16: Relative pck1 mRNA expression of diabetic C57BL/6 mice treated with metformin (50mg/kg) or compound Eso at two doses (3 and 15mg/kg). Values are normalized with regulatory gene GAPDH. **p<0.01 versus negative control, error bars represent ± s.e.m.

Subsequently, pck1 protein levels in diabetic mice were examined to further validate that Eso treatment lowers blood glucose levels partly through regulating hepatic gluconeogenesis, in which pck1 enzyme is responsible for the first rate-limiting step. As shown in figure 4.17, Eso treatment at 15mg/kg BW led to significantly decreased pck1 protein levels by approximately 34%. Met treatment, which is known to inhibit hepatic gluconeogenesis, also led to decreased pck1 protein levels by approximately 23%. However, there was no significant decrease in Eso treatment at 3mg/kg BW.

Figure 4.17: Pck1 protein expression of diabetic C57BL/6 mice treated with metformin (50mg/kg) or compound Eso at two doses (3 and 15mg/kg). Quantifications of western blots were done with ImageJ. Data are expressed as means ± s.e.m. **p<0.01 versus negative control.
Correspondingly, the nuclear FoxO-1 protein levels were decreased by approximately 20% and 40% by Eso treatment at 3 and 15mg/kg BW, respectively (Figure 4.18B). On the other hand, Met treatment in diabetic mice did not have any effect on FoxO-1 levels.

Figure 4.18: (A) cytosolic and (B) nuclear FoxO-1 protein expression of diabetic C57BL/6 mice treated with metformin (50mg/kg) or compound Eso at two doses (3 and 15mg/kg). Quantifications of western blots were done with ImageJ. Data are expressed as means ± s.e.m. **p<0.01 versus negative control.

In addition, similar to normal mice, there was an increase in the levels of serum gastrin and insulin in Eso treated diabetic mice. The Eso treatment at 3 and 15mg/kg BW showed approximately 2-fold and 11-fold increased serum gastrin levels, respectively (Figure 4.19A). As well, there was approximately 1.6 and 2.3 times greater serum insulin levels by Eso treatment at 3 and 15mg/kg BW, respectively (Figure 4.19B), indicating that the increase in gastrin levels by Eso treatment was sufficient to promote insulin secretion from pancreatic β-cells.

Met treatment in HFD/STZ induced diabetic mice differed from the Met treatment in normal mice; Met treatment in diabetic mice led to significantly increased serum insulin levels (approximately increased by 2.2 times) as shown in Figure 4.19B, which is consistent with the previous studies by Han et al who have shown increased insulin levels in both pancreatic tissues and serum in Met treated STZ-diabetic mice.
Figure 4.19: Serum (A) gastrin and (B) insulin levels of diabetic C57BL/6 mice treated with metformin (50mg/kg BW) or compound Eso at two doses (3 and 15mg/kg BW). Data are expressed as means ± s.e.m. *p<0.05, **p<0.01 versus negative control.
Chapter 5

5 Discussion

5.1 Zebrafish studies identified esomeprazole (Eso) as effective at lowering glucose levels through regulating pck1.

5.1.1 Ten lead compounds were narrowed down to three: Epi, Eso, and Tol.

The four independent experiments using zebrafish larvae, luciferase assay (Figure 4.1), fluorescence reading (Figure 4.2), qPCR (Figure 4.3), and glucose assay (Figure 4.4), were carried out to rank the effects of the ten lead compounds at down-regulating pck1 and lowering glucose levels. The ten lead compounds were ranked for each experiment; these rankings were then averaged to give each compound a final ranking number. From this aggregated ranking, three compounds, Epi, Eso, and Tol were selected for further comparison studies.

Through four independent experiments, we were able to rank the ten lead compounds with increased power of our study and reduced variability. Nonetheless, discrepancy between the four experiments was inevitable, which may partially be explained by lack of sample uniformity. While qRT-PCR and glucose assays used homogenized larvae, fluorescence intensity reading was performed on live larvae, potentially introducing more variables in the reading. Although lysis buffer was added prior to luciferase assay measurements, it does not completely lyse the larva in each well, impacting sample uniformity and increasing the variability. In addition, the reading method for luciferase assay was the center reading (in the interest of time), instead of the area scan reading which gives an averaged result of the entire area of the well. In contrast, center reading reads only the centre of the wells regardless of their homogeneity of signal throughout the well. Since area scanning takes a long time for each plate, this method of reading was not a good choice for luciferase assay as there was a time limit.
5.1.2 Results from fluorescence screening correspond to the fluorescence liver imaging.

Although there is a discrepancy in the magnitude of changes in pck1 expression between the two different methods of fluorescence measurements, the trends closely correspond to each other, confirming the utility of both methods to examine pck1 expression levels. The discrepancy might be explained by a different setting and sensitivity of the experiment.

It is important to note that the corresponding results from the two different fluorescence measurement methods validate our preliminary trial of measuring fluorescence in Tg(pck1:Venes) using a 96-well plate. This observation is useful, as this method will allow timely, efficient screening of a drug library in future.

5.1.3 Dose response assessment of the top three lead compounds.

Although Eso (esomeprazole) did not show dose-dependency in pck1 expression levels (luciferase assay, figure 4.7B), the results correspond to the previous luminescence and fluorescence measurements, where 1 larva was used for each well in a 96-well plate, in which pck1 expression was higher at 10μM than at 5μM (Figures 4.1 and 4.2). On the other hand, compound Eso lowered glucose levels in a dose dependent manner with the IC\textsubscript{50} of 5.22μM (Figure 4.8B). One possible explanation for this phenomenon might be activated compensatory pathways in response to overwhelming glucose lowering by compound Eso, which was observed in Gut et al. Gut and his colleagues utilized the same transgenic zebrafish pck1 reporter line, Tg(pck1:Luc2), to screen 2400 bioactive compounds, identifying two novel compounds that lowered glucose levels but paradoxically increased pck1 levels. They reasoned this phenomenon was a compensatory response to the low glucose levels induced by their two novel compounds.

Meanwhile, compound Tol (tolterodine) showed a dose-dependent linear reduction of pck1 expression while a dose-dependent increase in glucose level was observed with the EC\textsubscript{50} of 16 nM (Figures 4.7D and 4.8D). Compound Tol showed the most significant reduction in pck1 mRNA level (Figure 4.3) and from the dose response curve (Figure 4.7D), pck1 level was lower than that of negative control group at all concentrations. The discrepancy in the four independent experiments that were done to examine pck1 levels may be due to the differences in the experimental protocol. However, based on these four independent studies, it is more likely that
pck1 level in Tol treated larvae increases proportionally with the concentration. Increased glucose level may be attributed to its role as an antagonist of M₃ receptors, which are highly expressed in the pancreatic β-cells and are important regulators involved in glucose homeostasis (Gautam et al. 2006).

Among the three lead compounds, compound Epi (epirubicin) was found to be most effective at lowering both pck1 and glucose levels with IC₅₀ values of 2.51 and 1.28μM, respectively (Figure 4.7C and 4.8C).

By comparing the efficacy of the top three lead compounds on lowering pck1 and glucose levels, but taking into account the current use and possible adverse effects of the compounds, esomeprazole (Eso) was selected for further experiments to confirm its potential beneficial role in glycemic control and to study its possible mechanism of action using a higher vertebrate model, the mouse.

5.2 Mouse studies validated the potential anti-diabetic effects of esomeprazole (Eso).

5.2.1 Esomeprazole enhances glycemic control under hyperglycemic condition but does not cause hypoglycemia under normal condition.

Metformin monotherapy has low risk of hypoglycemia and low chance of weight gain (Chaudhury et al. 2017). As well, most of the negative results for the treatment with proton pump inhibitors (PPIs) with regards to glucose metabolism from the previous studies showed a tendency to involve the patients under good glycemic control (Takebayashi and Inukai 2015). Hence it was expected that the differences in blood glucose levels between saline and Met or Eso treated groups in normal mice would not be significant.

Our studies in both normal and diabetic mice correspond to the current view of these two classes of drugs; both Met and Eso treatments in normal mice did not show any difference in glycemic excursion demonstrated by iPTT while enhanced glycemic control was observed in both Met and Eso treatments in diabetic mice.

In contrast, both Met and Eso treatments in normal zebrafish larvae showed significantly lower glucose levels compared to negative control group (Figure 4.4). The difference between these
two different animal models can be explained partly by the different doses of the compound used in zebrafish and mouse studies. Since there is currently no calculation method available to convert the dose from zebrafish to mouse, the doses for Met and Eso for mouse studies were determined by referencing the existing literature. Also, the discrepancy can be attributed to the different administration routes of the compounds (transdermal vs. intraperitoneal injection), which may have influenced the biodistribution of the compound and thus affected the mechanism of action.

5.2.2 Esomeprazole promotes insulin secretion through increasing gastrin levels.

Proton pump inhibitor increases the level of gastrin due to the lack of feedback inhibition of gastric acid on gastrin release. Rooman et al. have demonstrated that administration of gastrin promotes β-cell neogenesis and expansion of the β-cell mass in rodent models. In agreement with the current view of the PPIs in promoting insulin secretion through increasing gastrin release, the current study demonstrated increased serum gastrin levels as well as insulin levels by Eso treatment (Figures 4.13B and 4.19B).

5.2.3 Eso treatment in normal mice suggests involvement of other factors in maintaining glucose homeostasis.

Although there was a significant decrease in pck1 protein levels (Figure 4.11) and an increase in serum gastrin and insulin levels (Figures 4.13A-B) by Eso treatment in normal mice, the corresponding iPTT results (Figures 4.9B-C) did not show a significant difference compared to saline group, indicating the involvement of compensatory pathways to maintain glucose homeostasis. The euglycemia observed in Eso treated normal mice, despite significantly decreased pck1 enzyme activity in the liver and increased serum gastrin and insulin levels, can be partly explained by the previous studies conducted by She et al. They have observed euglycemia in fasted mice with a liver-specific knockout of PEPCK and in fasted mice with 50, 90, and 95% PEPCK reduction as well. They concluded that there may be compensatory mechanisms for maintaining normal fasting blood glucose levels in mice with markedly diminished or absent hepatic PEPCK (She et al. 2003).
In addition, despite the significant decrease in pck1 protein levels in all treated mice, the corresponding western blot analysis of FoxO-1 did not show a significant difference compared to saline group (Figure 4.12B), suggesting that the significant pck1 reduction in all treatment groups was not due to the retention of FoxO-1 in the cytoplasm, which would lead to gluconeogenic gene suppression, but due to other mechanisms.

5.2.4 Eso treatment in diabetic mice enhances glucose control through regulating pck1.

Eso treatment in diabetic mice resulted in significantly improved glycemic control, which can be accredited to increased serum insulin levels (by gastrin) to suppress gluconeogenic gene expression (including pck1) at the transcription level, thereby suppressing gluconeogenesis. The pathway involves activation of Akt/PKB through phosphorylation by PI-3K. Upon insulin action, FoxO-1, an important transcription factor, becomes phosphorylated by Akt/PKB and is retained in the cytoplasm where it is unable to induce pck1 gene expression (Gross et al. 2008).

5.2.5 Eso treatment at 3mg/kg BW in diabetic mice suggests insulin-independent pathways in maintaining glucose homeostasis.

As shown in figures 4.17 and 4.18B, there were no significant changes in pck1 and nuclear FoxO-1 protein levels as well as serum insulin levels (Figure 4.19B) in Eso treatment at 3mg/kg BW in diabetic mice. This may be explained by the fact that the dose of Eso was insufficient to cause significant changes. Nonetheless, the corresponding iPTT result of these mice (Figure 4.15B) showed remarkably improved glycemic control similar to Eso treatment at 15mg/kg BW (Figure 4.15C), indicating that Eso treatment enhances glycemic control not only through increasing serum insulin levels to suppress expression of gluconeogenic gene (pck1), thereby reducing the rate of hepatic gluconeogenesis, but also through other pathways stimulated by Eso treatment to maintain glucose homeostasis.

5.3 Four lead compounds

5.3.1 Amlexanox (A - #1)

Amlexanox is an anti-inflammatory drug used for the treatment of aphthous ulcers and several inflammatory conditions. Reilly et al. have recently studied amlexanox for its potential anti-diabetic effect. Using both in vivo and in vitro models, they have demonstrated that amlexanox
induces an acute inhibition of IKKε (Inhibitor of nuclear factor kappa-B kinase subunit epsilon) and TBK1 (TANK-binding kinase 1), which have been identified as promising therapeutic targets for the treatment of obesity and insulin resistance, leading to an increase in cAMP levels in subcutaneous adipose depots of obese mice. This results in increased synthesis and secretion of cytokine IL-6 (interleukin 6) from both pre-adipocytes and adipocytes. IL-6 is essential for the activation of Stat3 by phosphorylation, which regulates glucose homeostasis through suppressing the expression of gluconeogenic genes in the liver (Reilly et al. 2015). Reilly et al. also have explored the potential effect of amlexanox by conducting a small cohort of obese-diabetic patients (n=6) treated with amlexanox. They noted that the complete results of this clinical study will be reported elsewhere. This independent report suggests that our zebrafish based drug screening strategy is a valid method for identifying novel anti-diabetic compounds.

Although it has already been demonstrated that treatment with amlexanox suppresses expression of the genes involved in hepatic gluconeogenesis, its full mechanism of action has not been completely understood. Further studies on amlexanox will elucidate the mechanism of action associated with glucose homeostasis. However, confirmation of its role in maintaining glucose homeostasis through suppressing gluconeogenic gene expression would not be as significant as other compounds that have never been studied in this aspect. Therefore, we excluded amlexanox for further validation study using mouse models.

5.3.2 Epirubicin (Epi - #2)

We are not aware of any studies on compound Epi (epirubicin), anthracycline drug for chemotherapy, used to control glucose homeostasis. However, doxorubicin, which shares similar molecular structure with epirubicin, was found to inhibit PPARγ, which regulates fatty acid storage and glucose metabolism, resulting in hyperglycemia and hyperlipidemia. PPARγ is involved in glucose metabolism through improving insulin sensitivity, and PPARγ activators and/or agonists are clinically used to counteract hyperglycemia (Arunachalam et al. 2013). Whether or not epirubicin also exhibits inhibitory effects on PPARγ needs further investigation.

Our data suggest that epirubicin lowers pck1 expression and glucose levels. Hence further studies on compound epirubicin would clarify its potential anti-diabetic effect. If further studies confirm its beneficial role in glucose regulation, it may become a therapeutic option for people
with diabetes (or in the stage of developing diabetes), chemotherapy patients, and people simultaneously in both groups.

5.3.3 Esomeprazole (Eso - #3)

Esomeprazole is a proton pump inhibitor (PPI) that reduces stomach acid secretion through H,K-ATPase inhibition in the parietal cells of the stomach, thereby increasing gastrin release due to the lack of feedback inhibition of gastric acid on gastrin release. PPIs are used to treat acid-related disorders such as gastro-esophageal reflex disease, gastritis, and gastric ulcers (Patil and Shirure 2017), as well as for primary prevention of gastroduodenal toxicity (Sheen 2011).

Gastrin is a peptide hormone that is mainly produced by G cells of the pyloric antrum of the stomach (Takebayashi et al. 2015). It is released by various stimuli including distension of the stomach, vagal stimulation, the presence of food (especially protein, peptides, and amino acids in the stomach), and high pH in the stomach cavity (Burkitt et al. 2009 and Schubert and Makhlouf 1992).

The main function of gastrin is promoting the secretion of gastric acid from the parietal cells of the stomach. It also acts as a growth factor and stimulates gastric cell proliferation (Burkitt et al. 2009 and Hansen et al. 1976). It binds to cholecystokinin B (CKK-B) receptor, which is abundantly expressed on enterochromaffin-like (ECL) cells in the stomach (Chen et al. 2004).

The binding of gastrin to CKK-B receptor on ECL cells stimulates histamine secretion that leads to subsequent promotion of gastric acid release from parietal cells of the stomach, which may be the core mechanism of gastrin-induced acid secretion (Dufresne et al. 2006 and Bitziou and Patel 2012).

Importantly, studies by Saillan-Barreau et al. have demonstrated that the gastrin receptor, CCK-B receptor, is involved in glucose homeostasis in adult humans and promotes islet differentiation and growth in the fetal pancreas (Saillan-Barreau et al. 1999).

Gastrin was an early incretin candidate as oral glucose induces its secretion which potentiates the glucose-induced insulin secretion (Rehfeld and Stadil 1973). However, because oral glucose-induced gastrin release does not significantly increase insulin secretion, major efforts have not yet been made to investigate its association with glucose metabolism (Boj-Carceller 2013).
Strikingly, recent studies by Rooman *et al.* have shown that administration of gastrin stimulates β-cell neogenesis and expansion of the β-cell mass in rodent models. Furthermore, Mefford *et al.* and Boj-Carceller *et al.* have observed a significant improvement in glycemic control in patients with T2DM taking PPIs. It has been shown that the beneficial effects of PPIs on glycemic control are more pronounced under hyperglycemic conditions (Takebayashi and Inukai 2015). Although some studies showed no significant difference in T2D patients with and without PPIs, our data suggest that esomeprazole (Eso) plays a positive role in glycemic control.

5.3.4 Tolterodine (Tol - #4)

Tolterodine is an anti-muscarinic drug that is extensively metabolized to 5-hydroxymethyl tolterodine (5-HMT) in the liver following oral administration, which then is excreted in the urine and feces (Andersson *et al.* 1998). It works by acting as an antagonist of M_2_ and M_3_ subtypes of muscarinic receptors.

M_3_ receptors are highly expressed on the pancreatic β-cells and, by modulating insulin secretion, are critical regulators of glucose homeostasis (Gautam *et al.* 2006). Gautam *et al.* have shown impaired glucose tolerance and significantly reduced insulin secretion in mice lacking M_3_ receptors in pancreatic β-cells while overexpression of M_3_ receptors in pancreatic β-cells significantly enhanced glucose tolerance and increased insulin secretion. Paradoxically, whole body M_3_ receptor knockout (KO) mice showed significantly improved glucose tolerance and insulin sensitivity. Also, M_3_ receptor KO mice were hypophagic and lean relative to the control group.

Using zebrafish larvae as a model organism to explore the effects of Tol treatment on glucose homeostasis, we demonstrate that Tol treatment most likely increases pck1 expression, increasing the rate of gluconeogenesis and thereby increasing glucose levels.

Other compounds are described in appendices.
5.4 Limitations

5.4.1 Zebrafish studies

Since the initial screening of a drug library and the subsequent re-screening to identify potential candidate compounds were done by only looking at the changes in pck1 levels using transgenic zebrafish pck1 reporter lines, Tg(pck1:Luc2) and Tg(pck1:Venus), our methods would not capture compounds with anti-diabetic effects potentiated through non-pck1 pathways. In addition, compounds we tested may have been evaluated outside of their effective dose range.

Another drawback of using these two transgenic zebrafish reporter lines is that they do not possess physiological characteristics associated with diabetes such as, increased blood glucose levels. Thus, the effects observed in healthy zebrafish larvae may be different under hyperglycemic condition. Also, as drug delivery in zebrafish was done transdermally due to their permeability to small molecules in the water, the effects observed in zebrafish larvae may not reflect drug biodistribution in mammalian models.

Ideally, we would have measured the homeostatic effects of the lead compounds on blood glucose levels instead of whole body free glucose levels. However, measuring blood glucose levels in larval zebrafish is impossible with current technology due to their small size and miniscule blood volumes. Although it is feasible to measure blood glucose levels in adult zebrafish, it requires large numbers of fish as it requires sacrificing one fish per each measurement, introducing variability. A recent publication by Zhang et al. has demonstrated repeated blood sampling in adult zebrafish, which increases the utility of zebrafish as a model organism. However, such blood collection requires advanced technical skills and still requires anesthesia, which may restrict the use of this technique. For example, this would not enable our experiments that require multiple blood samplings such as glucose or pyruvate tolerance tests, which measure blood glucose levels at 15- to 30-minute intervals (Ayala et al. 2010).

5.4.2 Mouse studies

It would have been more accurate to examine the hepatic glucose production through isotopic labeling for nuclear magnetic resonance (NMR) spectroscopy. However, as isotope labeling requires high technical skills and efforts, intraperitoneal pyruvate tolerance test (iPTT) was done to reflect the glycemic excursion based on simple intraperitoneal injections of pyruvate.
Due to practical difficulties, serum gastrin and insulin levels reflect single time point, since cardiac puncture was performed after mice received appropriate treatment (saline, metformin, or esomeprazole) just before sacrificing the mice. To better reflect the effects of Eso treatment on insulin secretion and action, insulin tolerance test (ITT) and blood collection at each time point during iPTT, to examine insulin levels over time, would be preferable. In addition, due to the number of animals for each condition (n=5), we were unable to study the insulin-dependent Akt/PKB pathway, which requires insulin injection prior to sacrificing the animals. Akt/PKB is activated through a signaling cascade via glucose-induced insulin activity. Subsequently, FoxO-1 becomes phosphorylated and retained in the cytoplasm, where it is incapable of inducing gluconeogenic gene expression including pck1 (Gross et al. 2008). Hence, measuring the activation of Akt/PKB would further validate the hypothesized pathway of Eso treatment demonstrated in the current study.
Chapter 6

6 Conclusion

People with type 2 diabetes mellitus (T2DM) are characterized by fasting (endogenous glucose production) and postprandial (after meal ingestion) hyperglycemia due to insulin resistance and impaired insulin secretion (Rizza 2010).

The fasting hyperglycemia in type 2 diabetes is mostly due to increased glucose production, which is likely the consequence of increased rate of hepatic glycogenolysis and/or gluconeogenesis. Previous studies by Consoli et al. have revealed that the increase in hepatic gluconeogenesis is the predominant factor (Consoli et al. 1989). However, the method to quantitatively measure the overall gluconeogenesis using [2-\(^{14}\)C] acetate has been questioned for its validity. Later studies by Korytkowski et al. suggested that gluconeogenesis is the predominant factor for fasting hyperglycemia through measuring the hepatic glucose output, in which [\(^{3}\)H] glucose indicates the rate of glycogenolysis, and [\(^{14}\)C] glucose is an index of gluconeogenesis. They have found nearly a 3-fold increase in [\(^{14}\)C] glucose while there was no increase in [\(^{3}\)H] glucose (Korytkowski et al. 1992).

The major gluconeogenic precursors are lactate (Kreisberg 1972), amino acids (such as alanine (Felig 1973) and glutamine (Bucci et al. 1992)), and glycerol (Nurjhan et al. 1992). Their approximate contributions to gluconeogenesis are as follow: 50-70% from lactate, 10-15% from alanine, 15-20% from glutamine, and 5-10% from glycerol (Ostenson et al. 1993). Previous studies have observed increased plasma lactate, alanine, and glycerol in type 2 diabetes (Consoli et al. 1990, Nurjhan et al. 1992, and Virkamaki et al. 1990), indicating increased gluconeogenesis, and therefore increased glucose levels.

The cytosolic form of phosphoenolpyruvate kinase (pck1), which commits the first rate-limiting step in gluconeogenesis has been extensively studied and identified as a potential therapeutic target for type 2 diabetes due to its close association with T2D (Beale et al. 2007).

Through examining the ten lead compounds, which were identified from screening a drug library consisting of 727-FDA approved drugs by a previous student (Ji Dong Bai), using two transgenic
pck1 zebrafish reporter lines, we selected compound Eso (esomeprazole) for validation studies in mouse models.

We showed that Eso treatment in normal mice does not cause hypoglycemia despite significantly reduced hepatic pck1 levels and increased serum insulin levels, indicating the involvement of compensatory pathways in maintaining euglycemia. On the other hand, Eso treatment in diabetic mice results in significantly improved glycemic control with reduced hepatic pck1 and nuclear FoxO-1 levels, and increased serum gastrin and insulin levels.

Eso is a proton pump inhibitor that increases gastrin release, and gastrin has been reported to promote pancreatic β-cell neogenesis (Rooman et al. 2002). Based on our data, Eso treatment leads to increased serum gastrin and insulin levels, suggesting that Eso treatment manages glucose homeostasis through regulating hepatic gluconeogenesis via an insulin dependent pathway. Whether or not Eso treatment is actually responsible for the increase in insulin release through promoting pancreatic β-cell neogenesis by gastrin requires further examination, which would include histological examination and comparison of pancreatic insulin levels between Eso treated and non-treated groups.

Importantly, the fact that Eso treatment does not cause hypoglycemia under normal glycemic condition allows it to serve its original purpose as proton pump inhibitor.

In the current study, we present a possible pathway by which Eso maintains glucose homeostasis under hyperglycemic condition: the down-regulation of pck1 gene expression at its transcription level, by the FoxO-1 transcription factor, via an insulin-dependent pathway to control the rate of hepatic gluconeogenesis.

In conclusion, our findings that compound Eso can exhibit anti-hyperglycemic effects at much lower doses than Met (3 or 15mg/kg vs. 50mg/kg BW), and that it does not cause weight gain, are notable characteristics for Eso as a therapeutic compound. Nonetheless, it is necessary to study the long-term effects of Eso treatment in appropriate preclinical models to ensure that the benefits of Eso outweigh the possible risks, and investigate other pathways by which Eso enhances glucose metabolism.
Chapter 7

7 Future directions

As demonstrated by Eso treatment in diabetic mice, the iPTT results at two Eso doses (3 and 15mg/kg BW) showed similar enhancement in glycemic control despite significant discrepancies observed in other parameters, specifically pck1, FoxO-1, and serum insulin levels. This suggests that Eso treatment not only enhances glycemic control through regulating hepatic gluconeogenesis via insulin-dependent pathways under hyperglycemic condition, but also through other insulin-independent mechanisms. In addition, since there have been no studies on the mechanism of action of PPIs for its anti-diabetic effects other than through an increase in gastrin release as shown in figure 7.1, further studies on esomeprazole (PPI) to unravel the other pathways are warranted.

Furthermore, previous studies have demonstrated synergistic effects of gastrin with other hormones such as transforming growth factor-α (TGF-α, Wang et al. 1993), epidermal growth factor (EFG, Suarez-Pinzon et al. 2005), and glucagon-like peptide-1 (GLP-1, Suarez-Pinzon et al. 2008). Additionally, recent studies by Patel et al. have observed improvements in insulin sensitivity and antioxidant activity in the liver in type 1 diabetic mice (STZ induced) following a combination therapy of omeprazole (PPI) with GLP-1 agonist (exendin-4) (Patel et al. 2013). Diabetic mice were treated twice a day through subcutaneous injection with either exendin-4 (8μg/kg BW) alone or exendin-4 (8μg/kg BW) and omeprazole (30mg/kg BW). Considering the frequency and the dose, esomeprazole may be more efficient than omeprazole in glucose control as Eso treatment in diabetic mice showed enhanced glycemic control at 3mg/kg BW (once per day through intraperitoneal injection). However, further preclinical investigations of esomeprazole are necessary to confirm the potential beneficial effects of esomeprazole as a new therapeutic for diabetes.

Consistent with our findings, a small case-control study by Hove et al. showed improved HbA1c (glycated hemoglobin) levels in patients with T2D taking esomeprazole (PPI). Importantly, the beneficial effects of esomeprazole were more pronounced in patients with poor glycemic control, corresponding to our Eso treatment of diabetic vs. normal mice.
The current study demonstrated that esomeprazole monotherapy can improve glucose control similarly to metformin monotherapy in HFD/STZ induced diabetic mice. Whether or not esomeprazole or other PPIs can be used as first-line anti-diabetic medication or as an adjunctive therapy will be of interest (Patil and Shirure 2017). At present, the duration of PPI-induced formation of pancreatic β-cells, and their contribution to insulin production, are unclear; this would be important to know to determine the duration of treatment.

Consistency in observing beneficial effects of the proton pump inhibitors (PPIs) on glycemic control led to interest in understanding the potential role of PPIs in promoting β-cell regeneration and expansion for T1D, and insulin secretion for T2D (Boj-Carceller 2013). If such efforts reveal significant beneficial effects of PPIs in maintaining glucose homeostasis under hyperglycemic condition with minimal side effects and well-understood mechanisms, this will open the possibility of a new therapeutic avenue for diabetes.

Figure 7.1: The possible mechanisms of proton pump inhibitors (PPIs) on the improvement of glycemic control (Takebayashi and Inukai 2015).


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Appendix 1: Work flow of zebrafish studies.
Normal C57BL/6 mice

Ad libitum access to food (chow diet) and water

HFD/STZ induced diabetic mice (C57BL/6)

Ad libitum access to food (HFD) and water

Appendix 2: Work flow of (A) normal and (B) diabetic mouse studies.
Appendix 3: Compounds #5-10.

Levofloxacin (C - #5)

There is evidence that fluoroquinolone class antibiotics such as levofloxacin cause dysglycemia in patients with (Chou et al. 2013), or without T2DM (Kabbara et al. 2015), and in STZ-diabetic rats (Absi et al. 2012). Chou et al. have performed a national diabetes cohort study and found an association between fluoroquinolones and a high risk of dysglycemia among diabetic patients in Taiwan. Also, Kabbara et al. studied 118 patients receiving broad-spectrum fluoroquinolones (levofloxacin, ciprofloxacin, and moxifloxacin). They have found patients receiving levofloxacin have the highest incidence of hyperglycemia followed by ciprofloxacin and moxifloxacin. It was emphasized that all fluoroquinolones can cause blood glucose fluctuation. In addition, Aspinall et al. have conducted a retrospective inception cohort study of outpatients with and without diabetes with a prescription for gatifloxacin, levofloxacin, ciprofloxacin, or azithromycin. They observed that the odds of severe hypo- and hyperglycemia are significantly greater with gatifloxacin and levofloxacin than in ciprofloxacin or azithromycin (which is an antibiotic used to treat various bacterial infections).

In contrast, Absi et al. have observed a hypoglycemic effect of levofloxacin after 3 hours and 14 days of intraperitoneal injection in STZ-diabetic rats. However, they have also observed that levofloxacin treatment accompanies bradycardia in STZ-diabetic rats, which underscores that drug benefits must outweigh the risks to be approved for medical uses. The opposing findings in levofloxacin treatment in patients vs. rats may be partially explained by species specificity or different administration routes (oral vs intraperitoneal, respectively).

Dicloxacillin (L - #6)

Dicloxacillin is a β-lactam penicillin class antibiotic for Gram-positive bacteria (Sherertz et al. 1989). There is currently no literature on a potential anti-diabetic effect of dicloxacillin. Although dicloxacillin was ranked 6th among our 10 compounds and was not studied further, further examination of its role in glucose regulation may be warranted.
Fluorocytosine (Fluo - #6)

Fluorocytosine is an anti-fungal used for infections caused by *Candida* or *Cryptococcus neoformans*. We found no studies on the effect of fluorocytosine on glycemic control.

Daunorubicin (Dau - #8)

Similar to compound Epi (epirubicin), we are not aware of any studies on compound Dau (daunorubicin), anthracycline drug for chemotherapy, used to control glucose homeostasis. Although epirubicin and daunorubicin share similar molecular structure, the effects of daunorubicin on pck1 expression and glucose levels were not as pronounced as seen in epirubicin treatment.

Naproxen (I - #9)

Motawi *et al.* have shown anti-diabetic effects of naproxen, which is a non-steroidal anti-inflammatory drug (NSAID) used for a variety of conditions including fever, menstrual cramps, and migraines. The drug was studied in diabetes and obesity induced Balb/C mouse models. There was a decrease in plasma glucose concentrations and obesity, but an increase in insulin levels in a dose-dependent manner in naproxen treated mice. It is thought that naproxen exhibits anti-diabetic and anti-obesity effects by acting as glycogen synthase kinase 3β inhibitor (GSK-3β) (Motawi *et al.* 2013). According to our data, compound I (naproxen) showed a reduction in pck1 and glucose levels but not as pronounced as the selected top three lead compounds.

Zolpidem (Zol - #10)

Zolpidem is a short-acting nonbenzodiazepine sedative or hypnotic that is one of the most commonly prescribed to treat insomnia. It acts on GABA A receptors, which are widely expressed in the brain, to potentiate GABA transmission (Berdyyeva *et al.* 2014). Lai *et al.* showed that patients who actively use zolpidem have 7-fold increased risk of acute pancreatitis, but it is not yet clear whether zolpidem is directly toxic to the pancreas. Among the ten lead compounds, zolpidem was our least effective compound and, unlike the other compounds, actually increased pck1 expression compared to the negative control group.