THE POTENTIAL ROLE OF A NOVEL METFORMIN-METHYLGLYOXAL IMIDAZOLINONE METABOLITE (IMZ) IN ALLEVIATING COMPLICATIONS FROM T2DM

by

Tiffanie L. Hargraves

Copyright © Tiffanie L. Hargraves 2019

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2019
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Tiffanie L. Hargraves, titled “The Potential Role of a Novel Metformin-methylglyoxal Imidazolinone Metabolite (IMZ) in Alleviating Complications from T2DM” and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

John W. Regan, Ph.D.
Date: 09/27/19

Nathan J. Cherrington, Ph.D.
Date: 09/27/19

Georg T. Wondrak, Ph.D.
Date: 09/27/19

Terrence J. Monks, Ph.D.
Date: 09/27/19

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

John W. Regan, Ph.D.
Dissertation Committee Chair
Pharmacology and Toxicology

Date: 09/27/19
Acknowledgements

First and foremost, I would like to thank my mentor, Dr. Serrine Lau, for her guidance and support throughout the beginning of my graduate career. Dr. Lau provided me with an inspiring and challenging environment that allowed me to advance as a critical thinker and an independent scientist. I am grateful for having the opportunity to train under her supervision at the University of Arizona.

I would also like to thank Dr. Terrence Monks for serving as my acting advisor following our relocation to Wayne State University. Dr. Monks provided consultation and research funding throughout the remainder of my graduate training.

In addition, I would like to thank my dissertation committee members, Dr. John Regan, Dr. Nathan Cherrington, Dr. Georg Wondrak, Dr. Craig Stump, and Dr. Terrence Monks. Their advice and guidance throughout the years were fundamental in the development of my dissertation project. I am thankful for the time and effort each one of them put into me and my dissertation work. Moreover, I would like to thank Dr. Regan for agreeing to serve as my University of Arizona Dissertation Committee Chair subsequent to our move to Wayne State University.

I would like to thank the staff at the proteomics, NMR (University of Arizona), animal, histology, and genomics (Wayne State University) cores. A special thanks to Dr. Jiemei Wang at Wayne State University. Without their expertise and technical support, much of my data would not exist.

I am sincerely grateful for the numerous past and present members of the Lau and Monks lab for their training, friendship, and support. To Dr. Owen Kinsky, thank you for providing the groundwork for this project and being instrumental to me during my early years as a graduate student. A special thanks to Dr. Nick Mastrandrea, Dr. Argel Islas Robles, Dr. Aram Cholanians, and Dr. Deepthi Yedlapudi for making work in the lab and life in general more enjoyable. Making my way through this program would have not been as pleasant without all of you there with me.

Finally, thank you to my family, friends, Kate, and Nick for being a constant source of support and encouragement over these challenging six years. Your unconditional love and support have helped shape me into the person I am today. I would have not been able to make it to this point in my life without help from you all.
Table of Contents

List of Abbreviations .................................................................................. 10
Abstract ......................................................................................................... 15
Chapter 1: Introduction................................................................................. 18
  1.1 General Comments ............................................................................... 18
  1.2 Mechanisms of Hyperglycemia Mediated Injury ................................ 22
  1.3 The Formation of Advanced Glycation End Products ...................... 25
  1.4 Methylglyoxal and Diabetic Complications ..................................... 29
  1.5 Therapeutic Target of Dicarbonyls ..................................................... 32
  1.6 Metformin Directly Scavenges Methylglyoxal .................................. 33
  1.7 Pharmacological Properties of Imidazolines ..................................... 37
  1.8 Imidazoline Receptors ....................................................................... 40
  1.9 Proposed I\textsubscript{1}R Cell Signaling Mechanism ......................... 44
  1.10 PC12 Cells, MIN6 Cells, HEPG2 Cells, and db/db Mice as Models for I\textsubscript{1}R Signaling and T2DM ....................................................... 47
  1.11 Dissertation Aims ............................................................................ 49
Chapter 2: A Metformin-methylglyoxal Imidazolinone Metabolite Enhances Insulin Action and Secretion \textit{in vivo} .................................................. 53
  2.1 Introduction ......................................................................................... 53
  2.2 Materials and Methods ..................................................................... 56
    2.2.1 Chemicals and antibodies ............................................................. 56
    2.2.2 Cell culture ................................................................................... 56
    2.2.3 Insulin sensitization in PC12 cells ................................................. 57
    2.2.4 Nischarin siRNA-mediated knockdown in PC12 cells ................ 58
    2.2.5 Insulin resistant HEPG2 cell model ............................................ 58
    2.2.6 Western blot analysis .................................................................. 59
    2.2.7 Insulin secretion ........................................................................... 60
    2.2.8 Nischarin siRNA in MIN6 cells .................................................... 60
    2.2.9 Statistical analysis ....................................................................... 61
  2.3 Results ................................................................................................. 62
    2.3.1 IMZ sensitized PC12 cells to insulin via modulation of ERK1/2 and AKT phosphorylation ................................................................. 62
    2.3.2 I\textsubscript{1}R antagonists efaroxan and idazoxan decreased IMZ induced insulin sensitization in PC12 cells ........................................... 64
2.3.3 siRNA-mediated knockdown of nischarin activated ERK1/2 in PC12 cells ................................................................. 66
2.3.4 IMZ improved insulin resistance (IR) in IR HEPG2 cells via activation of AKT .......................................................... 68
2.3.5 IMZ promoted insulin secretion in MIN6 cells ......................... 68
2.3.6 The I$_3$R antagonist KU14R reversed IMZ stimulated insulin secretion in MIN6 cells ..................................................... 71
2.3.7 Silencing nischarin muted IMZ induced insulin secretion in MIN6 cells ........................................................................... 71

2.4 Discussion ............................................................................. 74

Chapter 3: A Metformin-methylglyoxal Imidazolinone Metabolite Exerts Antidiabetic Effects in db/db Mice ................................................. 78

3.1 Introduction ............................................................................. 78
3.2 Materials and Methods ................................................................. 81
  3.2.1 Compound ........................................................................ 81
  3.2.2 Animals and experimental design ........................................... 81
  3.2.3 Biochemical analysis .............................................................. 82
  3.2.4 Histological analysis .............................................................. 83
  3.2.5 Western blot analysis ............................................................. 84
  3.2.6 RNA extraction and quantitative real time PCR analysis .......... 85
  3.2.7 Statistical analysis ................................................................. 86
3.3 Results ....................................................................................... 87
  3.3.1 IMZ improved insulin resistance in db/db mice after 7 days of treatment ................................................................. 87
  3.3.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 7 days of treatment .......................................................... 89
  3.3.3 IMZ reduced adipocyte size in EWAT from db/db mice after 7 days of treatment ............................................................. 91
  3.3.4 IMZ alleviated renal histopathological changes in db/db mice after 7 days of treatment ..................................................... 91
  3.3.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 7 days of treatment ..................................................... 95
  3.3.6 IMZ decreased hepatic markers of lipogenesis in db/db mice after 7 days of treatment ..................................................... 95
3.4 Discussion ................................................................................. 99

Chapter 4: Long Term Treatment of a Metformin-methylglyoxal Imidazolinone Metabolite Improves Metabolic Anomalies in db/db Mice ...104

4.1 Introduction ............................................................................. 104
4.2 Materials and Methods ................................................................. 107
4.2.1 Compounds ................................................................. 107
4.2.2 Animals and experimental design .................................. 107
4.2.3 Biochemical analysis .................................................... 109
4.2.4 Histological analysis .................................................... 109
4.2.5 Western blot analysis .................................................... 110
4.2.6 RNA extraction and quantitative real time PCR analysis ...... 111
4.2.7 Statistical analysis ....................................................... 112

4.3 Results ............................................................................. 113
4.3.1 IMZ improved insulin resistance and hyperglycemia in db/db mice after 60 days of treatment ........................................... 113
4.3.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 60 days of treatment .................................................... 115
4.3.3 IMZ reduced adipocyte size in EWAT from db/db mice after 60 days of treatment ..................................................... 117
4.3.4 IMZ alleviated renal histopathological changes in db/db mice after 60 days of treatment ................................................ 117
4.3.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 60 days of treatment ............................................. 118
4.3.6 IMZ decreased hepatic lipogenic and fibrotic markers in db/db mice after 60 days of treatment ................................. 122

4.4 Discussion ........................................................................ 124

Chapter 5: Concluding Remarks ............................................ 129
5.1 Insulin Sensitizing Properties of IMZ in PC12 Cells ............. 130
5.2 Insulin Sensitizing Properties of IMZ in Insulin Resistant HEPG2 Cells ............................................................................. 132
5.3 Insulin Secretagogue Activity of IMZ in MIN6 Cells ............. 134
5.4 Antidiabetic Properties of Short-term IMZ Treatment in db/db Mice ................................................................. 136
5.5 Antidiabetic Properties of Long-term IMZ Treatment in db/db Mice ............................................................................. 138

Chapter 6: Future Directions .................................................. 141
6.1 Effect of IMZ on Intracellular Calcium Influx in MIN6 Cells ....................................................................................... 141
6.1.1 Introduction ................................................................... 141
6.1.2 Methods ...................................................................... 142
6.1.3 Expected results ........................................................... 143

6.2 Effect of Small Molecule Inhibition of IRs and α2-ARs on IMZ’s Insulin Sensitization in Insulin Resistant HEPG2 Cells ............................................................................. 145
6.2.1 Introduction ................................................................... 145
6.2.2 Methods ...................................................................... 145
6.2.3 Expected results ........................................................... 147
6.3 Effect of IMZ on Activation of \( I_2R \)s in Skeletal Muscle Cells .......... 148
  6.3.1 Introduction ......................................................................................... 148
  6.3.2 Methods............................................................................................... 148
  6.3.3 Expected results .................................................................................. 151

6.4 Evaluation of the Affinity of IMZ for IRs and \( \alpha \)-ARs ..................... 152
  6.4.1 Introduction .......................................................................................... 152
  6.4.2 Methods............................................................................................... 152
  6.4.3 Expected results .................................................................................. 156

6.5 Evaluation of the Metformin Effect on Methylglyoxal in Patients with Type 2 Diabetes (MET) ................................................................. 157
  6.5.1 Introduction .......................................................................................... 157
  6.5.2 Study aims ........................................................................................... 158
  6.5.3 Inclusion criteria .................................................................................. 159
  6.5.4 Exclusion criteria ................................................................................ 159
  6.5.5 Methods .............................................................................................. 160
  6.5.6 Expected results .................................................................................. 161

References ........................................................................................................ 162
<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Four major pathways of hyperglycemia induced damage</td>
<td>24</td>
</tr>
<tr>
<td>1.2</td>
<td>Formation of Advanced Glycation End Products <em>in vivo</em></td>
<td>27</td>
</tr>
<tr>
<td>1.3</td>
<td>The major precursors and AGEs identified <em>in vivo</em></td>
<td>28</td>
</tr>
<tr>
<td>1.4</td>
<td>The formation, glycation, and detoxification of MGO</td>
<td>31</td>
</tr>
<tr>
<td>1.5</td>
<td>Traditional Mechanism of Metformin Drug Efficacy</td>
<td>35</td>
</tr>
<tr>
<td>1.6</td>
<td>Metformin-methylglyoxal Scavenging Mechanism</td>
<td>36</td>
</tr>
<tr>
<td>1.7</td>
<td>Imidazolines in Therapeutic Use</td>
<td>38</td>
</tr>
<tr>
<td>1.8</td>
<td>Proposed Pathway for Clinical Use of Imidazolines</td>
<td>41</td>
</tr>
<tr>
<td>1.9</td>
<td>Proposed I$_3$R Cell Signaling Mechanism</td>
<td>46</td>
</tr>
<tr>
<td>2.1</td>
<td>IMZ sensitized PC12 cells to insulin via modulation of AKT and ERK1/2 phosphorylation</td>
<td>63</td>
</tr>
<tr>
<td>2.2</td>
<td>I$_3$R antagonists efaroxan and idazoxan decreased IMZ induced insulin sensitization in PC12 cells</td>
<td>65</td>
</tr>
<tr>
<td>2.3</td>
<td>siRNA-mediated knockdown of niscahrin activated ERK1/2 in PC12 cells</td>
<td>67</td>
</tr>
<tr>
<td>2.4</td>
<td>IMZ improved insulin resistance (IR) in IR HEPG2 cells via activation of AKT</td>
<td>69</td>
</tr>
<tr>
<td>2.5</td>
<td>IMZ promoted insulin secretion in MIN6 cells</td>
<td>70</td>
</tr>
<tr>
<td>2.6</td>
<td>The I$_3$R antagonist KU14R reversed IMZ stimulated insulin secretion in MIN6 cells</td>
<td>72</td>
</tr>
<tr>
<td>2.7</td>
<td>Silencing nischarin muted IMZ induced insulin secretion in MIN6 cells</td>
<td>73</td>
</tr>
<tr>
<td>3.1</td>
<td>IMZ improved insulin resistance in db/db mice after 7 days of treatment</td>
<td>88</td>
</tr>
<tr>
<td>3.2</td>
<td>IMZ inhibited pancreatic islet hypertrophy in db/db mice after 7 days of treatment</td>
<td>90</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>IMZ reduced adipocyte size in EWAT from db/db mice after 7 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>IMZ alleviated renal histopathological changes in db/db mice after 7 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 7 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>IMZ decreased hepatic markers of lipogenesis in db/db mice after 7 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>IMZ improved insulin resistance and hyperglycemia in db/db mice after 60 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>IMZ inhibited pancreatic islet hypertrophy in db/db mice after 60 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>IMZ reduced adipocyte size in EWAT from db/db mice after 60 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>IMZ alleviated renal histopathological changes in db/db mice after 60 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 60 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>IMZ decreased hepatic lipogenic and fibrotic markers in db/db mice after 60 days of treatment</td>
<td></td>
</tr>
</tbody>
</table>
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3DG</td>
<td>3-deoxyglucosone</td>
</tr>
<tr>
<td>3DG-H1</td>
<td>3-deoxyglucosone-hydroimidazolone 1</td>
</tr>
<tr>
<td>α-ARs</td>
<td>α-adrenergic receptors</td>
</tr>
<tr>
<td>α2-ARs</td>
<td>α-2 adrenergic receptors</td>
</tr>
<tr>
<td>ACACA</td>
<td>acetyl-CoA carboxylase alpha</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACTB</td>
<td>actin beta</td>
</tr>
<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine transaminase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate activated protein kinase</td>
</tr>
<tr>
<td>ANS</td>
<td>autonomic nervous system</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEL</td>
<td>Ne-(carboxyethyl)-lysine</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ChREBP</td>
<td>carbohydrate-response element-binding protein</td>
</tr>
<tr>
<td>CLB</td>
<td>cell lysis buffer</td>
</tr>
<tr>
<td>CML</td>
<td>Ne-(carboxymethyl)-lysine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COL1A1</td>
<td>alpha-1 type 1 collagen</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular receptor kinase 1 and 2</td>
</tr>
<tr>
<td>EWAT</td>
<td>epididymal white adipose tissue</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FASN</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>FN1</td>
<td>fibronectin 1</td>
</tr>
<tr>
<td>fura-2</td>
<td>fluorescent dye fura 2-acetoxymethyl ester</td>
</tr>
<tr>
<td>G</td>
<td>glyoxal</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-H1</td>
<td>glyoxal-derived hydroimidazolone 1</td>
</tr>
<tr>
<td>GLO-1</td>
<td>glyoxalase 1</td>
</tr>
<tr>
<td>GLO-2</td>
<td>glyoxalase 2</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter type 4</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPG2</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>homeostasis model assessment of insulin resistance</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IMZ</td>
<td>(E)-1,1-dimethyl-2-(5-methyl-4-oxo-4,5-dihydro-1H-imidazol-2-yl)guanidine</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IRs</td>
<td>imidazoline receptors</td>
</tr>
<tr>
<td>I₁Rs</td>
<td>imidazoline-1 receptors</td>
</tr>
<tr>
<td>I₂Rs</td>
<td>imidazoline-2 receptors</td>
</tr>
<tr>
<td>I₃Rs</td>
<td>imidazoline-3 receptors</td>
</tr>
<tr>
<td>IRAS</td>
<td>imidazoline receptor antisera-selected</td>
</tr>
<tr>
<td>IR-HEPG2</td>
<td>insulin resistant</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>KRBH-BSA</td>
<td>Krebs-ringer modified HEPES buffer + bovine serum albumin</td>
</tr>
<tr>
<td>MAO-A</td>
<td>monoamine oxidase A</td>
</tr>
<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MF</td>
<td>metformin</td>
</tr>
<tr>
<td>MG-H1</td>
<td>methylglyoxal-derived hydroimidazolone 1</td>
</tr>
<tr>
<td>MGO</td>
<td>methylglyoxal</td>
</tr>
<tr>
<td>MIN6</td>
<td>mouse pancreatic insulinoma</td>
</tr>
<tr>
<td>MLXIPL</td>
<td>MLX interacting protein like</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
</tbody>
</table>

12
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NISCH</td>
<td>nischarin</td>
</tr>
<tr>
<td>ORO</td>
<td>oil red o</td>
</tr>
<tr>
<td>pAKT</td>
<td>phospho-protein kinase B</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>pheochromocytoma</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PC-PLC</td>
<td>phosphatidylcholine selective phospholipase C</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>phospho-extracellular receptor kinase 1 and 2</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidyl-inositol-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Rn18s</td>
<td>ribosomal RNA 18s</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RVLM</td>
<td>rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference ribonucleic acid</td>
</tr>
<tr>
<td>SIRT1</td>
<td>sirtuin 1</td>
</tr>
<tr>
<td>SREBF1</td>
<td>sterol regulatory element binding transcription factor 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SREBP1</td>
<td>sterol regulatory element-binding protein 1</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TC</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>triglycerides</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>transforming growth factor beta 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Abstract

Reactive dicarbonyls, such as methylglyoxal (MGO), are elevated in the plasma of patients with type-two diabetes mellitus (T2DM). These endogenous electrophiles covalently modify proteins, which may contribute to diabetic complications. The T2DM first-line therapy, metformin (MF), significantly reduces adverse diabetic endpoints and mortality more effectively than other anti-hyperglycemic agents, the mechanism(s) of which remain unclear.

We previously identified and characterized the product of the MF and MGO reaction as a novel imidazolinone metabolite (IMZ) in plasma and urine from patients with T2DM. The formation of IMZ, via scavenging of MGO by MF, represents a possible alternative mechanism of MF drug efficacy, in addition to its antigluconeogenesis properties. Imidazoline receptors (I₁R-I₃R) are novel targets for drug development in disorders associated with T2DM because they are involved in insulin secretion/sensitization and glucose homeostasis. Thus, we examined the ability of IMZ to modulate insulin-mediated cell signaling pathways in PC12 (express high levels of I₁R and lack the α₂-adrenergic receptor) and insulin resistant HEPG2 (a well-established model for assessing human liver metabolism) cells. In addition, the effects of IMZ on insulin secretion in MIN6 (best reflect normal pancreatic β-cell physiological conditions) cells were explored. Combination treatment of insulin and IMZ at physiologically relevant concentrations (1 pM & 1 nM) increased AKT and ERK1/2 phosphorylation above levels seen with insulin treatment alone in PC12 cells. This potentiation was not
observed in the presence of the I₁R antagonists efaroxan and idazoxan. IMZ also restored insulin sensitivity in insulin resistant HEPG2 cells via the AKT signaling pathway. Moreover, insulin secretion was increased in MIN6 cells exposed to various concentrations of IMZ (1 pM-1 µM) and this augmentation was inhibited by the I₃R antagonist KU14R and siRNA mediated knockdown of nischarin (mouse homolog of imidazoline receptor antisera-selected protein).

In addition, we explored the antidiabetic effects and possible intracellular signaling mechanisms engaged by IMZ in the well-established genetically obese db/db diabetic mouse model. Our results revealed that seven-day treatment of IMZ significantly alleviated insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes in db/db mice. In addition, IMZ promoted hepatic SIRT1 expression and the downregulation of SREBP1, ChREBP, ACC, and FAS at the protein and mRNA level in db/db mice.

Finally, we investigated the long-term protective effects and potential mechanisms of activity exerted by IMZ in db/db mice alongside a clinically relevant dose of MF. The results from these studies demonstrated that IMZ significantly alleviated hyperglycemia, insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes similarly to MF in db/db mice treated with IMZ for 60 days. In addition, IMZ promoted hepatic downregulation of the lipogenesis and fibrosis markers ACACA, FASN, FN1, COL1A1, and TGFβ1 at the protein and mRNA level in db/db mice comparably to MF.
The work in this dissertation therefore describes for the first time the protective effects of a MF metabolite *in vitro* and *in vivo*. Taken together, these findings support the hypothesis that the formation of IMZ may contribute to the antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM. Although the precise underlying mechanism(s) of IMZ’s pharmacological action remain to be fully elucidated, the present work opens an intriguing window on the mechanism of the antidiabetic properties of MF.
Chapter 1: Introduction

1.1 General Comments

Due to modern changes in dietary habits and substantial increases in a sedentary lifestyle, the global prevalence of diabetes and glucose intolerance has reached epidemic proportions. Diabetes is the seventh leading cause of death and has a devastating impact on healthcare and the economy. Currently, 30.3 million people in the United States are considered to have diabetes. Medical costs for patients with diabetes are more than two times higher than for people without diabetes, resulting in $245 billion in annual total health care costs. In addition, according to the International Diabetes Federation, there are an estimated 451 million people with diabetes worldwide, a figure which is predicted to increase to 693 million by 2045. The total global diabetes healthcare expenditure is estimated to be $850 billion annually and is expected to increase to $958 billion by 2045.\(^1,2\)

Type 2 diabetes mellitus (T2DM) is the predominant form of diabetes and accounts for ~95% of all diabetes cases. T2DM is a long-term progressive and chronic metabolic disorder, characterized by hyperglycemia and altered lipid metabolism resulting from irregular insulin secretion and/or insulin resistance. T2DM can be prevented or delayed by consciously making lifestyle alterations such as maintaining a healthy diet, being active, and losing weight. If lifestyle changes fail to have an impact on T2DM symptoms, pharmaceutical intervention is utilized. However, if not treated properly a large number of organs and tissues
can be affected by microvascular (neuropathy, nephropathy, and retinopathy) and macrovascular (coronary artery disease, peripheral arterial disease, and stroke) complications. T2DM complications are a result of sustained hyperglycemia, increasingly affect patients with T2DM, and can result in premature death.\textsuperscript{3-6}

Complications from T2DM has made the disease one of the most prevailing public health concerns due to increased rates of morbidity and mortality. Four main molecular mechanisms have been attributed to the underlying cause and progression of T2DM vascular complications. Namely, the elevation of reactive dicarboxyls and the non-enzymatic formation of stable advanced glycation end products (AGEs). The most reactive dicarbonyl, methylglyoxal (MGO), is highly elevated in patients with T2DM and is responsible for a large portion of AGEs. The importance of MGO-derived AGEs in the pathogenesis of diabetic complications is illustrated by the large body of work in the literature detailing that alleviation of diabetic complications are observed with inhibition of elevated MGO and thus MGO-derived AGE levels.\textsuperscript{7-12}

Strategies to reduce MGO and the resulting AGEs is still an evolving research field, with an emphasis on decreasing both MGO and AGEs, and thus lessening diabetic complications. Decreasing circulating levels of MGO, represents a practical therapeutic approach for reducing diabetic complications, but direct pharmacological sequestration of MGO has not been successfully pursued. While aminoguanidine is a known scavenger of MGO, it is not a food and drug administration (FDA) approved drug due to lack of efficacy and associated
toxicity.\textsuperscript{13-18} In addition, no drug has been specifically developed to scavenge dicarboxyls as a therapeutic approach for treating T2DM. The first line therapy for T2DM, metformin (MF), is similar in structure to aminoguanidine and has been FDA approved since 1994 with an exceptional safety profile.\textsuperscript{4,19,20} In addition, in basic research and clinical studies MF has been reported to scavenge MGO and to reduce MGO and resulting AGEs.\textsuperscript{21-25}

We previously investigated the MGO scavenging properties of MF and identified and characterized the reaction product of as a novel imidazolinone metabolite (IMZ) in the plasma and urine of patients with T2DM. Identification of IMZ reveals that MF possesses a potential alternative mechanism for lessening the adverse effects of MGO and AGEs, in addition to its antihyperglycemic properties. Although it is probable that the initial reduction of MGO and AGEs following MF treatment is due to improved glycemic control, the chemical structure of MF facilitates direct scavenging of MGO and thereby potentially eliminates detrimental MGO-derived AGEs that contribute to the progression of T2DM complications.\textsuperscript{24}

The formation of IMZ may also have significant therapeutic implications in the efficacy of MF’s antidiabetic properties. Imidazolines show promise in not only reducing hypertension, but also improving glucose tolerance, reducing hyperinsulinemia, and decreasing insulin resistance. In addition, imidazoline receptors play an important role in maintaining glucose homeostasis, insulin sensitization, and insulin secretion.\textsuperscript{26-33} Moreover, MF reduces plasma glucose and increases glucose uptake through activation of imidazoline receptors in \textit{in}}
vitro and in vivo models.\textsuperscript{34,35} The drug-like structure of IMZ suggests it may play a role in MF’s ability to reduce diabetic complications and might represent a key component in MF drug efficacy. Thus, the potential pharmacological activity of IMZ warrants further investigation.

The research described in this thesis explores the potential pharmacological properties and intracellular signaling mechanism of IMZ relating to imidazoline receptor interaction \textit{in vitro}. In addition, this work details the short-term and long-term antidiabetic properties of IMZ \textit{in vivo}. Results from this study suggest for the first time that IMZ may contribute to the antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM.
1.2 Mechanisms of Hyperglycemia Mediated Injury

Numerous studies have indicated that there is a strong relationship between hyperglycemia and the pathogenesis of microvascular and macrovascular complications in patients with T2DM. Thus, understanding how such diverse complications all arise from elevated levels of blood glucose has become a central research focus. In a diabetic state, excess glucose-derived pyruvate from the tricarboxylic acid cycle is oxidized, causing an influx of electron donors that donate electrons to molecular oxygen and produce superoxide. Consequently, the metabolic abnormalities in T2DM patients lead to highly elevated mitochondrial production of reactive oxygen species (ROS). This overproduction of ROS causes inhibition of the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase, which subsequently leads to an increase in upstream glycolytic intermediates that influx into four major pathways that cause tissue damage (Figure 1.1). The first is an increase in glucose which enters the polyol pathway; the second is increased activity of the hexosamine pathway; the third is an activation of protein kinase C; and the fourth is an increase of intracellular advanced glycation end products (AGEs). Although inhibition of these pathways improves several diabetic abnormalities, a commonality linking them together has not been fully understood until recently. The consensus that activation of all four mechanisms are attributed to the sole hyperglycemia induced processes of mitochondrial overproduction of ROS has provided a rationale. Thus, the breakthrough that a single hyperglycemia-induced activity links the four principal mechanisms
implicated in the pathogenesis of diabetic complications presents an innovative conceptual basis for future research.
A considerable amount of data in the literature has produced four key hypotheses on how hyperglycemia causes diabetic microvascular and macrovascular complications. The four hypotheses are: increased polyol pathway flux, augmented hexosamine pathway flux, activation of protein kinase C isoforms, and elevated formation of advanced glycation end-products.7
1.3 The Formation of Advanced Glycation End Products

The term glycation has been coined for the non-enzymatic reaction of sugars covalently modifying proteins or lipids. The discovery and structural identification of glycated hemoglobin (HbA1c) led to focused studies on post-translational glycated proteins due the numerous adverse side effects associated with compounds of this nature. Although HbA1c is now the standard biomarker for assessing average blood glucose levels and is used as a diagnostic test for T2DM, it does not appear to be an accurate predictor of diabetic complications. Recent studies have shown that despite maintaining good glycemic control (HbA1c < 6.5%), detrimental diabetic complications arise and are not proposed to be alleviated by targeting HbA1c. Glycation of proteins disrupts their molecular formation, which causes functional alteration, irregular enzymatic activity, reduced degradation, and receptor recognition interference. The AGE pathway is believed to be the primary pathway involved in the development of diabetic vascular complications. Further, diabetic vascular disease is improved with a reduction in AGEs.

Three different in vivo pathways have been implicated in the endogenous formation of AGEs; the non-enzymatic Maillard reaction, the polyol-pathway, and lipid peroxidation (Figure 1.2). The production of AGEs is dependent upon the concentration of glucose, the amount of precursors, as well as the availability of free amino groups on plasma proteins. The classic view of the Maillard reaction involves the non-enzymatic reaction between carbonyl groups of reducing sugars, or other carbonyl compounds, with the free amino groups on
proteins. In the early stage of this reaction, reducing sugars, such as glucose, react with the free amino group on biological amines, forming an unstable product referred to as a Schiff base, which further rearranges to a relatively stable Amadori product. In the transitional stage, reactive dicarbonyls, such as glyoxal (G), methylglyoxal (MGO), and 3-deoxyglucosone (3DG) are formed from the degradation of the Amadori product. These highly reactive intermediates can also be formed from the sorbitol pathway or from lipid peroxidation, but the products of these pathways are less explored. In the late stage of glycation, the direct reaction between the amino acid arginine and these dicarbonyls forms irreversible AGEs through oxidation, dehydration, and cyclization reactions.\textsuperscript{54,55}

Dicarbonyls are relevant glycating agents due to the fact that they are twenty thousand times more reactive with amino groups on biomolecules than glucose.\textsuperscript{53,56} G, MGO, and 3DG derived AGEs are the major AGEs formed \textit{in vivo} and play a significant role in the development and progression of diabetic complications (\textbf{Figure 1.3}). In addition, these AGEs are present at much higher levels in T2DM patients in relative to their precursors, which results in accelerated rates of diabetic complications. AGEs have recently been identified as biomarkers for diabetic complications. More importantly, AGEs derived from G, MGO, and 3DG proportionately reflect fluctuations in postprandial glucose better than the standard biomarker, HbA1c.\textsuperscript{10} Thus, research focused on dicarbonyl derived AGEs and their precursors could lead to the discovery of several functions underlying the metabolic pathways associated with T2DM complications, and aid in the treatment of patients with T2DM.
Figure 1.2 Formation of Advanced Glycation End Products *in vivo*

Three different pathways *in vivo* lead to the endogenous production of advanced glycation end products (AGEs): the non-enzymatic Maillard reaction, the Polyol-Pathway, and lipid peroxidation. The formation of reactive dicarbonyls such as glyoxal, methylglyoxal, and 3-desoxyglucoson precede that of AGEs in all three scenarios. In addition, if detoxification by the glyoxalase system is impaired, these compounds react further to form irreversible AGEs.⁸
Figure 1.3 The major precursors and AGEs identified in vivo

Displayed are the predominant precursors for the AGEs identified in vivo: CML: Ne-(carboxymethyl)-lysine; G-H1: glyoxal-derived hydroimidazolone 1; MG-H1: methylglyoxal-derived hydroimidazolone 1; CEL: Ne-(carboxyethyl)-lysine; 3DG-H1: 3-Deoxyglucosone-hydroimidazolone 1.9
1.4 Methylglyoxal and Diabetic Complications

MGO is the most reactive dicarbonyl and is produced as a byproduct of glycolysis. Research on the characterization and chemical reactions of MGO has been ongoing since the late 19th century. However, within the last few decades, the awareness of MGOs widespread distribution and high reactivity has driven more interest in MGO amongst the research community. The rate of MGO formation and its associated adducts is relatively higher than other precursors of AGEs and the strong interaction of MGO with functional arginine groups on proteins highlights its reactive nature. In addition, the MGO-derived AGE, methylglyoxal-derived hydroimidazolone 1 (MG-H1) rapidly forms at the guanidine group of arginine and is reported as the most abundant and largely irreversible AGE. Currently, several research groups have taken numerous approaches to establishing the physiological significance of MGO and its role in metabolic disorders such as diabetes.

The normal physiological concentration of MGO in human plasma is between 100 and 120 nM. However, this concentration increases rapidly depending on the amount of glucose and endogenous/exogenous sources of MGO. In addition, it is apparent that accelerated levels of MGO augment glycation of proteins, which lead to metabolic abnormalities in the human body, including diabetic complications. Certain foods, beverages, and smoke contain concentrations of MGO in the micromolar range and contribute to the endogenous supply of MGO. Fragmentation of glycolytic triose phosphates, catabolism of ketone bodies/threonine, and lipid peroxidation also result in the
endogenous production of MGO. However, glucose is responsible for the majority of MGO formation (Figure 1.4).\textsuperscript{67-71}

Simultaneously, endogenous and exogenous sources of MGO significantly increase intracellular and extracellular MGO concentrations alongside elevations in blood glucose. Under normal biological conditions, MGO is primarily detoxified through the glyoxalase system (GLO-1 and GLO-2 enzymes). However, during a diabetic state, detoxification by the glyoxalase system is impaired because these enzymes are not proportionately elevated along with the rapid increase in MGO production. Furthermore, levels of MG-H1 vary accordingly with the glyoxalase system. Thus, the dysfunction of MGO detoxification may lead to life-threatening circumstances wherein elevated levels of MGO contribute to more abundant and severe complications of T2DM. Moreover, recent studies have considered MGO as a biomarker for assessment of diabetic complications, because it is a main contributor to the glycation of proteins and has a direct impact on obesity and insulin resistance.\textsuperscript{10,72-74} In this regard, MGO is emphasized as a more suitable target for diabetes prevention and treatment relative to AGEs.
MGO is formed enzymatically and non-enzymatically from diverse origins, but glucose is the major contributor to the formation of MGO. Simultaneously, endogenous and exogenous sources of MGO production significantly increase intracellular and extracellular MGO concentration alongside elevations in blood glucose. Under normal biological conditions, MGO is primarily detoxified through the glyoxalase system (GLO-1 and GLO-2 enzymes). However, during a diabetic state, detoxification by the glyoxalase system is impaired. Accelerated levels of MGO augment glycation of proteins (AGEs), which lead to metabolic abnormalities in the human body such as diabetic complications.10
1.5 Therapeutic Target of Dicarbonyls

Based on the negative consequences of hyperglycemia induced elevations in reactive dicarbonyls and AGEs, targeting dicarbonyls is a practical therapeutic strategy for the treatment of T2DM. Direct scavengers of dicarbonyls were the first drugs to be developed and tested in clinical trials for preventing elevations in reactive dicarbonyls and thus dicarbonyl derived AGE production.\textsuperscript{75}

Aminoguanidine was the initial prototype and most promising inhibitor of AGEs. Aminoguanidine rapidly react with G, MGO, and 3DG via its guanidine group, thereby sequestering all three dicarbonyl compounds and forming a stable cyclized reaction product. In addition, in preclinical studies, aminoguanidine has an effect on albuminuria, diabetic nephropathy, and diabetic retinopathy, but these findings are not consistent across all studies. Although preclinical data revealed some potentially intriguing activity of aminoguanidine, several clinical trials in diabetic patients failed to reach their primary endpoint and were terminated due to adverse events and overall unfavorable risk-to-benefit ratio.\textsuperscript{13-18}

Similarly, the B6 vitamer pyridoxamine scavenges dicarbonyls \textit{in vitro} and has been examined as an inhibitor of dicarbonyl derived AGE formation. Pyridoxamine is also a metal chelator and could potentially prevent the production of AGEs via inhibition of the metal catalyzed autoxidation of glucose. Treatment with pyridoxamine in diabetic animal models alleviated albuminuria and retinopathy in parallel with a reduction of AGEs. However, clinical trials in diabetic patients were not successful and were prematurely terminated.\textsuperscript{76-81}
1.6 Metformin Directly Scavenges Methylglyoxal

The first line therapy for T2DM and 4th most prescribed drug in the US, MF, is structurally similar to aminoguanidine and significantly reduces levels of MGO in patients with T2DM.\textsuperscript{21,25,82} Unlike aminoguanidine, MF is FDA approved and proven to have therapeutic efficacy, with a good safety profile.\textsuperscript{4} MF is traditionally known to reduce hepatic gluconeogenesis through inhibition of the mitochondrial chain complex 1 with subsequent 5’ adenosine monophosphate activated protein kinase (AMPK) activation, thus reducing blood glucose levels (Figure 1.5).\textsuperscript{83} In addition, MF is unique because it notably decreases adverse diabetic endpoints and fatality more successfully than other antihyperglycemic agents.\textsuperscript{20} MF also appears to have an effect on both early and late stages of glycation, and effectively reduces AGEs derived from G, MGO, and 3DG in patients with T2DM.\textsuperscript{84-86}

Although MF has been used for the treatment of T2DM for over 60 years, its pleiotropic antidiabetic action remains elusive. It is probable that the initial reduction of MGO and dicarbonyl derived AGEs following MF treatment is due to improved glycemic control; however, the chemical structure of MF facilitates direct scavenging of MGO. Previous work exploring the MGO scavenging mechanism by MF proposed a seven-membered triazepinone based structure as the primary reaction product.\textsuperscript{87} While this structure may indeed exist, based on very limited data, the expected instability of the triazepinone product, and disagreement with other similar types of reactions with MGO, raised our concern. For example, one of the major products resulting from the reaction of MGO with
arginine is MG-HI, the stable five-membered ring structured hydroimidazolone.\textsuperscript{88} Moreover, the similarity in structure of MF to arginine led to the deduction that a five-membered ring structure was the more likely product of the MF–MGO reaction.

We further explored the MGO scavenging properties of MF and unequivocally identified and characterized the reaction product as a novel five membered ring structured imidazolinone metabolite (IMZ) in the plasma and urine of patients with T2DM (Figure 1.6).\textsuperscript{24} Thus, in addition to lowering hepatic gluconeogenesis, MF also scavenges the highly reactive MGO, forming a novel IMZ metabolite \textit{in vivo} thereby reducing potentially detrimental MGO protein adducts, which likely contributes to a reduction in diabetic complications. In addition, the formation of IMZ may also have significant therapeutic implications in the efficacy of MF’s antidiabetic properties, due to its drug-like structure, and deserves further investigation.
Figure 1.5 Traditional Mechanism of Metformin Drug Efficacy

MF primarily acts through a transient inhibition of the mitochondrial chain complex 1 (known as the principal target of MF) that sequentially decreases hepatic gluconeogenesis. Alteration of hepatic energy status, via increase in the AMP/ATP ratio, consequently, activates AMPK which in turn shifts the metabolic status of the cells towards inhibition of glucose, lipid, fatty acid, and protein synthesis leading to glucose uptake and lower blood glucose levels.
Figure 1.6 Metformin-methylglyoxal Scavenging Mechanism

MF (B) directly scavenges the reactive dicarbonyl (MGO) to form a novel 5 membered imidazolinone (C) metabolite and not the theorized 7 membered triazepinone (D), in patients with T2DM.²⁴
1.7 Pharmacological Properties of Imidazolines

Imidazolines are five-membered heterocyclic compounds found in nature and medicinal chemistry. The discovery of pharmacologically active imidazolines can be dated back to the 1930s. The imidazolines naphazoline (Privine®) and tetrahydrozoline (Tyzine®) became the first imidazoline drugs used medically; they both have activity at α-adrenergic receptors (α-ARs) and possess vasoconstricting properties that provide relief for nasal congestion. Another breakthrough for the therapeutic development of imidazoline drugs was the discovery of clonidine in the 1960s. Clonidine was originally developed as a nasal decongestant but was later found to concomitantly lower blood pressure to a greater extent, and subsequently became the model for centrally acting antihypertensive therapeutics. Clonidine also improves psychiatric disorders, pain disorders, drug withdrawal, metabolic disorders, glaucoma, defective thermoregulation, and sickle cell anemia. It was traditionally believed that clonidine exerted its effects solely through the activation of the α2-ARs. However, given the multitude and diversity of clonidine’s pharmacological activity, other sites of activity have been explored. Recent studies have shown that clonidine also has activity mediated through various subtypes of α-ARs and imidazoline receptors, suggesting that clonidine has promiscuous binding specificity. Subsequent to clonidine, many other imidazolines have been development and comprise an assortment of over the counter and prescribed medications, used for a variety of indications (Figure 1.7).
Figure 1.7 Imidazolines in Therapeutic Use

Shown are the chemical structures of an assortment of over the counter and prescribed imizadoline drugs used for a variety of indications. These medications express varying affinities for α-ARs and/or imidazoline receptors.¹⁰³
The finding that clonidine is able to activate imidazoline receptors resulted in a newfound interest in the identification of additional imidazolines that bind imidazoline receptors and exert a therapeutic response. The second-generation antihypertensives, moxonidine and rilmenidine, are structural analogs of clonidine, but exhibit decreased frequency and severity of adverse side effects. In addition, both compounds selectively activate imidazoline receptors, leading to an emphasis on the beneficial effects of imidazoline receptor agonists not only on hypertension, but also on disorders associated with T2DM, such as insulin resistance, impaired glucose tolerance, and hyperlipidemia.²⁶,¹⁰⁴-¹⁰⁸
1.8 Imidazoline Receptors

It is generally accepted that the antihypertensive properties of selective imidazolines are mediated in part by a nonadrenergic mechanism. This consensus is based on several studies revealing that inhibition studies with $\alpha_2$-AR antagonists that also have affinity for imidazoline receptors (idazoxan and efaroxan) have a potent inhibitory effect on the hypotensive properties of the agonist. On the other hand, $\alpha_2$-AR antagonists that are not selective for imidazoline receptors, such as yohimbine and SK&F 86466, only partially inhibit the antihypertensive activities of imidazolines.\textsuperscript{108-112} Since the discovery of imidazoline receptors in the 1960s, further work based on binding affinity, function, and location has provided evidence that there are three subtypes of imidazoline receptors (I\textsubscript{1}R-I\textsubscript{3}R), which are located in various tissues, have diverse functions, and have differential ligand binding specificity (Figure 1.8). \textsuperscript{113-116}
I₁Rs may act synergistically with α₂-ARs, and thus stimulation of one leads to the activation of the other; however, the hypotension and bradycardia is more likely due to activation of I₁Rs. I₂Rs are allosteric binding sites for MAO-A and MAO-B and may affect the autonomic nervous system (ANS) which have together been proposed for its ability to influence psychiatric disorders, glucose homeostasis, withdrawal, and antinociception. I₃Rs are present in pancreatic β-cells and induce insulin secretion.¹⁰³
I_1Rs are plasma membrane bound and located in the brain, heart, kidneys, liver, and pancreas. High densities of I_1R have been found in many regions of the brain, such as the rostral ventrolateral medulla (RVLM), medulla oblongata, cerebral cortex, and hypothalamus. In addition, I_1Rs have also been localized in peripheral tissue, such as the adrenal medulla, kidney, liver, and platelets. Clonidine, moxonidine, and rilmenidine have a higher affinity for I_1Rs than α_2-ARs. Moreover, the sympathoinhibitory activity of all three compounds have been associated with activation of I_1R, thus implicating I_1R involvement in regulation of the cardiovascular system. In addition, agmatine (a guanidine), harmane, and imidazole acetic acid have been identified as endogenous I_1R ligands.

I_2Rs, originally referred to as the idazoxan-preferring receptor, are allosteric binding sites for monoamine oxidase-A and B (MAO-A, MAO-B) and have been localized to the outer membrane of the mitochondria. Binding sites of I_2Rs are widely distributed in the CNS and peripheral tissues and are 100 times more abundant than I_1R binding sites. Imidazoline and guanidine compounds bind I_2Rs, but clonidine, moxonidine, and rilmenidine have a much lower affinity for I_2Rs than I_1Rs. Thus, I_2Rs are less likely to contribute to the pharmacological activity of clonidine, moxonidine, and rilmenidine. Most research on I_2Rs has focused on their role in depression, suggesting possible antidepressant activity of imizazolines. The newer imidazoline, idazoxan, has been under investigation as an antidepressant, and exhibits high affinity for I_2Rs. Another interesting expansion with I_2Rs is on their role in inhibiting nociception and addiction. Studies show that this effect may be due to a synergistic effect involving α_2-ARs,
I₂Rs, and opioid receptors. Finally, agmatine, MF, and moxonidine all have beneficial effects on glucose homeostasis and insulin resistance through I₂R activation.

I₃Rs are the least characterized of all three imidazoline receptor subtypes and have only been identified in pancreatic beta cells. I₃R activation increases insulin secretion, possibly through involvement with ATP sensitive potassium channels. Ligand binding and specificity of I₁Rs and I₃Rs seem to be similar, but compounds show distinct patterns of efficacy, as demonstrated with an investigational imidazoline, efaroxan, which is a selective antagonist of I₁Rs but is an agonist of I₃Rs. It is further proposed that I₃Rs are primarily responsible for control of hormone secretion. Harmane, an endogenous imidazoline agonist, directly elevates cytosolic calcium and increases insulin secretion through I₃R activation in pancreatic β-cells.
1.9 Proposed I₁R Cell Signaling Mechanism

The discovery and characterization of a functional I₁R protein, imidazoline receptor antisera- selected (IRAS) and the mouse homologue nischarin (NISCH), has led to a better understanding of the distribution of imidazoline receptors and their functional structure-activity association with their ligands. IRAS was initially isolated from the human hippocampus and is found at high levels in brain tissue. IRAS has also been detected in tissue from the testes, liver, kidney, spleen, lung, and skeletal muscle. This is consistent with previous radioligand binding studies that revealed I₁R binding sites. Overexpression of IRAS in Chinese hamster ovary (CHO) and pheochromocytoma (PC12) cells resulted in high affinity and specific imidazoline ligand binding. The mouse homologue of human IRAS, NISCH, has 80% homology to IRAS, but lacks the N-terminal 244 amino acid residues. Stable expression of IRAS has been linked to an antiapoptotic effect and prolonged cell survival; these studies also confirmed that this effect is potentially mediated through the downstream activation of the mitogen activated protein kinase (MAPK) member, extracellular receptor kinase 1 and 2 (ERK1/2).¹²⁰,¹⁴⁰-¹⁴³ This data is consistent with earlier experiments conducted in PC12 cells and spontaneously hypertensive rats that attempted to explain downstream cell signaling in response to moxonidine. In addition, studies on IRAS in human embryonic kidney 293 (HEK293) cells revealed that imidazoline receptors are associated with insulin receptor substrates (IRS 1-4) and play a role in activation of several components of the insulin signaling cascade, including ERK1/2 and protein kinase B (AKT). This finding was confirmed with moxonidine...
and rilmenidine treatment in IRAS transfected HEK293 and parental cells.\textsuperscript{144} Moxonidine has also been shown clinically to improve elements of metabolic syndrome in obese patients with mild hypertension. From these studies, it was demonstrated that moxonidine treatment has positive effects on blood pressure, dyslipidemia, hyperglycemia, and has even reduced insulin levels to a similar extent seen with MF treatment.\textsuperscript{133} Thus, moxonidine treatment in humans enhances insulin sensitivity, which could prevent or delay the development of T2DM.

The proposed model for I\textsubscript{1}R receptor signaling is shown in Figure 1.9. This model illustrations that the interaction of moxonidine with I\textsubscript{1}R results in a downstream activation of IRS1-4, phosphatidyl-inositol-3-kinase (PI3K), ERK1/2, AKT, translocation of glucose transporter type 4 (GLUT4) to the plasma membrane, and eventual glucose uptake. In addition, these activities can be blocked by the I\textsubscript{1}R antagonists efaroxan and idazoxan. This signaling cascade has been proposed to be linked to activation of phosphatidylincholine selective phospholipase C (PC-PLC), which utilizes phosphatidylincholine (PC) to generate the lipid second messenger diacylglycerol (DAG). DAG accumulation in turn activates protein kinase C (PKC) which provides a connection between DAG production and ERK1/2 activation.\textsuperscript{145,146} Taken together, the hypothesis is that there is crosstalk between insulin receptor and I\textsubscript{1}R mediated signaling, which leads to an improvement in hypertension, glucose tolerance, insulin resistance, and hyperinsulinemia. Thus, imidazoline receptors are novel targets for the development of drugs for the treatment of disorders associated with T2DM.
Figure 1.9 Proposed I1R Cell Signaling Mechanism

The interaction of moxonidine with I1R results in a downstream activation of IRS1-4, PI3K, ERK1/2, AKT, GLUT4 translocation, and eventual glucose uptake. Thus, I1R activation results in improved glucose tolerance, insulin resistance, hyperinsulinemia, and hypertension.
1.10 PC12 Cells, MIN6 Cells, HEPG2 Cells, and db/db Mice as Models for I\(_1\)R Signaling and T2DM

Cell line models provide an ideal method for analyzing responses produced by receptor-ligand interactions. Rat-derived pheochromocytoma (PC12) cells are the predominant in vitro model for assessing I\(_1\)R activation and coupled downstream signaling cascades. As shown by radioligand binding studies and mRNA expression levels, these cells constitutively express a high density of I\(_1\)R binding sites and lack \(\alpha_2\)-adrenergic receptors (\(\alpha_2\)-ARs), for which many imidazolines exhibit activity with. Further, epinephrine and guanabenz, non-imidazoline structured \(\alpha_2\)-AR agonists, have no pharmacological effect in PC12 cells. Thus, experiments to assess signaling in response to imidazoline agonists and antagonists can be carried out in PC12 cells without the need to inhibit \(\alpha_2\)-ARs in advance. Experiments performed in PC12 cells with I\(_1\)R agonists clonidine, moxonidine, and rilmenidine, as well as the I\(_1\)R antagonists efaroxan and idazoxan have shaped our understanding of imidazoline receptor-ligand mediated pharmacological activity.\(^{26,146,147}\)

The MIN6 mouse pancreatic (insulinoma) B-cell line displays characteristics of glucose metabolism and glucose stimulated insulin release similar to those of normal human islets. More importantly, MIN6 cells best reflect normal physiological conditions because they respond to glucose at physiologically relevant concentrations and express glucokinase, unlike some of the other B-cell lines available. In addition, several studies have shown that imidazolines have I\(_3\)R mediated insulinotropic properties in the MIN6 pancreatic
β-cell line. Thus, MIN6 cells are a valuable tool for understanding the molecular events underlying β-cell function and dysfunction as well as I_{3}R mediated insulin secretion.\textsuperscript{137,148-152}

HEPG2 cells are a human liver (hepatocellular carcinoma) cell line that is well established as a reliable model for assessing liver metabolism and insulin signaling. In addition, numerous studies have demonstrated insulin resistant HEPG2 cells as a dependable model for measuring liver metabolism and signaling in an insulin resistant state. Therefore, HEPG2 cells are a useful model for understanding liver metabolism in T2DM because resistance to insulin in hepatocytes reduces glycogen synthesis and results in hyperglycemia.\textsuperscript{153-155}

T2DM is a complex metabolic disorder caused by either the abnormal secretion of insulin from the pancreas and/or by the body not effectively utilizing/responding to circulating insulin, the consequences of which are a prolonged hyperglycemia, obesity, and numerous severe diabetic complications. The db/db mouse model is a genetically derived diabetic animal caused by mutations in the gene that encodes for the leptin receptor. Due to the lack of leptin signaling, the pathogenesis of T2DM in db/db mice exhibits metabolic characteristics that are comparable to human T2DM, such as hyperinsulinemia, hyperglycemia, obesity, and dyslipidemia. Thus, in recent years, db/db mice have been widely used as an accepted animal model to replicate human T2DM.\textsuperscript{156-159}
1.11 Dissertation Aims

Our laboratory has previously identified and characterized a novel metformin-methylglyoxal derived imidazolinone (IMZ) metabolite in the plasma and urine of patients with T2DM. MF, the first-line therapy for T2DM, is traditionally known for inhibiting hepatic gluconeogenesis and thus reducing hyperglycemia. However, MF’s biological activity is multifaceted, and its precise mechanisms of action remain an enigma. Imidazolines have shown promise in not only reducing hypertension, but also improving glucose tolerance, reducing hyperinsulinemia, and decreasing insulin resistance. In addition, imidazoline receptors play an important role in maintaining glucose homeostasis, insulin sensitization, and insulin secretion. Moreover, MF reduces plasma glucose and increases glucose uptake through activation of imidazoline receptors in an *in vitro* and an *in vivo* model. The chemical structure of IMZ suggests it may aid in the ability of MF to activate imidazoline receptors and may be a fundamental element in MF drug efficacy. Thus, the potential pharmacological activity of IMZ *in vitro* and *in vivo* merits further investigation.

We initially explored the possible pharmacological properties of IMZ *in vitro* (Chapter 2; aim 1). More specifically, these studies assessed the ability of IMZ to improve insulin sensitization in PC12 and insulin resistant HEPG2 cells. In addition, the effects of IMZ on insulin secretion in MIN6 cells were explored. The results from these studies describe for the first time the potential intracellular signaling mechanisms behind IMZ action *in vitro*. In addition, my results suggest
that the ability of IMZ to sensitize PC12 cells to insulin and induce insulin secretion in MIN6 cells may be mediated through I_1R and I_3R, respectively. Moreover, my findings show that IMZ is able to sensitize insulin resistant HEPG2 cells to insulin through modulation of AKT phosphorylation, which may be mediated through imidazoline or α-adrenergic receptors.

We subsequently explored the antidiabetic effects and possible intracellular signaling mechanisms engaged by IMZ in genetically obese db/db diabetic mice (Chapter 3; aim 2). More specifically, this study assessed the ability of IMZ to improve insulin resistance, pancreatic islet morphology and function, and adipocyte and renal histopathological changes. In addition, the effects of IMZ on hepatic steatosis and lipogenesis mediated through sirtuin 1 (SIRT1) activation were explored. The results from these studies describes for the first time the protective effects of IMZ in vivo. Moreover, these results show that IMZ significantly alleviated insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes in db/db mice treated for 7 days. IMZ also promoted hepatic SIRT1 expression and downregulation of SREBP1, ChREBP, ACC, and FAS at the protein and mRNA level in db/db mice, which may contribute to the antidiabetic effects exerted by MF, which itself can ameliorate hyperglycemia, dyslipidemia, hepatic steatosis, insulin resistance, and lower microvascular and macrovascular complications associated with T2DM.

Finally, we investigated the long-term protective effects and potential mechanisms of activity exerted by IMZ in the well-established genetically obese
db/db diabetic mouse model alongside a clinically relevant dose of MF (Chapter 4; aim3). In particular, these studies evaluated the ability of IMZ to recover hyperglycemia, insulin resistance, irregular pancreatic islet morphology and function, and adipocyte and renal histopathological abnormalities similarly to MF. In addition, the effects of IMZ on markers of hepatic steatosis, lipogenesis, and fibrosis were studied. Herein, I describe for the first time the antidiabetic properties of long-term IMZ treatment in vivo and offer awareness into a potential innovative mechanism behind the protective effects of MF. The results from these studies demonstrated that IMZ significantly alleviated hyperglycemia, insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes in db/db mice treated with IMZ for 60 days. In addition, IMZ promoted hepatic downregulation of the lipogenesis and fibrosis markers ACACA, FASN, FN1, COL1A1, and TGFβ1 at the protein and mRNA level in db/db mice. These findings are in agreement with the fact that MF itself can ameliorate hyperglycemia, dyslipidemia, hepatic steatosis, insulin resistance, and lower microvascular and macrovascular complications associated with T2DM.

Taken together, the findings detailed in this dissertation support the hypothesis that the formation of IMZ may contribute to the antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM. Although the precise underlying mechanism(s) of IMZ’s pharmacological action remain to be fully elucidated, the
present work opens an intriguing window on the mechanism of the antidiabetic properties of MF.
Chapter 2: A Metformin-methylglyoxal Imidazolinone Metabolite Enhances Insulin Action and Secretion in vivo

2.1 Introduction

Diabetes has reached epidemic proportions worldwide due to substantial alterations in modern diet and lifestyle. The number of people with diabetes is projected to increase from an estimated 451 million to 693 million by 2045.\(^1\) Diabetes is a long-term metabolic disease characterized by hyperglycemia resulting from impaired insulin action and glucose homeostasis. If left untreated, severe health complications develop such as hypoglycemia, hypertension, dyslipidemia, stroke, foot ulcers, cardiomyopathy, neuropathy, nephropathy, retinopathy, and premature death. The precise pathophysiological mechanisms behind diabetic complications are not well understood, however, insulin resistance, beta cell dysfunction, and beta cell apoptosis resulting in lower circulating insulin levels have been implicated.\(^2,3,5,6\) Thus, a crucial therapeutic approach for treating diabetes is reversing pancreatic islet dysfunction and insulin resistance as they both play a vital role in the pathogenesis of diabetes.

Imidazoline receptors are considered novel targets for drug development in disorders associated with type-two diabetes mellitus (T2DM) because they are involved in glucose homeostasis and insulin sensitization/secretion. Three main subtypes of imidazoline receptors (I\(_1\)R- I\(_3\)R) have been identified and located in the mitochondria, brain, heart, kidney, liver, and pancreas. Moreover, the evolving consensus is that I\(_1\)R activation plays an important role in insulin sensitization, I\(_2\)R facilitates glucose uptake, and the I\(_3\)R subtype, located only in
pancreatic B-cells, leads to improved insulin secretion. In addition, imidazoline like drugs have been shown to stimulate insulin secretion, enhance insulin action, and help maintain glycemic control.\textsuperscript{2,6,26,27,29-33,160} However, no compound from this class of drugs has yet come into clinical use as an effective antidiabetic agent due to the fact that the molecular characteristics of imidazoline receptor binding and the exact structure-activity association of their ligands remains unclear.

Our laboratory has identified and characterized a novel metformin-methylglyoxal derived imidazolinone (IMZ) metabolite in the plasma and urine of patients with T2DM.\textsuperscript{24} Metformin (MF), the first-line therapy for T2DM, is classically known for inhibiting gluconeogenesis in the liver and thus reducing hyperglycemia.\textsuperscript{4,161} However, MF’s biological activity is multifaceted, and its precise mechanisms of action remain an enigma. MF has recently been shown to reduce plasma glucose and increase glucose uptake through activation of imidazoline receptors in an \textit{in vitro} and an \textit{in vivo} model.\textsuperscript{34,35} The chemical structure of IMZ suggests it may aid in the ability of MF to activate imidazoline receptors and may be a fundamental element in MF drug efficacy. Thus, the potential pharmacological activity of IMZ \textit{in vitro} merits further investigation.

Herein, we explored the possible pharmacological properties of IMZ \textit{in vitro}. More specifically, this study assessed the ability of IMZ to improve insulin sensitization in PC12 and insulin resistant HEPG2 cells. In addition, the effects of IMZ on insulin secretion in MIN6 cells were explored. The present study describes for the first time the potential intracellular signaling mechanisms
behind IMZ *in vitro* and provides insight into a potential alternative mechanism by which MF exerts its antidiabetic properties.
2.2 Materials and Methods

2.2.1 Chemicals and antibodies

A metformin-methylglyoxal metabolite, (E)-1,1-dimethyl-2-(5-methyl-4-oxo-4,5-dihydro-1H-imidazol-2-yl)guanidine (IMZ), was synthesized in our laboratory as previously described by Kinsky et al. D-glucose, human recombinant insulin, moxonidine, efaroxan, idazoxan, and canavanine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tocris Bioscience™ KU14R was purchased from Fisher Scientific (Hampton, NH, USA). Primary antibodies used were as follows: p44/42 MAPK (ERK1/2, 9107S), Phospho-p44/42 (Thr202/Tyr204) MAPK (pERK1/2, 9101S), protein kinase B (AKT, 2920S), phospho-protein kinase B (S473, pAKT, 4060S), Nischarin (85124S) (Cell Signaling Technologies, Danvers, MA, USA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, ab8245).

2.2.2 Cell culture

Rat-derived pheochromocytoma cells (PC12, ATCC, Manassas, VA, USA) were cultured in Corning™ RPMI 1640 medium (Fisher Scientific) supplemented with 10% Gibco™ heat inactivated horse serum (Thermo Fisher Scientific, Waltham, MA, USA) and 5% fetal bovine serum (FBS, Serum Source International, Charlotte, NC, USA). Human hepatocellular carcinoma cells (HEPG2, ATCC) were
cultured in Corning™ Eagle's MEM (Fisher Scientific) supplemented with 10% FBS. Mouse pancreatic β-cells (MIN6, AddexBio, San Diego, CA, USA) were cultured in AddexBio advanced DMEM-based medium optimized to culture MIN6 cells. All cell lines were maintained in the presence of 5% CO2 at 37°C.

2.2.3 Insulin sensitization in PC12 cells

PC12 cells were plated in 6-well plates at a density of 2 million cells per well and grown to 80% confluency. On the day of the experiment, the cells were rinsed twice with phosphate-buffered saline and the culture medium was replaced with serum free medium for 2 hours. After serum starvation, PC12 cells were then treated with either moxonidine (1 µM), insulin (1 µM), IMZ at various concentrations (1 pM - 100 nM), or a combination of IMZ (1 pM, 1 nM) and insulin (1 µM) for 10 minutes. Another group of cells were pretreated with idazoxan (50 µM) or efaroxan (10 µM) and then treated with insulin (1 µM) alone or a combination of IMZ (1 nM) and insulin (1 µM) in the presence of idazoxan (50 µM) or efaroxan (10 µM) for 10 minutes. Treated cells were lysed using ice cold Cell Lysis Buffer 10X (CLB, Cell Signaling Technologies) containing 1 mM Pefabloc® SC, cOmplete™ protease inhibitor cocktail, and PhosSTOP™ phosphatase inhibitor cocktail (Roche, South San Francisco, CA, USA), centrifuged at 13,000 rpm for 10 min. at 4°C, and the supernatant was stored at −80°C for western blot analysis.
2.2.4 Nischarin siRNA-mediated knockdown in PC12 cells

PC12 cells were plated in 6-well plates and grown to ~40% confluency. Small interference ribonucleic acid (siRNA) targeting rat nischarin (NISCH; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used (6 µL per sample). Pooled non-targeting scramble siRNA was used (6 µL per sample) as a negative control (Santa Cruz Biotechnology, Inc.). Lipofectamine 2000 (Thermo Fisher Scientific) was used (4 µL per sample) and transfection was performed according to the manufacturer’s instructions. After 48 hrs. of transfection, NISCH silenced and scramble control cells were treated with insulin (1 µM), IMZ (1nM), or a combination of insulin (1 µM) and IMZ (1nM) for 10 minutes. After treatment, cells were lysed in CLB, centrifuged at 13,000 rpm for 10 min. at 4°C, and the supernatant stored at −80°C for western blot analysis.

2.2.5 Insulin resistant HEPG2 cell model

HEPG2 cells were plated in 6-well plates at a density of 1 million cells per well and grown to 65-70% confluency. Once cells reached confluency, the medium was replaced with serum free medium and incubated overnight (~16 hrs) in the presence of 5% CO2 at 37°C. On the next day, the medium was replaced with glucose and serum free Gibco™ DMEM (Fisher Scientific) supplemented with either a low glucose concentration (5.5 mM), a high glucose concentration (25
mM), or a high concentration of glucose (25 mM) and insulin (1 μM) for 24 hours. After 24 hrs, cells were serum starved for 2 hrs. and subsequently dosed with insulin (100 nM) or various concentrations of IMZ (1 nM, 100 nM, and 1 μM) for 10 minutes. After treatment, cells were lysed in CLB, centrifuged at 13,000 rpm for 10 min. at 4°C, and the supernatant stored at ~80°C for western blot analysis.

2.2.6 Western blot analysis

Concentrations of protein in cell lysates were determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). Protein (40-100 μg) was separated on an 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA) for 2 hrs. at 50 volts in 4°C. PVDF membranes were blocked in Odyssey® Blocking Buffer in tris-buffered saline (LI-COR, Lincoln, NE, USA) for 1 hr. at room temperature (RT), followed by incubation with specific primary antibodies, diluted 1:500 or 1:1000 in 5% bovine serum albumin (BSA), at 4°C overnight. Membranes were then incubated with corresponding near-infrared fluorescent secondary antibodies (LI-COR), diluted in blocking buffer 1:15000, for 1 hr. at RT. Targeted proteins were detected using an Odyssey® FC imaging system and band intensity was analyzed using Image Studio Lite Ver 5.2 (LI-COR).
2.2.7 Insulin secretion

Min6 cells were plated in 24-well plates at a density of 500,000 cells per well. On the next day, the culture medium was replaced with glucose and serum free Gibco™ DMEM (Fisher Scientific) supplemented with 0.5 mM glucose and 0.1% BSA and incubated overnight (~12 hrs.). On the day of the experiment, the cells were starved for 4 hrs. in glucose free Krebs-ringer modified HEPES buffer containing 0.1% BSA (KRBH-BSA) at 37°C with 5% CO2. Following the 4hr. incubation, cells were treated with canavanine (10 µM), efaroxan (100 µM), and various concentrations of IMZ (1 pM-1 µM) for 15 minutes in KRBH-BSA supplemented with 7 mM glucose. Another group of cells were pretreated with KU14R (1 µM) for 30 min. and then treated under the same conditions in the presence of KU14R for 15 minutes. After treatment, the medium was collected, centrifuged at 13,000 rpm for 10 min. at 4°C, and stored at -80°C for insulin measurement. Insulin release was measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions. Protein concentrations were determined using the DC™ Protein Assay (Bio-Rad).

2.2.8 Nischarin siRNA in MIN6 cells

Min6 cells were plated in 6-well plates and grown to ~40% confluency. According to a previous method (Lin, M. H. et al DOI: 10.1111/1440-1681.12815)
siRNA targeting human NISCH (GE Healthcare Dharmaco Inc., Lafayette, CO, USA), a mouse homologue of the human imidazoline receptor antisera-selective (IRAS) protein, was used (25 nM siRNA). Pooled non-targeting scramble siRNA was used as a negative control (Santa Cruz Biotechnology, Inc.). Lipofectamine 2000 (Thermo Fisher Scientific) was used and transfection was performed according to the manufacturer’s instructions. After 48 hrs. of transfection, NISCH silenced and scramble control cells were treated with canavanine (10 μM) and IMZ (10 nM) in KRBH-BSA supplemented with 7 mM glucose for 15 minutes. After treatment, the medium was collected, cells were lysed in CLB, both medium and cells were centrifuged at 13,000 rpm for 10 min. at 4°C, and the supernatant was stored at −80°C for further analysis. Collected medium was used for insulin measurement via ELISA and NISCH knockdown was validated in cell lysate via western blot analysis.

2.2.9 Statistical analysis

All data were analyzed using GraphPad Prism 5 software (San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). A one-way ANOVA, or a two-way ANOVA for multiple comparisons, followed by Bonferroni’s post-test was used for analysis. A p-value less than 0.05 was considered statistically significant at the 95% confidence level.
2.3 Results

2.3.1 IMZ sensitized PC12 cells to insulin via modulation of ERK1/2 and AKT phosphorylation

Combination treatment of IMZ (1pM, 1nM) and insulin (1 µM) resulted in a greater phosphorylation of AKT and ERK1/2 than insulin treatment alone in a dose-dependent manner. Peak phosphorylation of AKT and ERK1/2 was observed with combination treatment of insulin and 1nM IMZ. IMZ (1 pM-100 nM) treatment alone did not cause an increase in phosphorylation of AKT or ERK1/2 above basal level in PC12 cells. However, the known I₁R agonist moxonidine (1 µM) potentiated AKT and ERK1/2 phosphorylation in comparison to control (Figure 2.1).
Figure 2.1 IMZ sensitized PC12 cells to insulin via modulation of AKT and ERK1/2 phosphorylation

Representative immunoblots of pAKT, AKT, pERK1/2, ERK1/2, and GAPDH (A) and western blot analysis of AKT (B) and ERK1 (C, white bars), ERK2 (C, black bars) activation in PC12 cells treated with insulin (1 μM), IMZ (1 pM-100 nM), a combination of IMZ (1 pM, 1nM) and insulin (1 μM), or the I1R agonist moxonidine (mox, 1 μM) for 10 minutes. AKT activation was determined by the ratio of AKT phosphorylation on Ser473 to the total AKT protein level (B). ERK activation was determined by the ratio of ERK1/2 phosphorylation on Thr202/Tyr204 to the total ERK1/2 protein level (C). All western blot analysis was quantified using Image Studio Lite and normalized to the loading control GAPDH. Data are presented as fold change of insulin treated PC12 cells and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (* P < 0.05, **** P < 0.0001 indicates a significant difference between moxonidine treated and control vehicle treated PC12 cells; # P < 0.05, #### P < 0.0001 indicates a significant difference between insulin treated and combination of IMZ and insulin treated PC12 cells).
2.3.2 \( \beta_1 \)R antagonists efaroxan and idazoxan decreased IMZ induced insulin sensitization in PC12 cells

The increase in phosphorylation of AKT and ERK1/2 induced by combination treatment of IMZ and insulin in PC12 cells was reduced in the presence of the \( \beta_1 \)R antagonists efaroxan and idazoxan. In addition, efaroxan and idazoxan treatment alone did not significantly modulate the phosphorylation of AKT and ERK1/2 above basal level. Interestingly, the combination treatment of insulin with efaroxan and idazoxan augmented AKT and ERK1/2 phosphorylation greater than insulin treatment alone (Figure 2.2).
Figure 2.2 I\(_1\)R antagonists efaroxan and idazoxan decreased IMZ induced insulin sensitization in PC12 cells

Representative immunoblots and western blot analysis of AKT and ERK1/2 activation in PC12 cells treated with efaroxan (10 µM), idazoxan (50 µM), IMZ (1 nM), insulin (1 µM), and a combination of IMZ and insulin with and without the presence of efaroxan or idazoxan for 10 minutes. AKT activation was determined by the ratio of AKT phosphorylation on Ser473 to the total AKT protein level (B, C). ERK activation was determined by the ratio of ERK1/2 phosphorylation on Thr202/Tyr204 to the total ERK1/2 protein level (D, E). All western blot analysis was quantified using Image Studio Lite and normalized to the loading control GAPDH. Data are presented as fold change of insulin treated PC12 cells and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (**** P < 0.0001 indicates a significant difference between insulin treated and combination of IMZ and insulin treated PC12 cells; # P < 0.05 indicates a significant difference between combination of IMZ and insulin treated and combination of IMZ, insulin, and efaroxan or idazoxan treated PC12 cells).
2.3.3 siRNA-mediated knockdown of nischarin activated ERK1/2 in PC12 cells

In PC12 cells treated with NISCH siRNA for 48 hrs, there was a greater than 75% reduction in the expression of nischarin protein level in comparison to cells treated with control siRNA (Figure 2.3A). Insulin treatment in control siRNA PC12 cells increased phosphorylation of AKT and ERK1/2 above the level of the control at 10 minutes. However, combination treatment of insulin and IMZ for 10 min. in control siRNA cells did not result in augmentation of AKT and ERK1/2 phosphorylation in comparison to insulin treatment alone (Figure 2.3C, D). In contrast, PC12 cells treated with NICH siRNA showed an increase in the phosphorylation of ERK1/2 following 10 min. treatment with insulin and combination treatment of insulin and IMZ, compared to control siRNA cells treated in the same manner (Figure 2.3D). In contrast, in PC12 cells treated with NISCH siRNA, there was no effect on AKT phosphorylation, compared to treated cells without NISCH siRNA (Figure 2.3C). IMZ treatment alone did not have an effect on the phosphorylation of ERK1/2 and AKT in either control or NISCH siRNA treated cells (Figure 2.3C, D).
Figure 2.3 siRNA-mediated knockdown of niscahrin activated ERK1/2 in PC12 cells

Representative immunoblots and western blot analysis of NISCH, pAKT, AKT, pERK1/2, ERK1/2, and GAPDH in control siRNA PC12 cells treated with, insulin (1 µM), IMZ (1 nM), or IMZ and insulin, and NISCH siRNA PC12 cells treated with, insulin (1 µM), IMZ (1 nM), or IMZ and insulin for 10 minutes (A). NISCH knock down was determined by the ratio of NISCH to GAPDH total protein level (B). AKT activation was determined by the ratio of AKT phosphorylation on Ser473 to the total AKT protein level (C). ERK activation was determined by the ratio of ERK1/2 phosphorylation on Thr202/Tyr204 to the total ERK1/2 protein level (D). All western blot analysis was quantified using Image Studio Lite and normalized to the loading control GAPDH. Data are presented as fold change of insulin treated PC12 cells and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (### P < 0.0001 indicates a significant difference between scramble and si-NISCH treated PC12 cells; **** P < 0.0001 indicates a significant difference between insulin treated control siRNA cells and insulin or insulin+IMZ treated si-NISCH PC12 cells).
2.3.4 IMZ improved insulin resistance (IR) in IR HEPG2 cells via activation of AKT

Insulin (100 nM) treatment for 10 min. of HEPG2 (IR HEPG2) cells cultured in high glucose (25 mM) and high insulin (1 μM) containing medium resulted in a significant decrease in the phosphorylation of AKT in comparison to insulin treatment in HEPG2 cells cultured in either low glucose (5 mM) or high glucose (25 mM) containing medium (Figure 2.4A). Combination treatment of insulin (100 nM) and varying concentrations of IMZ (100 nM, 1 μM) in IR HEPG2 cells resulted in a greater phosphorylation of AKT than treatment with insulin alone for 10 minutes. However, IMZ treatment alone had no effect on AKT phosphorylation in IR HEPG2 cells (Figure 2.4B).

2.3.5 IMZ promoted insulin secretion in MIN6 cells

In MIN6 cells, IMZ increased insulin secretion in a biphasic dose-response manner at 15 minutes. Maximal insulin secretion was reached with treatment of 10 nM IMZ. In addition, IMZ increased insulin secretion at a lower concentration in comparison to the effectiveness of the positive controls canavinine and efaroxan. However, all compounds showed similar maximal effects on insulin secretion in MIN6 cells at the treatment concentration (Figure 2.5).
Figure 2.4 IMZ improved insulin resistance (IR) in IR HEPG2 cells via activation of AKT

Representative immunoblots and western blot analysis of AKT activation in HEPG2 cells cultured in low glucose (LG, 5 mM), high glucose (HG, 25 mM) or high glucose and high insulin (HGHI, 25 mM, 1 μM) containing medium for 24 hrs. and subsequently treated with insulin (100 nM) for 10 minutes. AKT activation was determined by the ratio of AKT phosphorylation on Ser473 to the total AKT protein level (A). Representative immunoblots and western blot analysis of AKT activation in HEPG2 cells cultured in high glucose and high insulin containing medium for 24 hrs. and subsequently treated with insulin (100 nM), IMZ (1 pM-1 μM), or a combination of insulin and IMZ for 10 minutes. AKT activation was determined by the ratio of AKT phosphorylation on Ser473 to the total AKT protein level (B). All western blot analysis was quantified using Image Studio Lite and normalized to the loading control GAPDH. Data are presented as fold change control and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (**** P < 0.0001 indicates a significant difference between LG+insulin treated and HGHI+insulin treated HEPG2 cells; ## P < 0.005 indicates a significant difference between insulin treated and combination of IMZ and insulin treated HEPG2 cells).
Insulin secretion induced by canavanine (10 µM), efaroxan (100 µM), and varying concentrations of IMZ (1pM-10 µM) were determined in MIN6 cells cultured in buffer containing glucose (7 mM) via ELISA. Data are presented as fold change of vehicle treated control in MIN6 cells and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (* P < 0.05, *** P < 0.0005, **** P < 0.0001 indicates a significant difference between drug treated and vehicle treated control MIN6 cells).
2.3.6 The I$_3$R antagonist KU14R reversed IMZ stimulated insulin secretion in MIN6 cells

The I$_3$R antagonist KU14R treatment alone did not significantly influence insulin secretion above basal level. However, the increase in insulin secretion induced by IMZ was reduced in the presence of KU14R. In addition, the effects of efaroxan on insulin secretion were also attenuated by KU14R (Figure 2.6).

2.3.7 Silencing nischarin muted IMZ induced insulin secretion in MIN6 cells

NISCH protein expression level was decreased by 50% in MIN6 cells treated with NISCH siRNA for 48 hrs. in comparison to the scrambled siRNA negative control (Figure 2.7A). The increase in insulin secretion induced by IMZ was markedly reduced in NISCH silenced MIN6 cells. Similarly, insulin secretion induced by the positive control canavanine was inhibited in the same manner (Figure 2.7B).
Figure 2.6 The I$_3$R antagonist KU14R reversed IMZ stimulated insulin secretion in MIN6 cells

Insulin secretion induced by efaroxan (100 µM) and IMZ (10 nM) were determined in MIN6 cells cultured in buffer containing glucose (7 mM) via ELISA. In addition, the effect of KU14R (1 µM) on insulin secretion induced by efaroxan and IMZ were determined in MIN6 cells cultured in buffer containing glucose (7 mM) via ELISA. Data are presented as fold change of vehicle treated control in MIN6 cells and mean ± SEM. P values were calculated using a two-way ANOVA or a two-way ANOVA for multiple comparisons, followed by Bonferroni’s post-test. (*** P < 0.0005, **** P < 0.0001 indicates a significant difference between drug treated and vehicle treated control MIN6 cells; #### P < 0.0001 indicates a significant difference between IMZ treated and IMZ treated in the presence of KU14R MIN6 cells; $ P < 0.05 indicates a significant difference between efaroxan treated and efaroxan treated in the presence of KU14R MIN6 cells).
Figure 2.7 Silencing nischarin muted IMZ induced insulin secretion in MIN6 cells

Representative immunoblots and western blot analysis of NISCH normalized to GAPDH in control siRNA (scramble) MIN6 cells and NISCH siRNA (si-NISCH, 25 nM) MIN6 cells (A). Insulin secretion induced by canavanine (10 µM) and IMZ (10 nM) were determined in scramble and si-NISCH MIN6 cells cultured in buffer containing glucose (7 mM) via ELISA (B). Data are presented as fold change of scramble control siRNA treated MIN6 cells and mean ± SEM. P values were calculated using a one-way ANOVA or a two-way ANOVA for multiple comparisons, followed by Bonferroni’s post-test. (** P < 0.005, * P < 0.05 indicates a significant difference between drug treated and vehicle treated scramble MIN6 cells; # P < 0.05 indicates a significant difference between IMZ treated scramble MIN6 cells and IMZ treated si-NISCH MIN6 cells; $ P < 0.05 indicates a significant difference between canavanine treated scramble MIN6 cells and canavanine treated si-NISCH MIN6 cells ).
2.4 Discussion

We previously identified and characterized a novel metformin-methylglyoxal derived imidazolinone metabolite in the plasma and urine of patients with T2DM. The chemical structure of IMZ and the awareness that all three imidazoline receptor subtypes appear to play an important role in regulating insulin sensitivity and/or insulin secretion raised the possibility that IMZ may be a fundamental element in MF’s antidiabetic action. Herein, we demonstrated that IMZ exerts pharmacological activity in PC12, insulin resistant HEPG2, and MIN6 cells. Thus, this study provides evidence of the potential intracellular signaling mechanisms behind IMZ in vitro and provides insight that MF’s therapeutic response may be mediated in part by the production of IMZ.

PC12 (pheochromocytoma) cells are the predominant in vitro model for assessing I₁R activation because they express a high density of I₁R binding sites and lack α₂- adrenergic receptors (α₂-AR), which many imidazoline compounds also interact with. The discovery and characterization of a functional I₁R protein, IRAS and the mouse homologue NISCH, lead to the understanding that imidazoline receptors are associated with insulin receptor substrates and play a role in activation of several components of the insulin signaling cascade. The present study was designed to explore whether IMZ has the ability to activate two key proteins in insulin signaling, AKT and ERK1/2, via I₁R stimulation at physiologically relevant concentrations. Our results reveal that IMZ treatment, in combination with insulin, was able to sensitize PC12 cells to
insulin in a concentration dependent manner via activation of AKT and ERK1/2. However, IMZ treatment alone did not increase phosphorylation of AKT and ERK1/2 in comparison to the I$_1$R agonist moxonidine (Figure 2.1). Combination treatment of IMZ and insulin in the presence of known I$_1$R antagonists, efaroxan and idazoxan, resulted in a reduction in insulin sensitization (Figure 2.2). Interestingly, knocking down NISCH via siRNA augmented insulin action, a finding consistent with recent reports that NISCH inhibition alters energy metabolism (Figure 2.3). Thus, our data is consistent with the fact that there is crosstalk between insulin and imidazoline receptor signaling and that the ability of IMZ to enhance insulin action in PC12 cells may be mediated through I$_1$R activation but does not conclusively establish I$_1$R activation via IMZ.\textsuperscript{26}

Insulin resistance underpins T2DM and is defined by insufficient cellular response to normal circulating insulin levels, which leads to irregular nutrient metabolism and glucose uptake in insulin sensitive tissues, including the liver. Resistance to insulin in hepatocytes reduces glycogen synthesis and results in hyperglycemia.\textsuperscript{162} Thus, eliminating insulin resistance by re-sensitizing liver cells to insulin could help improve T2DM. Numerous studies have demonstrated insulin resistant human liver HEPG2 cells as a reliable model for measuring liver metabolism and signaling at an insulin resistant state.\textsuperscript{153-155} Results from this study indicate that an insulin resistant HEPG2 cell line was successfully established via markedly decreased activation of AKT after insulin treatment (Figure 2.4A). In addition, we explored whether IMZ is able to sensitize insulin resistant HEPG2 cells to insulin. Our results indicate that treatment with 100 nM
IMZ in combination with insulin sensitized insulin resistant HEPG2 cells to insulin via modulation of AKT phosphorylation (Figure 2.4B). This data suggests that IMZ may have the ability to recover insulin resistance mediated through AKT activation. Moreover, this sensitization may be activated through either the $I_1\text{R}$ or $\alpha_2\text{AR}$ since HEPG2 cells express both receptors.

Insulin is secreted from pancreatic $\beta$-cells in response to an increase in plasma glucose levels to decrease glucose to physiological levels. Insulin resistance and prolonged hyperglycemia lead to $\beta$-cell dysfunction and loss of mass, which are critical to the development of T2DM. Several studies have shown that imidazoline compounds have $I_3\text{R}$ mediated insulinotropic properties in the MIN6 pancreatic $\beta$-cell line. Herein, we used MIN6 cells to investigate the ability of IMZ to stimulate insulin release and discover the possible intracellular mechanism. Findings from this study revealed that insulin secretion was increased in MIN6 cells exposed to various concentrations of IMZ to a similar level as the positive controls canavanine and efaroxan (Figure 2.5). Our results also indicate that IMZ induced insulin secretion was inhibited by the known $I_3\text{R}$ antagonist KU14R to a level similar to that by which KU14R inhibited efaroxan induced secretion (Figure 2.6). Furthermore, similar to canavanine, the secretagogue activity of IMZ was significantly reduced in MIN6 cells with siRNA-mediated NISCH knockdown (Figure 2.7). Thus, the ability of IMZ to induce insulin secretion in pancreatic $\beta$-cells may occur primarily through activation of $I_3\text{R}$. 
In summary, our results suggest that the ability of IMZ to sensitize PC12 cells to insulin and induce insulin secretion in MIN6 cells may be mediated through I₁R and I₃R, respectively. In addition, our findings show that IMZ is able to sensitize insulin resistant HEPG2 cells to insulin through modulation of AKT phosphorylation. However, due to the fact that HEPG2 cells express both α₂AR receptors and imidazoline receptors, the mechanism behind this pharmacological activity needs to be further explored. Taken together, the findings in the present study support the hypothesis that the formation of IMZ may contribute to the antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM. Although the mechanism(s) underlying the pharmacological activity of IMZ remain to be elucidated, the present study opens an intriguing window on the mechanism of the antidiabetic properties of MF.
3.1 Introduction

Diabetes is a chronic endocrine disorder, characterized by hyperglycemia and altered lipid metabolism resulting from irregular insulin secretion and/or insulin resistance. Due to modern changes in dietary habits and increases in a sedentary lifestyle, the global prevalence of diabetes has been increasing rapidly for decades, especially that of type 2 diabetes mellitus (T2DM) which accounts for ~95% of all diabetes cases.\textsuperscript{2,3,164} According to the International Diabetes Federation, there were an estimated 451 million people (18–99 years of age) worldwide with diabetes in 2017, and this figure was projected to increase to 693 million people by 2045.\textsuperscript{1} As a consequence of sustained hyperglycemia, complications such as pancreatic dysfunction, nonalcoholic fatty liver disease (NAFLD), abnormal lipid levels, retinal damage, renal failure, and cardiovascular disease are increasingly affecting patients with diabetes.\textsuperscript{6,165,166} Because of the variety and severity of diabetic complications, fully elucidating the pharmacological properties of current therapies could provide a rationale for an expansion of their clinical use.

Metformin (MF), the first-line therapy for T2DM, significantly reduces adverse diabetic endpoints and mortality more effectively than other anti-hyperglycemic agents, the mechanism(s) of which remain enigmatic.\textsuperscript{4,20,161,167}
Reactive dicarbonyls, such as methylglyoxal (MGO), are elevated in the plasma of patients with T2DM. These endogenous electrophiles covalently modify proteins, which may contribute to diabetic complications. MF is also unique in comparison to other glucose lowering drugs because it has been linked to lowering plasma levels of MGO and MGO-derived advanced glycation end products.\textsuperscript{11,21,22,25,53,65,72,168} We previously identified and characterized the product of the MF and MGO reaction as a novel imidazolinone metabolite (IMZ) in plasma and urine from patients with T2DM.\textsuperscript{24} Imidazoline receptors (I\textsubscript{1}R-I\textsubscript{3}R) are located in the brain and kidney as well as insulin sensitive tissues and are classically known to play a role in the sympathetic nervous system, cardiovascular function, monoamine turnover, and insulin secretion. However, more recently, all thee subtypes have been shown to play an important role in either maintaining glucose homeostasis, insulin sensitization, or insulin secretion. In addition, imidazolines show promise in not only reducing hypertension, but also improving glucose tolerance, reducing hyperinsulinemia, and decreasing insulin resistance.\textsuperscript{26-33,160} The drug-like structure of IMZ suggests it may play a role in MF’s ability to reduce diabetic complications, and might represent a key component in MF drug efficacy. Thus, the potential pharmacological activity of IMZ \textit{in vivo} warrants further investigation.

Herein, we explored the antidiabetic effects and possible intracellular signaling mechanisms engaged by IMZ in genetically obese db/db diabetic mice. More specifically, this study assessed the ability of IMZ to improve insulin resistance, pancreatic islet morphology and function, and adipocyte and renal
histopathological changes. In addition, the effects of IMZ on hepatic steatosis and lipogenesis mediated through sirtuin 1 (SIRT1) activation were explored. The present study describes for the first time the protective effects of IMZ \textit{in vivo} and provides insight into a possible new mechanism by which MF exerts its antidiabetic properties.
3.2 Materials and Methods

3.2.1 Compound

(E)-1,1-dimethyl-2-(5-methyl-4-oxo-4,5-dihydro-1H-imidazol-2-yl)guanidine (IMZ), a metformin-methylglyoxal metabolite, was synthesized in our laboratory as previously described by Kinsky et al.24

3.2.2 Animals and experimental design

Seven-week-old male mice homozygous for the diabetes spontaneous mutation (Lepr<sup>db</sup>, B6.BKS(D)-Lepr<sup>db</sup>/J, #000697) and age and sex matched healthy wild type (WT) mice (C57BL/6J, #000664) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under standard conditions with a 12-hour light-dark cycle, suitable relative humidity and temperature, and with water and standard mouse chow ad libitum. All animal experimentation described in the present study were performed in accordance with Wayne State University Institutional Animal Care and Use Committee guidelines (#IACUC-17-11-0416).

After one week of acclimatization, mice (8 weeks of age) were randomly assigned into the following three groups: WT control group (C57BL/6, n = 6), control db/db group (db/db, n = 6), and IMZ treated db/db group (db/db+IMZ, n
The db/db+IMZ group was treated with 20 mg/kg IMZ (dissolved in phosphate-buffered saline (PBS)) twice a day for seven days via intraperitoneal injection, and the C57BL/6 and db/db control groups were treated with PBS correspondingly. The dosage of IMZ was given based on a preliminary pharmacokinetic pilot study conducted in our laboratory (data not shown). After fasting mice for 6 hrs., fasting blood glucose was measured from tail vein blood using a standard blood glucose meter and body weight was recorded daily. On day seven of treatment, all animals were euthanized forty minutes after their last treatment with either PBS or IMZ. Whole blood was collected, serum was isolated and stored at -80 °C for biochemical analysis. Kidney, liver, pancreas, and adipose tissue were isolated, rinsed with PBS, and stored in two portions: half of each tissue was immediately frozen in liquid nitrogen and stored at -80 °C for western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analysis, and the other half of each tissue was immediately fixed in 4% paraformaldehyde (PFA; Santa Cruz Biotechnology, Inc. (SCB), Dallas, TX, USA) overnight at 4°C for histological analysis.

3.2.3 Biochemical analysis

Whole blood was used to measure fasting blood glucose via a glucometer (Contour-Next Bayer, Parsippany, NJ, USA) and glycated hemoglobin (HbA1c; Crystal Chem, Elk Grove Village, IL, USA) using a commercial kit according to the manufacturer’s instructions. Heparin-containing whole blood was centrifuged at
13,000 rpm for 10 min at 4°C to obtain plasma. Fasting plasma levels of glucose (Invitrogen, Carlsbad, CA, USA) insulin (Mercodia, Uppsala, Sweden), free fatty acids (FFA), triglycerides (TG), and total cholesterol (TC) (Abcam, Cambridge, MA, USA) were measured using commercial colorimetric or enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions. Homeostasis model assessment of insulin resistance (HOMA-IR) value was calculated using the following formula: HOMA-IR = fasting plasma insulin (mU/L) \times \text{fasting blood glucose (mg/dL)} / 405.

### 3.2.4 Histological analysis

PFA fixed kidney, liver, pancreas, and adipose tissue was embedded in paraffin, sectioned at 5 µm, and mounted onto glass slides. Sectioned tissue slides were then processed and stained with hematoxylin and eosin (H&E; Sigma-Aldrich) using standard lab protocols. For immunohistochemistry (IHC) analysis of insulin, pancreatic tissue paraffin sections were deparaffinized with a xylene-ethanol gradient. Endogenous peroxides were removed by a methanol/1.2% hydrogen peroxide solution and heat-induced epitope antigen retrieval was performed with a citrate buffer (pH 6.0) and Decloaking Chamber™ (Biocare Medical, Pacheco, CA, USA), followed by blocking with SuperBlock™ blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA) for forty minutes prior to adding the primary antibody. After rinsing with PBS, sections were immunostained with insulin (1:1000) overnight (Abcam, ab181547). Detection
was obtained using a 3,3’-diaminobenzidine chromogen kit (GBI Labs, Bothell, WA, USA) and sections were counterstained with hematoxylin. In order to perform Oil red O (ORO) staining, liver tissue was frozen in Fisher Healthcare™ Tissue-Plus™ O.C.T. compound (Thermo Fisher Scientific) at -20°C for forty-five minutes. Sections of 5 µm thickness were mounted onto slides and then fixed in 10% neutral buffered formalin (Sigma-Aldrich) for one minute at room temperature (RT). ORO staining was performed as suggested by the manufacturer’s instructions (Abcam) and slides were counterstained with hematoxylin. All histology images were acquired using an Invitrogen™ EVOS™ FL Auto Imaging System (Thermo Fisher Scientific) and analyzed using ImageJ software (NIH, Bethesda, Maryland, USA).

3.2.5 Western blot analysis

Tissue lysates were generated using ice cold Cell Lysis Buffer 10X (Cell Signaling Technologies (CST), Danvers, MA, USA) containing 1 mM Pefabloc® SC, cOmplete™ protease inhibitor cocktail, and PhosSTOP™ phosphatase inhibitor cocktail (Roche, South San Francisco, CA, USA). Concentrations of protein were determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). Protein (40-100 µg) was separated on an 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA) for 2 hrs. at 50 volts in 4°C. PVDF membranes were blocked in Odyssey® Blocking
Buffer in tris-buffered saline (LI-COR, Lincoln, NE, USA) for 1 hr. at RT, followed by incubation with specific primary antibodies, diluted 1:500 or 1:1000 in 5% bovine serum albumin, at 4°C overnight. Primary antibodies used were as follows: sirtuin 1 (SIRT1, CST, 8469S), acetyl-CoA carboxylase (ACC, CST, 3676S), fatty acid synthase (FAS, CST, 3180S), sterol regulatory element-binding protein 1 (SREBP1, SCB, sc-13551), carbohydrate-response element-binding protein (ChREBP, SCB, sc-515922), and Vinculin (CST, 13901S; SCB, sc-73614). Membranes were then incubated with corresponding near-infrared fluorescent secondary antibodies (LI-COR), diluted in blocking buffer 1:15000, for 1 hr. at RT. Targeted proteins were detected using an Odyssey® FC imaging system and band intensity was analyzed using Image Studio Lite Ver 5.2 (LI-COR).

3.2.6 RNA extraction and quantitative real time PCR analysis

Frozen liver tissue was lysed and ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer’s instructions. RNA concentration and purity were determined using the Take3™ Micro-Volume Plate and Gen5 quantification software (BioTek Instruments, Winooski, VT, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 µg of total RNA using the Applied Biosystems™ TaqMan™ High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Quantitative RT-PCR was conducted using 20 ng of cDNA and the Applied Biosystems™ TaqMan™ Gene Expression Master Mix (Thermo
Fisher Scientific) for each reaction. The following qRT-PCR amplification conditions were used: 48°C for 15 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were run in triplicate on the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) by the Applied Genomics Technology Center at Wayne State University, School of Medicine. Predesigned TaqMan® Gene Expression Assays (Thermo Fisher Scientific) comprised of gene specific primers and fluorescent dye labeled minor groove binding probes were used for the following genes: SIRT1 (Mm00490758_m1), sterol regulatory element binding transcription factor 1 (SREBF1, Mm00550338_m1), MLX interacting protein like (MLXIPL, Mm02342723_m1), fatty acid synthase (FASN, Mm00662319_m1), acetyl-CoA carboxylase alpha (ACACA, Mm01304257_m1), actin beta (ACTB, Mm00607939_s1), and ribosomal RNA 18s (Rn18s, Mm03928990_g1). Relative mRNA expression fold changes were normalized to the housekeeping genes Rn18s and Actb and quantified using the $2^{-\Delta\Delta CT}$ method.

3.2.7 Statistical analysis

All data were analyzed using GraphPad Prism 5 software (San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). A one-way ANOVA followed by Bonferroni’s post-test was used for analysis. A p-value less than 0.05 was considered statistically significant at the 95% confidence level.
3.3 Results

3.3.1 IMZ improved insulin resistance in db/db mice after 7 days of treatment

Fasting blood glucose levels of db/db control mice were significantly higher than in the C57BL/6 WT mice, but short-term IMZ treatment of db/db mice had no effect on their blood glucose levels (Figure 3.1A). Compared to C57BL/6 control mice, HbA1c and fasting blood glucose in db/db control mice were drastically higher; however, IMZ treatment of db/db mice only slightly decreased fasting blood glucose and HbA1c in comparison to control db/db mice (Figure 3.1B, C). Fasting plasma insulin and HOMA-IR in db/db mice were markedly elevated compared to C57BL/6 WT controls. Interestingly, plasma insulin levels and HOMA-IR were significantly reduced after administration of IMZ to db/db mice compared to untreated db/db control mice (Figure 3.1D, E).
Figure 3.1 IMZ improved insulin resistance in db/db mice after 7 days of treatment

Fasting blood glucose taken from the tail vein of C57BL/6 (white squares, n = 6), db/db (black squares, n = 6), and IMZ treated db/db mice (gray squares, n = 10) was determined daily by a blood glucose meter (A). Biochemical analysis of HbA1c (B), fasting plasma glucose (C), and fasting plasma insulin (D) of C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), and IMZ treated db/db mice (gray bars, n = 10) was measured via a colorimetric kit or ELISA. HOMA-IR (E) was calculated using the following formula: HOMA-IR = fasting insulin (mU/L) x fasting glucose (mg/dL) \( /405 \). Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (*** P < 0.0005, ** P < 0.005 indicates a significant difference between db/db and C57BL/6 mice; ## P < 0.005, # P < 0.05 indicates a significant difference between db/db and IMZ treated db/db mice).
3.3.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 7 days of treatment

H&E staining of pancreatic tissue from the three groups of animals displayed differences in the size of the pancreatic islets (Figure 3.2A). These images revealed that the pancreatic islets of the db/db model control group were irregular in shape, hypertrophic, and lacked organization of islet cells when compared to the WT C57BL/6 control group. However, administration of IMZ to db/db mice for seven days reversed the abnormal histopathologic changes of pancreatic islets. The corresponding average pancreatic islet areas were calculated (Figure 3.2C). In comparison to the C57BL/6 control mice, the average islet area of the db/db control mice was significantly larger. However, IMZ treatment significantly decreased pancreatic islet area in db/db mice to an area indistinguishable from that of the C57BL/6 mice. IHC staining of insulin (Figure 3.2B) revealed insulin storage in pancreatic islets was considerably decreased in db/db mice in comparison to C57BL/6 mice. Conversely, the administration of IMZ ameliorated these morphological changes in pancreatic islets. The insulin intensity in pancreatic islets was calculated to semi-quantify insulin storage in islets (Figure 3.2D). Db/db mice displayed a significant decrease in insulin intensity in comparison to C57BL/6 mice, and this decrease was reversed by IMZ treatment of db/db mice.
Figure 3.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 7 days of treatment

Representative images of hematoxylin and eosin (H&E) staining (A) and immunohistochemical (IHC) staining for insulin (B) in consecutive cross sections of pancreatic islets from C57BL/6, db/db, and IMZ treated db/db mice. Magnification: 40x, scale bar: 100 µm. Pancreatic islet area (C) and insulin stain intensity (D) of C57BL/6 (white bars), db/db (black bars), and IMZ treated db/db mice (gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group; *** P < 0.0005, ** P < 0.005 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005, ## P < 0.005 indicates a significant difference between db/db and IMZ treated db/db mice).
3.3.3 IMZ reduced adipocyte size in EWAT from db/db mice after 7 days of treatment

Epididymal white adipose tissue (EWAT) stained with H&E from the three groups of mice showed differences in the size of epididymal adipocytes (Figure 3.3A, B) revealing exacerbated epididymal adipocyte inflammation and hypertrophy in the db/db model control group compared to the WT C57BL/6 mice. However, seven-day treatment with IMZ alleviated these morphological changes in adipocytes in db/db mice. Average adipocyte area and cell count were quantified (Figure 3.3C, D). Histological analysis showed that the average adipocyte area of the db/db control mice was significantly larger, and the average cell count significantly lower compared to the C57BL/6 mice. Remarkably, IMZ treatment significantly decreased adipocyte size and significantly increased cell number in db/db mice.

3.3.4 IMZ alleviated renal histopathological changes in db/db mice after 7 days of treatment

H&E staining of renal tissue from the three groups of animals presented differences in the size of the glomerulus (Figure 3.4A). The images revealed that glomerular hypertrophy and inflammation were increased in the db/db model control group in comparison to the WT C57BL/6 group. IMZ administration reversed these renal histopathological changes in db/db mice. Average
glomerular area, Bowman’s space area, and proximal tubule area were quantified (Figure 3.4B, C, D). The db/db group displayed a significantly larger average glomerulus, Bowman’s space, and proximal tubule area than that of the C57BL/6 WT mice, and IMZ treatment significantly reversed these changes to a nearly healthy state.
Figure 3.3 IMZ reduced adipocyte size in EWAT from db/db mice after 7 days of treatment

Representative images of hematoxylin and eosin (H&E) staining of epididymal white adipose tissue (EWAT) from C57BL/6, db/db, and IMZ treated db/db mice. Magnification: 10x, scale bar: 400 µm (A). Magnification: 20x, scale bar: 200 µm (B). Adipocyte area (C) and cell count (D) of C57BL/6 (white bars), db/db (black bars), and IMZ treated db/db mice (gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group; *** P < 0.0005 indicates a significant difference between db/db and C57BL/6 mice; #### P < 0.0005, ## P < 0.005 indicates a significant difference between db/db and IMZ treated db/db mice).
Figure 3.4 IMZ alleviated renal histopathological changes in db/db mice after 7 days of treatment

Representative images of hematoxylin and eosin (H&E) staining of renal tissue from C57BL/6, db/db, and IMZ treated db/db mice (A). Magnification: 40x, scale bar: 100 µm. Glomerular area (B), Bowman’s space area (C), and proximal tubule area (D) from C57BL/6 (white bars), db/db (black bars), and IMZ treated db/db mice (gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group ** P < 0.005 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005, ## P < 0.005 indicates a significant difference between db/db and IMZ treated db/db mice).
3.3.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 7 days of treatment

Histological analyses of H&E stained liver sections from the three groups of mice revealed excessive lipid droplets in the db/db control group in comparison to the C57BL/6 group and was strikingly ameliorated by IMZ treatment (Figure 3.5A). Consistent with the H&E staining results, oil red O (ORO) staining displayed decreased lipid accumulation in db/db mice treated with IMZ in comparison to db/db control mice (Figure 3.5B). In addition, IMZ treatment slightly decreased plasma free fatty acid (FFA) and triglyceride (TG) plasma levels, although not statistically significant (Figure 3.5C, D). However, total cholesterol (TC) plasma levels were significantly decreased in IMZ treated db/db mice in comparison to db/db control mice.

3.3.6 IMZ decreased hepatic markers of lipogenesis in db/db mice after 7 days of treatment

Western blot analysis revealed a significant decrease in SIRT1 relative protein levels in the db/db control mice in comparison to C57BL/6 WT mice, whereas SIRT1 protein levels were significantly increased in IMZ treated db/db mice compared to db/db controls. In addition, IMZ treatment significantly reduced protein levels of the lipogenic markers SREBP1, ChREBP, ACC, and FAS in comparison to db/db mice, which were significantly increased in control db/db
mice in comparison to the C57BL/6 mice (Figure 3.6 A, B). Consistent with the western blot analysis, relative mRNA levels displayed significantly decreased gene expression of the lipogenic genes SREBF1, MLXIP1, ACACA, and FASN in db/db mice treated with IMZ in comparison to db/db control mice (Figure 3.6C).
Figure 3.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 7 days of treatment

Representative images of hematoxylin and eosin (H&E) and Oil red O (ORO) staining of hepatic tissue from C57BL/6, db/db, and IMZ treated db/db mice. Magnification: 20x, scale bar: 200 µm (A). Magnification: 40x, scale bar: 100 µm (B). Plasma FFA (C), TG (D), and TC (E) levels from C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), and IMZ treated db/db mice (gray bars, n = 10) was measured with a commercial colorimetric kit. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (*** P < 0.0005, * P < 0.05 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005 indicates a significant difference between db/db and IMZ treated db/db mice).
Figure 3.6 IMZ decreased hepatic markers of lipogenesis in db/db mice after 7 days of treatment

Representative immunoblots of SIRT1, SREBP1, ChREBP1, ACC, FAS, and vinculin in the liver from C57BL/6, db/db, and IMZ treated db/db mice (A). Western blot analysis of the relative protein levels in liver tissue from C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), and IMZ treated db/db mice (gray bars, n = 10) was quantified using Image Studio Lite and normalized to vinculin (B). Relative mRNA levels in liver tissue from C57BL/6 (white bars, n = 3), db/db (black bars, n = 3), and IMZ treated db/db mice (gray bars, n = 3) were detected by qRT-PCR, normalized to the housekeeping genes Rn18s and Actb, and quantified using the 2^{-ΔΔCT} method (C). Data are presented as fold change of the C57BL/6 control group and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (* P < 0.05, ** P < 0.005, *** P < 0.0005 indicates a significant difference between db/db and C57BL/6 mice; # P < 0.05, ## P < 0.005, ### P < 0.0005 indicates a significant difference between db/db and IMZ treated db/db mice).
3.4 Discussion

We recently identified and characterized a metformin-methylglyoxal metabolite (IMZ) in T2DM patient urine and plasma. The drug-like structure of IMZ and the knowledge that all three imidazoline receptor subtypes appear to have a functional role in regulating insulin sensitivity and/or insulin secretion, raised the possibility that IMZ may contribute to the antidiabetic action of MF. Herein we demonstrate that IMZ possesses pharmacological activity in diabetic db/db mice, and thereby provide evidence that the therapeutic efficacy of MF may be mediated in part via the formation of IMZ.

T2DM is a complex metabolic disorder caused by either the abnormal secretion of insulin from the pancreas and/or by the body not effectively utilizing/responding to circulating insulin, the consequences of which are a prolonged hyperglycemia, obesity, and numerous severe diabetic complications. The db/db mouse model is a genetically derived diabetic animal caused by mutations in the gene that encodes for the leptin receptor. Due to the lack of leptin signaling, the pathogenesis of T2DM in db/db mice exhibits metabolic characteristics that are comparable to human T2DM, such as hyperinsulinemia, hyperglycemia, obesity, and dyslipidemia. Thus, in recent years, db/db mice have been widely used as an accepted animal model to replicate human T2DM.

To investigate the possible biological effects of IMZ on T2DM in vivo, db/db mice were treated with IMZ twice a day for 7 days. Measuring fasting blood
glucose levels, fasting plasma insulin levels, and calculating the HOMA-IR index are commonly used methods for clinical assessment of insulin action in vivo. Our initial data showed that IMZ reduced fasting plasma insulin levels and HOMA-IR value to levels comparable to that of the healthy WT C57BL/6 mice, although IMZ did not effectively reduce blood glucose or HbA1c in db/db mice (Figure 3.1). Moreover, a vital therapeutic approach for treating diabetes is reversing pancreatic islet dysfunction, because islet beta cell damage plays a crucial role in the pathogenesis of diabetes. Histopathological assessment of the pancreas revealed that IMZ completely reversed islet hypertrophy, and increased islet insulin IHC staining to a level indistinguishable from that of WT C57BL/6 mice (Figure 3.2). These results indicate that IMZ has pancreatic protective effects and the ability to reduce hyperinsulinemia, insulin resistance, pancreatic dysfunction, and enhance insulin sensitivity in db/db mice.

Inflammation and dysregulation of adipose tissue also contribute to the progression of insulin resistance and T2DM. Our results revealed that IMZ successfully reduced adipocyte area, cell number, and the morphology of adipocytes in EWAT from db/db mice (Figure 3.3), which is consistent with a reduction in insulin resistance. The conventional view of insulin action focuses on studies in insulin-sensitive tissues such as the pancreas, skeletal muscle, adipose tissue, and the liver. However, insulin also has an effect on kidney tissue that regulates growth, hypertrophy, as well as microcirculatory pathways, which in turn, impact glomerular function. Our data reveals that IMZ effectively reduced glomerular, Bowman’s space, and proximal tubule area (Figure 3.4) in
db/db mice to a level similar to that of healthy WT C57BL/6 mice. Together these results suggest that IMZ is able to improve EWAT and renal tissue histopathological changes that occur in db/db mice.

T2DM is also accompanied by disorders in lipid metabolism, including obesity, dyslipidemia, and NAFLD. FFA, TG, and TC accumulation increase the risk of insulin resistance and contribute to the pathogenesis of NAFLD, which is considered an overlooked complication of T2DM. Further, dyslipidemia is a key contributor to cardiovascular disease, the leading fatal complication of diabetes.\textsuperscript{166,182,183} Thus, maintaining lipid homeostasis is an effective way to relieve symptoms of diabetes. In this study, IMZ significantly reduced plasma levels of TC and slightly reduced plasma levels of FFA and TG. In addition, histopathological examination of the liver revealed that IMZ suppressed lipid accumulation and reduced hypertrophy of hepatocytes (Figure 3.5) in db/db mice. Excess lipid accumulation in hepatocytes results in NAFLD, which has been linked to increased hepatic expression of several transcription factors involved in lipogenesis, such as SREBF1, MLXIPL, ACACA, and FASN.\textsuperscript{184,185} The activation of SIRT1 inhibits lipogenesis via deactivation of SREBP1 and ChREBP and has been shown to play a beneficial role in obesity, insulin resistance, T2DM, and NAFLD.\textsuperscript{186} Our db/db mice developed apparent hepatic steatosis, accompanied by the downregulation of SIRT1 expression in the liver, and subsequent upregulation of the lipogenic markers SREBP1, ChREBP, ACC, and FAS expression at the protein and mRNA level. Importantly, IMZ treatment significantly increased SIRT1 and inhibited SREBP1, ChREBP, ACC, and FAS expression at the protein and mRNA
level (Figure 3.6). This data is consistent with the fact that upregulation of SIRT1 expression can successfully induce antidiabetic effects.\textsuperscript{186,187} Thus, these results reveal that IMZ suppressed hepatic steatosis, lipogenesis, and has ability to control lipid metabolism disorders in db/db mice, which may be due to the activation of SIRT1.

In summary, IMZ significantly alleviated insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes in db/db mice treated for 7 days. IMZ promoted hepatic SIRT1 expression and downregulation of SREBP1, ChREBP, ACC, and FAS at the protein and mRNA level in db/db mice, which may contribute to the antidiabetic effects exerted by MF, which itself can ameliorate hyperglycemia, dyslipidemia, hepatic steatosis, insulin resistance, and lower microvascular and macrovascular complications associated with T2DM.\textsuperscript{4,20,161,167} For such a relatively simple molecule, MF exhibits an intriguing array of biological effects, the mechanism(s) of which remain unclear. Recent studies have shown that imidazolines can alleviate T2DM symptoms by reducing insulin resistance, decreasing blood glucose levels, potentiating insulin secretion, improving pancreatic β-cell function, and protecting against cardio-metabolic disorders. In addition, Imidazoline receptors are considered novel targets for drug development in disorders associated with T2DM because they are involved in insulin secretion/sensitization, glucose homeostasis, and in ameliorating pancreatic damage.\textsuperscript{26-33,160} Taken together, the findings in the present study support the hypothesis that the formation of IMZ may contribute to the
antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM. Although the mechanism(s) underlying the pharmacological activity of IMZ remain to be elucidated, the present study opens an intriguing window on the mechanism of the antidiabetic properties of MF.
Chapter 4: Long Term Treatment of a Metformin-methylglyoxal Imidazolinone Metabolite Improves Metabolic Anomalies in db/db Mice

4.1 Introduction

The number of patients with diabetes is continually increasing in prevalence. Currently, 30.3 million people in the United States have diabetes. In addition, there are an estimated 451 million people with diabetes worldwide, which is predicted to increase to 693 million by 2045. Among all diabetes cases, \(-95\%\) are comprised of type 2 diabetes mellitus (T2DM). T2DM is a progressive and chronic metabolic disorder, caused by impairments in insulin action and/or insulin secretion, lipid metabolism, and glucose homeostasis. Complications from T2DM have made the disease one of the most prevalent public health issues, due to increased morbidity and mortality. Thus, understanding the pharmacological basis of current diabetic therapies could aid in development of their clinical utility.

Metformin (MF) has been prescribed as an antihyperglycemic for several decades and is now recommended by the American Diabetes Association and the European Association for the Study of Diabetes as the first-line treatment for patients with T2DM. These clinical guidelines are based on meta-analysis, prospective studies, and are reinforced by MF’s ability to maintain glycemic control and reduce cardiovascular related death without weight gain or hypoglycemic events. In addition, MF has been referred to as the aspirin of the
21st century and is the preferred antidiabetic therapy due to its minimal cost and tolerability without toxicity.\(^4,161,188\)

In the clinic, MF notably decreases adverse diabetic endpoints and death more successfully than other glucose lowering drugs.\(^20\) Although MF has been used for the treatment of T2DM for over 60 years, its pleiotropic action remains elusive. MF is also unique in comparison to other antihyperglycemic drugs because it has been associated with lowering plasma levels of methylglyoxal (MGO) and MGO-derived advanced glycation end products, which contribute to the progression of diabetic complications.\(^21,22,86\)

We formerly investigated the MGO scavenging properties of MF and identified and characterized, in T2DM patient plasma and urine, the reaction product of as a novel imidazolinone metabolite (IMZ).\(^24\) Imidazolines are classically known for reducing hypertension, but currently have been showing promise in also improving glucose tolerance, reducing hyperinsulinemia, and decreasing insulin resistance. In addition, imidazoline receptors (I\(_1\)R-I\(_3\)R) are located in insulin sensitive tissues, the brain, and the kidney; they are traditionally recognized as being involved in insulin secretion, the sympathetic nervous system, monoamine turnover, and cardiovascular function. However, all three receptor subtypes are implicated in playing a fundamental role in insulin sensitization, insulin secretion, and the maintenance of glucose homeostasis.\(^2,6,26,27,29-33,160\) Moreover, MF increases glucose uptake and reduces hyperglycemia through activation of imidazoline receptors in \textit{in vitro} and \textit{in vivo} models.\(^34,35\) The drug-like chemical composition of IMZ implies that it may be
essential to the blood glucose lowering activity of MF and the ability of MF to reduce diabetic complications. Thus, the probable pharmacological properties of long-term treatment of IMZ in vivo deserves further exploration.

In the present study, we investigated the protective effects and potential mechanisms of activity exerted by IMZ in the well-established genetically obese db/db diabetic mouse model alongside a clinically relevant dose of MF. In particular, this study evaluated the ability of IMZ to recover hyperglycemia, insulin resistance, irregular pancreatic islet morphology and function, and adipocyte and renal histopathological abnormalities similarly to MF. In addition, the effects of IMZ on markers of hepatic steatosis, lipogenesis, and fibrosis were studied. Herein, we describe for the first time the antidiabetic properties of long-term IMZ treatment in vivo and offer awareness into a potential innovative mechanism behind the protective effects of MF.
4.2 Materials and Methods

4.2.1 Compounds

The synthesis of a metformin-methylglyoxal metabolite, (E)-1,1-dimethyl-2-(5-methyl-4-oxo-4,5-dihydro-1H-imidazol-2-yl)guanidine (IMZ), was performed in our laboratory as previously described by Kinsky et al. 1,1-Dimethylbiguanide, hydrochloride (metformin) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

4.2.2 Animals and experimental design

Male mice (7 weeks of age) homozygous for the diabetes spontaneous mutation (Lepr\textsuperscript{db}, B6.BKS(D)-Lepr\textsuperscript{db}/J, #000697) and same sex/age healthy wild type (WT) mice (C57BL/6J, #000664) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained on a 12-hour light/dark cycle, with suitable relative humidity and temperature, and with standard mouse chow and water ad libitum. All animal experimentation described in the present study were approved by the Wayne State University Institutional Animal Care and Use Committee and performed in accordance to their ethical animal care and use guidelines (approval# IACUC-17-11-0416).
Mice were allowed to acclimate for 1 week and then were randomly assigned into the following groups: WT control group (C57BL/6, n = 6), control db/db group (db/db, n = 6), IMZ treated db/db group (db/db+IMZ, n = 6), and MF treated db/db group (db/db+MF, n = 4). The db/db+MF group was treated with a comparable dose to that of humans (250 mg/kg/day) for 60 days via intraperitoneal (IP) injection and used as a positive control. The db/db+IMZ group was treated twice a day with 20 mg/kg IMZ (dissolved in phosphate-buffered saline (PBS)) for 60 days by IP injection, and the C57BL/6 and db/db control groups were treated with PBS correspondingly. The dosage of IMZ was based on a preliminary pharmacokinetic pilot study conducted in our laboratory (data not shown). Fasting blood glucose was measured from tail vein blood using a standard blood glucose meter (Contour-Next Bayer, Parsippany, NJ, USA) and body weight was recorded weekly after 6 hrs. of fasting. At the end of the study, all animals were euthanized after their last treatment with either PBS, IMZ, or MF. Plasma was isolated from collected whole blood and both were stored at -80°C for biochemical analysis. Kidney, liver, pancreas, and adipose tissue were isolated, rinsed with PBS, and stored in two portions: half of each tissue was immediately fixed in 4% paraformaldehyde (PFA; Santa Cruz Biotechnology, Inc. (SCB)) overnight at 4°C for histological analysis and the other half of each tissue was rapidly frozen in liquid nitrogen and stored at -80°C for western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.
4.2.3 Biochemical analysis

Glycated hemoglobin (HbA1c; Crystal Chem, Elk Grove Village, IL, USA) was measured from whole blood using a commercial kit according to the manufacturer’s instructions. Plasma was obtained from heparin-containing whole blood by centrifugation at 13,000 rpm for 10 min. at 4°C. Insulin (Mercodia, Uppsala, Sweden), alanine transaminase (ALT) activity, Free fatty acids (FFA), triglycerides (TG), and total cholesterol (TC) (Abcam, Cambridge, MA, USA) were measured using commercial colorimetric or enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions. Homeostasis model assessment of insulin resistance (HOMA-IR) value was determine by the following formula: HOMA-IR = fasting plasma insulin (mU/L) x fasting blood glucose (mg/dL) /405.

4.2.4 Histological analysis

Kidney, liver, pancreas, and adipose tissue was fixed with PFA, embedded in paraffin, sectioned at 5 μm, and mounted onto glass slides. Slides were then processed and stained with hematoxylin and eosin (H&E; Sigma-Aldrich) using common laboratory procedures. For analysis of pancreatic insulin via immunohistochemistry (IHC), pancreatic tissue paraffin sections were deparaffinized, endogenous peroxides were removed, and heat-induced epitope
antigen retrieval was performed, followed by blocking with SuperBlock™ blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 40 min. prior to adding the primary antibody. After rinsing with PBS, sections were then immunostained overnight with insulin (1:1000, Abcam, ab181547). Sections were counterstained with hematoxylin and detection was achieved using a 3,3’-diaminobenzidine chromogen kit (GBI Labs, Bothell, WA, USA).

4.2.5 Western blot analysis

Ice-cold Cell Lysis Buffer 10X (Cell Signaling Technologies (CST), Danvers, MA, USA) containing 1 mM Pefabloc® SC, cOmplete™ protease inhibitor cocktail, and PhosSTOP™ phosphatase inhibitor cocktail (Roche, South San Francisco, CA, USA) was used to produce tissue lysates. Protein concentrations were determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA), 40-100 µg was separated on an 8, 10, or 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred to Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA) for 2 hrs. at 50 volts in 4°C. PVDF membranes were blocked for 1 hr. at RT in Odyssey® Blocking Buffer in tris-buffered saline (LI-COR, Lincoln, NE, USA), followed by incubation at 4°C overnight with specific primary antibodies, diluted 1:500 or 1:1000 in 5% bovine serum albumin. Primary antibodies used were as follows: acetyl-CoA carboxylase (ACC, CST, 3676S), fatty acid synthase (FAS,
CST, 3180S), fibronectin (abcam, ab2413), alpha-1 type 1 collagen (COL1A1, CST, 843365S), transforming growth factor beta (TGFB, CST, 3711S), and Vinculin (CST, 13901S; SCB, sc-73614). Membranes were then incubated for 1 hr. at RT with corresponding near-infrared fluorescent secondary antibodies (LI-COR), diluted in blocking buffer 1:15000. The Odyssey® FC imaging system was used to detect targeted proteins and band intensity was analyzed using Image Studio Lite Ver 5.2 (LI-COR).

4.2.6 RNA extraction and quantitative real time PCR analysis

Ribonucleic acid (RNA) was extracted from liver tissue lysate using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer’s instructions. The Take3™ Micro-Volume Plate and Gen5 quantification software (BioTek Instruments, Winooski, VT, USA) was used to determine RNA concentration and purity. Total RNA (1 µg) was used to synthesize complementary deoxyribonucleic acid (cDNA) using the Applied Biosystems™ TaqMan™ High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Quantitative RT-PCR was conducted using the Applied Biosystems™ TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific) and 20 ng of cDNA for each reaction. The following qRT-PCR amplification conditions were used: 48°C for 15 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) by the Applied Genomics Technology
Center at Wayne State University, School of Medicine was used for analysis and all reactions were run in triplicate. Predesigned TaqMan® Gene Expression Assays (Thermo Fisher Scientific) comprised of gene specific primers and fluorescent dye labeled minor groove binding probes were used for the following genes: fatty acid synthase (FASN, Mm00662319_m1), acetyl-CoA aarboxylase alpha (ACACA, Mm01304257_m1), FN1 (Mm01256744_m1), COL1A1 (Mm00801666_g1), TGFβ1 (Mm01178820_m1), actin beta (ACTB, Mm00607939_s1), and ribosomal RNA 18s (Rn18s, Mm03928990_g1). The housekeeping genes Rn18s and Actb were used for normalization and the relative mRNA expression fold changes were quantified using the $2^{-\Delta\Delta CT}$ method.

4.2.7 Statistical analysis

GraphPad Prism 5 software (San Diego, CA, USA) was used for statistical analysis and data are expressed as mean ± standard error of mean (SEM). A one-way ANOVA followed by Bonferroni’s post-test was used to examine statistical differences between groups. A $p$-value less than 0.05 was considered statistically significant at the 95% confidence level.
4.3 Results

4.3.1 IMZ improved insulin resistance and hyperglycemia in db/db mice after 60 days of treatment

Body weight of control model db/db mice was significantly elevated in comparison to control WT C57BL/6 mice throughout the entire study. Db/db mice treated with MF were significantly lower in weight than control db/db mice from 4-8 weeks of the study. However, IMZ treatment of db/db mice only slightly decreased body weight in comparison to db/db control mice (Figure 4.1A). Fasting blood glucose levels of MF treated db/db mice were significantly lower than in the db/db control mice from 2-8 weeks of the study and IMZ treatment of db/db mice significantly reduced blood glucose levels from 6-8 weeks (Figure 4.1B). Compared to control db/db mice, HbA1c was drastically lower in db/db mice dosed with MF; however, IMZ treatment of db/db mice only slightly decreased HbA1c in comparison to control db/db mice (Figure 4.1C). Fasting plasma insulin and HOMA-IR in db/db mice were markedly elevated compared to C57BL/6 WT controls. Interestingly, plasma insulin levels and HOMA-IR were significantly reduced after administration of IMZ or MF to db/db mice compared to untreated db/db control mice (Figure 4.1D, E).
Figure 4.1 IMZ improved insulin resistance and hyperglycemia in db/db mice after 60 days of treatment

Weight (A) and fasting blood glucose (B) taken from the tail vein of C57BL/6 (white squares, n = 6), db/db (black squares, n = 6), IMZ treated db/db mice (gray squares, n = 6), and MF treated db/db mice (dark gray bars, n = 4) was determined daily. Biochemical analysis of HbA1c (C) and fasting plasma insulin (D) of C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), IMZ treated db/db (gray bars, n = 10), and MF treated db/db mice (dark gray bars, n = 4) was measured via a colorimetric kit or ELISA. HOMA-IR (E) was calculated using the following formula: HOMA-IR = fasting insulin (mU/L) x fasting glucose (mg/dL) /405. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (**** P < 0.0001, *** P < 0.0005 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005 indicates a significant difference between db/db and IMZ treated db/db mice; $$$$ P < 0.0001, $$ P < 0.0005 indicates a significant difference between db/db and MF treated db/db mice ).
4.3.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 60 days of treatment

Pancreatic tissue stained with H&E from the four groups of animals displayed changes in the size of the pancreatic islets (Figure 4.2A). These images showed that the db/db model control group’s pancreatic islets were abnormal in shape, hypertrophic, and lacked organization of islet cells when compared to the WT C57BL/6 control group. However, administration of IMZ or MF to db/db mice for 60 days reversed the irregular histopathologic alterations of pancreatic islets. The average area of pancreatic islets were quantified (Figure 4.2C). This data revealed that the average islet area of the db/db control mice was significantly larger than the C57BL/6 control mice. Pancreatic islet area in db/db mice treated with IMZ or MF was significantly reduced to an area comparable to that of the C57BL/6 mice. Insulin IHC staining of the pancreas (Figure 4.2B) demonstrated insulin storage in pancreatic islets was considerably decreased in db/db mice in comparison to C57BL/6 mice. Conversely, the administration of IMZ or MF alleviated these morphological changes in pancreatic islets. The insulin intensity in pancreatic islets was calculated to semi-quantify insulin storage in islets (Figure 4.2D). Db/db mice displayed a significant reduction in insulin intensity in comparison to C57BL/6 mice, and this decrease was reversed by IMZ or MF treatment of db/db mice.
Figure 4.2 IMZ inhibited pancreatic islet hypertrophy in db/db mice after 60 days of treatment

Representative images of hematoxylin and eosin (H&E) staining (A) and immunohistochemical (IHC) staining for insulin (B) of pancreatic islets from C57BL/6, db/db, IMZ treated db/db, and MF treated db/db mice. Magnification: 40x, scale bar: 100 µm. Pancreatic islet area (C) and insulin stain intensity (D) of C57BL/6 (white bars), db/db (black bars), IMZ treated db/db (gray bars), and MF treated db/db mice (dark gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group; **** P < 0.0001 indicates a significant difference between db/db and C57BL/6 mice; #### P < 0.0001 indicates a significant difference between db/db and IMZ treated db/db mice; $$$ P < 0.0005 indicates a significant difference between db/db and MF treated db/db mice).
4.3.3 IMZ reduced adipocyte size in EWAT from db/db mice after 60 days of treatment

H&E stained epididymal white adipose tissue (EWAT) from all four groups of mice showed differences in the size of epididymal adipocytes (Figure 4.3A) and presented inflamed and hypertrophic epididymal adipocytes in the db/db model control group compared to the WT C57BL/6 mice. However, 60-day treatment with IMZ or MF in db/db mice ameliorated these morphological changes in adipocytes. Average adipocyte area and cell count were calculated (Figure 4.3B, C). The average adipocyte area of db/db control mice was significantly larger and the average cell number was significantly lower in comparison to C57BL/6 mice. Remarkably, IMZ or MF treatment significantly reduced adipocyte size and significantly increased cell count in db/db mice.

4.3.4 IMZ alleviated renal histopathological changes in db/db mice after 60 days of treatment

Renal tissue stained with H&E from the four groups of animals revealed variations in the size of the glomerulus (Figure 4.4A). The images showed that glomerular hypertrophy and inflammation were exacerbated in the db/db model control group in comparison to the WT C57BL/6 group. IMZ or MF administration reversed these renal histopathological changes in db/db mice. Average glomerular area, Bowman’s space area, and proximal tubule area were
determined (Figure 4.4B, C, D). The db/db group exhibited a significantly larger average glomerulus, Bowman’s space, and proximal tubule area than that of the C57BL/6 WT mice, and IMZ and MF treatment significantly reversed these changes to a nearly healthy state.

4.3.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 60 days of treatment

Liver sections stained with H&E and corresponding histological analysis from the four groups of mice revealed excessive lipid droplets in the db/db control group in comparison to the C57BL/6 group. Strikingly, this histopathological abnormality was ameliorated by IMZ or MF treatment in db/db mice (Figure 4.5A, B, C). In addition, IMZ treatment in db/db mice slightly decreased plasma ALT, FFA, and TG levels, although MF treatment statistically decreased ALT, FFA, and TG plasma levels in db/db mice (Figure 4.5D, E, F). However, TC plasma levels were significantly decreased in both IMZ and MF treated db/db mice in comparison to db/db control mice.
Figure 4.3 IMZ reduced adipocyte size in EWAT from db/db mice after 60 days of treatment

Representative images of hematoxylin and eosin (H&E) staining of epididymal white adipose tissue (EWAT) from C57BL/6, db/db, IMZ treated, and MF treated db/db mice. Magnification: 20x, scale bar: 200 µm (A). Adipocyte area (B) and cell count (C) of C57BL/6 (white bars), db/db (black bars), IMZ treated db/db (gray bars), and MF treated db/db mice (dark gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group; **** P < 0.0001 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005, # P < 0.05 indicates a significant difference between db/db and IMZ treated db/db mice; $$$$ P < 0.0001, $$ P < 0.005 indicates a significant difference between db/db and MF treated db/db mice).
Figure 4.4 IMZ alleviated renal histopathological changes in db/db mice after 60 days of treatment

Representative images of hematoxylin and eosin (H&E) staining of renal tissue from C57BL/6, db/db, IMZ treated db/db mice, and MF treated db/db mice (A). Magnification: 40x, scale bar: 100 µm. Glomerular area (B), Bowman’s space area (C), and proximal tubule area (D) from C57BL/6 (white bars), db/db (black bars), IMZ treated db/db (gray bars), and MF treated db/db mice (dark gray bars) was quantified using ImageJ software. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (n = 3-6 per group **** P < 0.0001 indicates a significant difference between db/db and C57BL/6 mice; #### P < 0.0001 indicates a significant difference between db/db and IMZ treated db/db mice, $$$$ P < 0.0001 indicates a significant difference between db/db and MF treated db/db mice).
Figure 4.5 IMZ ameliorated hepatic steatosis and plasma TC in db/db mice after 60 days of treatment

Representative images of hematoxylin and eosin (H&E) staining of hepatic tissue from C57BL/6, db/db, IMZ treated db/db mice, and MF treated db/db mice (A). Magnification: 20x, scale bar: 200 µm. Lipid droplet area (B) and lipid droplet count (C) from C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), IMZ treated db/db (gray bars, n = 6), and MF treated db/db mice (dark gray bars, n = 4) was quantified using ImageJ software. Plasma ALT (D), FFA (E), TG (F), and TC (G) levels from C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), IMZ treated db/db (gray bars, n = 6), and MF treated db/db mice (dark gray bars, n = 4) was measured via a commercial colorimetric kit. Data are presented as mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (**** P < 0.0001, *** P < 0.0005 indicates a significant difference between db/db and C57BL/6 mice; $$$ P < 0.0005, ## P < 0.005 indicates a significant difference between db/db and IMZ treated db/db mice; $$$$ P < 0.0001, $$ P < 0.005, $ P < 0.05 indicates a significant difference between db/db and MF treated db/db mice).
4.3.6 IMZ decreased hepatic lipogenic and fibrotic markers in db/db mice after 60 days of treatment

Western blot analysis revealed a significant increase in ACC, FAS, fibronectin, COL1A1, and TGFB relative protein levels in the db/db control mice in comparison to C57BL/6 WT mice, whereas these protein levels were significantly decreased in IMZ or MF treated db/db mice compared to db/db control mice. (Figure 4.6 A, B). Consistent with the western blot analysis, relative mRNA levels displayed significantly decreased gene expression of the lipogenic and fibrotic genes ACACA, FASN, FN1, COL1A1, and TGFB1 in db/db mice treated with IMZ or MF in comparison to db/db control mice (Figure 4.6C).
Figure 4.6 IMZ decreased hepatic lipogenic and fibrotic markers in db/db mice after 60 days of treatment

Representative immunoblots of ACC, FAS, fibronectin, COL1A1, TGFβ, and vinculin in the liver from C57BL/6, db/db, IMZ treated db/db mice, and MF treated db/db mice (A). Western blot analysis of the relative protein levels in liver tissue from C57BL/6 (white bars, n = 6), db/db (black bars, n = 6), IMZ treated db/db (gray bars, n = 6), and MF treated db/db mice (dark gray bars, n = 6) was quantified using Image Studio Lite and normalized to vinculin (B). Relative mRNA levels in liver tissue from C57BL/6 (white bars, n = 3), db/db (black bars, n = 3), IMZ treated db/db (gray bars, n = 3), and MF treated db/db mice (dark gray bars, n = 3) were detected by qRT-PCR, normalized to the housekeeping genes Rn18s and Actb, and quantified using the 2^ΔΔCT method (C). Data are presented as fold change of the C57BL/6 control group and mean ± SEM. P values were calculated using a one-way ANOVA followed by Bonferroni’s post-test. (*** P < 0.0005, ** P < 0.005, * P < 0.05 indicates a significant difference between db/db and C57BL/6 mice; ### P < 0.0005, ## P < 0.005, # P < 0.05 indicates a significant difference between db/db and IMZ treated db/db mice; $$$ P < 0.0005, $$ P < 0.005, $ P < 0.05 indicates a significant difference between db/db and MF treated db/db mice).
4.4 Discussion

We previously identified and characterized a metformin-methylglyoxal metabolite (IMZ) in urine and plasma from patients with T2DM. Because MF activates imidazoline receptors, the structure of IMZ is drug-like, and the understanding that imidazoline receptors have a functional role in regulating glucose homeostasis and insulin secretion/sensitization, we rationalized that IMZ may be a fundamental element in MF drug efficacy. In the present study, we show that long-term treatment with IMZ in diabetic db/db mice reverses marker of T2DM, and thereby provides evidence that the formation of IMZ may contribute to the antidiabetic properties of MF.

To examine the possible pharmacological effects of IMZ on T2DM in vivo, db/db mice were treated with IMZ twice a day for 60 days. Our initial data demonstrated that IMZ slightly reduced body weight, although not significantly, and effectively reduced fasting blood glucose levels from 6-8 weeks of the study. Measuring fasting blood glucose levels, fasting plasma insulin levels, and calculating the HOMA-IR index are well established clinical procedures for evaluation of insulin action in vivo. IMZ not only decreased elevated fasting blood glucose levels, but also reduced fasting plasma insulin levels and the HOMA-IR value to levels comparable to those achieved with MF, and to levels in healthy WT C57BL/6 mice (Figure 4.1). This is consistent with the ability of MF to clinically reduce obesity and hyperglycemia in patients with T2DM, which was also verified in our study. Furthermore, pancreatic islet beta cell damage plays
a critical role in the pathogenesis of diabetes, and thus diminishing pancreatic islet dysfunction is an imperative therapeutic strategy for treating diabetes. Histopathological assessment of the pancreas revealed that IMZ drastically reversed islet hypertrophy, and potentiated islet insulin IHC staining to a level comparable to that of WT C57BL/6 mice and indistinguishable from that of MF (Figure 4.2). This data aligns with the fact that MF has a direct effect on pancreatic beta cells, prevents desensitization of pancreatic islets, and restores insulin secretion. Thus, these results reveal that IMZ has protective effects on the pancreas and is able to reduce hyperinsulinemia, insulin resistance, pancreatic dysfunction, and enhance insulin sensitivity in db/db mice similarly to MF.

Dysregulation and inflammation of adipose tissue also contribute to the progression of insulin resistance and T2DM. Our results revealed that IMZ successfully reduced adipocyte area, cell number, and the morphology of adipocytes in EWAT from db/db mice to those seen in MF treated animals (Figure 4.3), which is consistent with MF’s insulin sensitizing properties and its ability to reduce insulin resistance at the adipose tissue level. The conventional view of MF’s effect on insulin action focuses on studies in insulin-sensitive tissues such as the pancreas, skeletal muscle, adipose tissue, and the liver. However, insulin also has an effect on the kidney that influences growth, hypertrophy, and glomerular function. In addition, diabetic nephropathy and kidney disease are severe complications of T2DM and recent studies have supported the renoprotective properties of MF. Our data demonstrated that IMZ
significantly reduced glomerular, Bowman’s space, and proximal tubule area (Figure 4.4) in db/db mice equivalent to the effects of MF. Together these results suggest that IMZ is able to improve EWAT and renal tissue histopathological changes that occur in db/db mice in a similar fashion to MF.

T2DM is associated with irregular lipid metabolism, obesity, dyslipidemia, and NAFLD. Accumulation of FFA, TG, and TC lead to insulin resistance and the pathogenesis of NAFLD, which is deemed an unrecognized T2DM complication. Furthermore, dyslipidemia is a significant contributor to cardiovascular disease, which is the leading fatal complication of diabetes. Clinically, aspartate aminotransferase and alanine aminotransferase (ALT) plasma levels are assessed as biomarkers of liver function and remain informative until more accurate noninvasive methods become available. Thus, measuring AST/ALT levels is a reliable way to examine liver function, and maintaining lipid homeostasis is an effective way to relieve symptoms of diabetes. IMZ significantly reduced plasma levels of TC in a manner similar to MF, and slightly reduced plasma levels of ALT, FFA, and TG. In addition, histopathological analysis of the liver revealed that IMZ suppressed lipid accumulation and reduced hypertrophy of hepatocytes (Figure 4.5) in db/db mice, again in a manner similar to MF. This data supports findings that MF is beneficial in the management of the spectrum of liver disease that occurs in T2DM patients, and that IMZ might contribute to some of these effects.

Excessive lipid accumulation and insulin resistance in hepatocytes results in NAFLD, which can progress to nonalcoholic steatohepatitis (NASH) and
fibrosis/cirrhosis, both of which have been linked to increased expression of several hepatic markers of fibrosis and lipogenesis, such ACACA, FASN, FN1, COL1A1, and TGFβ1.\textsuperscript{184-194} Our db/db mice developed apparent hepatic steatosis, accompanied by the upregulation of ACC, FASN, FN1, COL1A1, and TGFβ1 expression at the protein and mRNA level. Importantly, IMZ treatment replicated the effects of MF and significantly inhibited ACC, FASN, FN1, COL1A1, and TGFβ1 expression at the protein and mRNA level (Figure 4.6). This data is in agreement with MF’s hepatoprotective effects.\textsuperscript{191} Thus, these results reveal that IMZ suppressed hepatic steatosis, lipogenesis, and has ability to control lipid metabolism disorders and associated fibrosis in db/db mice in a fashion to MF.

In summary, IMZ significantly alleviated hyperglycemia, insulin resistance, islet hypertrophy, hyperlipidemia, hepatic steatosis, adipocyte hypertrophy, and renal histopathological changes in db/db mice. IMZ promoted hepatic downregulation of the lipogenesis and fibrosis markers ACACA, FASN, FN1, COL1A1, and TGFβ1 at the protein and mRNA level in db/db mice. These findings are consistent with the ability of MF to ameliorate hyperglycemia, dyslipidemia, hepatic steatosis, insulin resistance, and lower microvascular and macrovascular complications associated with T2DM. In addition, imidazoline receptors are novel targets for drug development in disorders associated with T2DM. Indeed, MF has recently been shown to activate imidazoline receptors, and imidazolines can alleviate symptoms of T2DM. Although the precise mechanism(s) underlying IMZ’s pharmacological action remain to be elucidated, the present study reveals an interesting aspect of the antidiabetic properties of MF. Taken together, these
results support the hypothesis that IMZ contributes to the antidiabetic properties of MF, the structure of which may provide a basis to develop other effective agents for the treatment of T2DM.
Chapter 5: Concluding Remarks

Although MF’s primary therapeutic effect is to lower hyperglycemia, which is considered to be mediated via inhibition of hepatic gluconeogenesis along with improved peripheral tissue insulin sensitivity.\(^4\) The precise mechanism(s) by which these effects are achieved remains uncertain and the subject of debate. In addition, MF notably decreases adverse diabetic endpoints and death more successfully than other glucose lowering drugs.\(^20\) MF is unique in comparison to other antihyperglycemic drugs because it has been associated with lowering plasma levels of MGO and MGO-derived AGEs, both of which contribute to the progression of diabetic complications.\(^21,22,86\) This reduction in MGO and MGO-derived AGEs is exclusive to MF and is perhaps the primary mechanism by which MF reduces the development and progression of diabetic complications. Although MF has been used for the treatment of T2DM for over 60 years, its pleiotropic action remains elusive.

Previously, we have unequivocally demonstrated that the primary product of the reaction between MF and MGO is a novel five-membered imidazolinone (IMZ) metabolite, and not the previously theorized triazepinone derivative.\(^24\) Our hypothesis that the therapeutic efficacy of MF is mediated, at least in part, by the formation of IMZ, provides a potential rationale for the therapeutic effectiveness of MF in the treatment of T2DM and diabetic complications. Thus, this work is unique in that, to the best of our knowledge, there are currently no other studies considering the role of MF metabolites in MF’s mechanism of action.
5.1 Insulin Sensitizing Properties of IMZ in PC12 Cells

MF reduces plasma glucose and increases glucose uptake through activation of imidazoline receptors (IRs) in \textit{in vitro} and \textit{in vivo} models.\textsuperscript{34,35} The chemical structure of IMZ suggests it may aid in the ability of MF to activate IRs and may be a fundamental element in MF drug efficacy. PC12 cells are the predominant \textit{in vitro} model for assessing IR activation because they express a high density of IR binding sites and lack \( \alpha_2 \)-ARs, which many imidazolines also interact with. In addition, the proposed model for IR signaling results in downstream activation of IRS1-4/PI3K/AKT/ERK1/2, GLUT4 translocation, and eventual glucose uptake. Furthermore, these signaling events can be blocked by the IR antagonists, efaroxan and idazoxan.\textsuperscript{26} Our results revealed that IMZ sensitized PC12 cells to insulin via modulation of AKT and ERK1/2 phosphorylation at physiologically relevant concentrations (\textbf{Figure 2.1}). Moreover, our data showed that this sensitization can be inhibited by the known IR antagonists, efaroxan and idazoxan (\textbf{Figure 2.2}). Interestingly, our results also demonstrated that knocking down nischarin (the mouse homolog of the human imidazoline receptor antisera-selective protein) via siRNA augmented insulin action (\textbf{Figure 2.3}).

In regard to insulin sensitization, these findings were anticipated based on currently known interactions of imidazolines with the IR and downstream signaling. In addition, these results are consistent with findings from
another novel imidazoline, S43126, which is selective for IIR; however, our results differ from this study with respect to siRNA mediated knockdown of nischarin. Another interesting result to highlight is that when the IIR antagonists efaroxan and idazoxan are combined with insulin alone in PC12 cells, insulin signaling is also enhanced. Although these results were not further explored, the new finding that nischarin inhibition alters energy metabolism in mice is consistent with our own results that indicate insulin action is increased via inhibition or knock down of nischarin in PC12 cells. Thus, our data are compatible with the fact that an interaction between imidazolines and IRs results in crosstalk between insulin and imidazoline receptor signaling. In addition, this data indicates that the ability of IMZ to enhance insulin action in PC12 cells may be mediated through IIR but does not conclusively establish IIR activation via IMZ. In fact, our data may infer that IMZ is an antagonist at IIR, due to the fact that IMZ mimics the effects of efaroxan and idazoxan when combined with insulin in PC12 cells. Finally, this data may shed light on a unique underlying mechanism behind MF’s insulin sensitizing properties, potentially through IIR, that have not yet been explored.
5.2 Insulin Sensitizing Properties of IMZ in Insulin Resistant HEPG2 Cells

The primary function of MF is to decrease hepatic gluconeogenesis and potentiate peripheral insulin sensitivity, thus reducing hyperglycemia and insulin resistance in T2DM patients. Insulin resistance is a complex pathological state of inappropriate cellular response to insulin in insulin sensitive cells, such as the liver. Several studies have established that insulin resistant HEPG2 cells are a dependable model for measuring liver metabolism and insulin signaling in an insulin resistant state. In addition, HEPG2 cells express both I₁R and α₂-ARs, which allows for assessment of IMZ’s activity in the liver under an insulin resistant state, to further understand how MF exerts its antigluconeogenic properties in the liver, where both receptors are present. Our results from this study indicate that an insulin resistant HEPG2 cell line was successfully established via markedly decreased activation of AKT after insulin treatment (Figure 2.4A). In addition, our data revealed that IMZ sensitized insulin resistant HEPG2 cells to insulin via modulation of AKT phosphorylation (Figure 2.4B).

The above data is consistent with studies showing that imidazoline drugs improve insulin sensitivity in models of glucose intolerance; however, this is the first report assessing the activity of an imidazoline in insulin resistant HEPG2 cells. Two other studies have shown that experimental imidazolines with I₁R selectivity, LNP509 and LNP599, increased phosphorylation of proteins involved in the insulin signaling pathway in normal HEPG2 cells. Moreover, this
modulation was markedly inhibited by the selective I₁R antagonist, efaroxan. Our data suggests that IMZ may have the ability to recover insulin resistance mediated through either the I₁R or α₂AR in insulin resistant HEPG2 cells and are consistent with the above mentioned I₁R activation in normal HEPG2 cells. Further studies need to be conducted in the presence of I₁R and α₂AR antagonists to provide evidence for activity at either receptor in this model. Given that it seems IMZ may have activity through I₁R in PC12 cells and other studies have confirmed I₁R activation in HEPG2 cells with imidazolines, I₁R is more likely the major player in IMZ’s ability to recover insulin resistance in insulin resistant HEPG2 cells. Additionally, this data may provide a further mechanistic basis underlying MF’s ability to improve hepatic insulin resistance through IR activation, which has yet to be studied.
5.3 Insulin Secretagogue Activity of IMZ in MIN6 Cells

Insulin is secreted from pancreatic β-cells in response to an increase in plasma glucose levels, which subsequently normalizes glucose to physiological levels. Insulin resistance and prolonged hyperglycemia lead to β-cell dysfunction and loss of mass, which are critical to the development of T2DM. MF has a direct effect on pancreatic β-cells, prevents desensitization of pancreatic islets, and restores insulin secretion.\textsuperscript{174,175} Several studies have shown that imidazolines possess I\textsubscript{3}R mediated insulinotropic properties in the MIN6 pancreatic β-cell line.\textsuperscript{30,33,152,163} Findings from our studies revealed that IMZ increased insulin secretion in MIN6 cells to a similar level as the positive controls, canavanine and efaroxan (Figure 2.5). Our results also indicate that IMZ induced insulin secretion was inhibited by the known I\textsubscript{3}R antagonist KU14R to a level similar to that by which KU14R inhibited efaroxan induced secretion (Figure 2.6). Furthermore, similar to canavanine, the secretagogue activity of IMZ was significantly reduced in MIN6 cells with siRNA mediated knockdown of the mouse homolog of the human imidazoline receptor antisera-selective protein, nischarin (Figure 2.7).

This data aligns with the current understanding of the interaction between imidazolines with I\textsubscript{3}R and their downstream signaling in pancreatic β-cells. More specifically, our results are consistent with studies on the prototypical I\textsubscript{3}R agonist, efaroxan, and the mechanism behind its stimulatory effects in
pancreatic B-cells.\textsuperscript{26} Moreover, our data demonstrates that IMZ may be more selective for \( I_3R \) than efaroxan and canavanine because IMZ increased insulin secretion to a similar level as both agonists, but at a lower concentration. However, the specific affinity of IMZ for \( I_3R \) still requires a direct binding study. Thus, the ability of IMZ to induce insulin secretion in pancreatic B-cells may occur primarily through activation of \( I_3R \). Finally, this data may add to the understanding of MF’s direct effect on pancreatic B-cells, which may be mediated through IRs.
5.4 Antidiabetic Properties of Short-term IMZ Treatment in db/db Mice

MF can ameliorate hyperglycemia, dyslipidemia, hepatic steatosis, insulin resistance, and lower microvascular and macrovascular complications associated with T2DM, the mechanism(s) of which remain unclear.\textsuperscript{4,174} To investigate the possible biological effects of short-term IMZ treatment on T2DM in vivo, genetically obese db/db mice were treated with IMZ twice a day for 7 days. Our initial data showed that IMZ reduced fasting plasma insulin levels and HOMA-IR value to levels comparable to that of the healthy WT C57BL/6 mice (Figure 3.1). Histopathological assessment of the pancreas illustrated that IMZ completely reversed islet hypertrophy, and increased islet insulin IHC staining to a level indistinguishable from that of WT C57BL/6 mice (Figure 3.2). In addition, our results revealed that IMZ successfully reduced adipocyte area, cell number, and the morphology of adipocytes in EWAT from db/db mice (Figure 3.3). Similarly, our data demonstrated that IMZ effectively reduced glomerular, Bowman’s space, and proximal tubule area in db/db mice to a level similar to that of healthy WT C57BL/6 mice (Figure 3.4). Moreover, histopathological examination of the liver illustrated that IMZ suppressed lipid accumulation and reduced hypertrophy of hepatocytes in db/db mice (Figure 3.5). More importantly, our results revealed that IMZ significantly increased hepatic SIRT1 and inhibited SREBP1, ChREBP, ACC, and FAS expression at the protein and mRNA level (Figure 3.6).
This data is consistent with current studies showing that imidazolines can alleviate T2DM symptoms by reducing insulin resistance, improving pancreatic β-cell function, and protecting against adiposity and obesity in *in vitro* and *in vivo* models. More specifically, our data suggests that IMZ directly targets adipose, pancreatic, kidney, and hepatic tissue to improve the T2DM phenotype, which is also apparent with reports exploring the activity of new I₁R selective imidazolines in Zucker fatty rats and spontaneously hypertensive rats. Thus, this data further supports our *in vitro* data detailing IMZ’s potential interaction with IRs in kidney, pancreas, and liver cells. Moreover, this data is compatible with the recent finding that MF can suppress diabetic driven metabolic abnormalities through induction of hepatic SIRT1 in db/db mice at the protein and mRNA level. More importantly, the fact that upregulation of SIRT1 expression can successfully induce antidiabetic effects, especially with regard to insulin resistance and fatty liver disease, leads to the suggestion that IMZ’s pharmacological activity may indeed be clinically relevant to MF’s therapeutic efficacy in T2DM patients. Therefore, this data may provide the rationale behind an additional mechanism of MF’s antidiabetic properties, independent of its glucose lowering capabilities, which may be mediated through IRs and SIRT1 regulation.
5.5 Antidiabetic Properties of Long-term IMZ Treatment in db/db Mice

MF reduces plasma glucose and increases glucose uptake through activation of IRs, and the interaction of imidazolines with IRs can alleviate symptoms of T2DM.\textsuperscript{26,34,35} To investigate the possible biological effects of long-term IMZ treatment and whether they mimic MF’s effects on T2DM \textit{in vivo}, genetically obese db/db mice were treated with IMZ twice a day for 60 days alongside the positive control MF at a clinically relevant dose. Our initial results revealed that long-term IMZ treatment not only decreased elevated fasting blood glucose levels, but also reduced fasting plasma insulin levels and the HOMA-IR value to levels comparable to those achieved with MF, and to levels similar to those measured in healthy WT C57BL/6 mice (Figure 4.1). Histopathological assessment of the pancreas illustrated that IMZ drastically reversed islet hypertrophy, and potentiated islet insulin IHC staining, again to a level comparable to that of WT C57BL/6 mice and indistinguishable from that of MF (Figure 4.2). Our data also revealed that IMZ successfully reduced adipocyte area, cell number, and the morphology of adipocytes in EWAT from db/db mice to the level seen in MF treated animals (Figure 4.3). Similarly, our data demonstrated that IMZ significantly reduced glomerular, Bowman’s space, and proximal tubule area in db/db mice equivalent to the effects of MF (Figure 4.4). Additionally, IMZ significantly reduced plasma levels of TC in a manner similar to MF, and slightly reduced plasma levels of ALT, FFA, and TG. Histopathological analysis of the liver illustrated that IMZ suppressed lipid accumulation and
reduced hypertrophy of hepatocytes in db/db mice, again in a manner similar to MF (Figure 4.5). Importantly, IMZ treatment replicated the effects of MF and significantly inhibited hepatic ACC, FASN, FN1, COL1A1, and TGFβ1 expression at the protein and mRNA level (Figure 4.6).

Our results are consistent with the ability of MF to clinically reduce obesity, hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, and hepatic steatosis in T2DM patients, all of which were verified in our study. Together, these results suggest that IMZ is able to improve the diabetic phenotype in a similar fashion to MF in db/db mice at a dose 12.5 times lower than MF. In addition, this difference in concentration of drug may be physiologically relevant because we previously showed that only ~0.02% of MF is consumed to form the levels of IMZ that are detected in T2DM patient urine. However, one distinguishing difference between MF and IMZ is the time it takes to reduce hyperglycemia. The fact that short-term treatment of IMZ does not have an effect on hyperglycemia reveals that IMZ’s ability to reduce hyperglycemia with long-term treatment is independent of MF’s traditional AMPK driven antihyperglycemic mechanism. Moreover, this data falls in line with the recent finding that MF can suppress disorders associated with T2DM through suppression of hepatic TGFβ in mice at the protein and mRNA level. TGFβ1, FN1, and COL1A1 are well known contributors to liver fibrosis. Interestingly current studies have shown that interaction of moxonidine and idazoxan with I1R and I2R, respectively, regulates the progression of liver fibrosis through pathways involving these same key players. In combination, the findings detailed in
This dissertation supports the hypothesis that the formation of IMZ may contribute to the antidiabetic properties of MF, with the potential to provide the structural basis of an effective agent in the management of T2DM.
Chapter 6: Future Directions

6.1 Effect of IMZ on Intracellular Calcium Influx in MIN6 Cells

6.1.1 Introduction

Imidazoline receptors (I₁R-I₃R) are considered novel targets for drug development in disorders associated with T2DM because they are involved in glucose homeostasis and insulin sensitization/secretion. The I₃R subtype is located only in pancreatic β-cells and its activation leads to improved insulin secretion. Electrophysiological studies indicate that the insulin secreting mechanism of the classic I₃R agonist, efaroxan, is mediated by calcium influx. In addition, it has also been shown that guanidine containing compounds, such as MF, can improve glucose homeostasis through IR activation.

We previously identified and characterized a novel metformin-methylglyoxal derived imidazolinone (IMZ) metabolite in the plasma and urine of patients with T2DM. The chemical structure of IMZ suggests it may aid in the ability of MF to activate imidazoline receptors. In addition, as outlined in this dissertation, we have shown that IMZ has insulinotropic properties in MIN6 cells, which may be mediated through I₃R. However, we have not yet explored whether IMZ’s insulin secreting properties in MIN6 cells are also mediated through an influx in intracellular calcium.
6.1.2 Methods

A. Cell culture

Mouse pancreatic β-cells (MIN6, AddexBio, San Diego, CA, USA) will be cultured in AddexBio advanced DMEM-based medium optimized to culture MIN6 cells and maintained in the presence of 5% CO2 at 37°C.

B. Insulin secretion measurement

MIN6 cells will be plated in 24-well plates at a density of 500,000 cells per well. On the next day, the culture medium will be replaced with glucose and serum free Gibco™ DMEM (Fisher Scientific) supplemented with 0.5 mM glucose and 0.1% BSA and incubated overnight (~12 hrs.). On the day of the experiment, the cells will be starved for 4 hrs. in glucose free Krebs-ringer modified HEPES buffer containing 0.1% BSA (KRBH-BSA) at 37°C with 5% CO2. Following the 4hr. incubation, cells will be treated with canavanine (10 µM), efaroxan (100 µM), and various concentrations of IMZ (1 pM-1 µM) for 15 minutes in KRBH-BSA supplemented with 7 mM glucose. Another group of cells will be pretreated with nifedipine (10 µM) for 30 min. and then treated under the same conditions in the presence of nifedipine for 15 mins. After treatment, the medium will be collected, centrifuged at 13,000 rpm for 10 min. at 4°C, and stored at -80°C for insulin measurement. Insulin release will be measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions. Protein concentrations will be determined using the DC™ Protein Assay (Bio-Rad).
C. Intracellular calcium measurement

Another group of cells will be used for calcium measurement. Intracellular calcium concentrations will be detected using the calcium-sensitive fluorescent dye fura 2-acetoxymethyl ester (fura-2; Molecular Probes, Eugene, OR, USA). Cells will be placed in a buffered physiological saline solution (PSS) containing 5 mM fura-2 and incubated for 30 mins at 37°C. Cells will then be washed gently 3 times with PSS and incubated in the dark at room temperature for 30 mins. Cells will be treated with canavanine, efaroxan, IMZ, and nifedipine as previously mentioned. Calcium will be continuously recorded using an emission wavelength of 520 nm and alternating excitatory wavelengths of 340 and 380 nm with a fluorescence microplate reader (BioTek, Winooski, VT, USA). The intracellular calcium ([Ca^{2+}]_i) levels will be calculated using the following equation: 

\[ [\text{Ca}^{2+}]_i = \frac{(R - R_{\text{min}})/(R_{\text{max}} - R)) \times (S_{r2}/S_{b2}) \times K_d}{R}\]

where R is the fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm, R_{min} is the minimum fluorescence ratio of approximately 0.768 and R_{max} is the maximum fluorescence ratio of approximately 35.1. The coefficient S_{r2} indicates the free dye measured at wavelength of 380 nm and S_{b2} indicates calcium bound dye at 380 nm. According to experimental data, S_{r2}/S_{b2} for fura-2 is approximately 15.3 and K_d is the effective dissociation constant of fura-2, which is approximately 135 nM.

6.1.3 Expected results

From these studies we expect to see similar effects in MIN6 cells with IMZ as shown with the known I_3R agonists canavanine and efaroxan with and without
the presence of the well known calcium channel blocker, nifedipine. Thus, we expect that in addition to IMZ’s ability to induce insulin secretion, IMZ will cause an influx in intracellular calcium in MIN6 cells. Moreover, we expect that IMZ’s induction in insulin secretion and intracellular calcium will be inhibited by nifedipine in MIN6 cells.
6.2 Effect of Small Molecule Inhibition of IRs and α2-ARs on IMZ’s Insulin Sensitization in Insulin Resistant HEPG2 Cells

6.2.1 Introduction

In this dissertation we demonstrated that IMZ sensitizes insulin resistant HEPG2 cells to insulin, which express both the I_1R and α2-AR. However, we have not yet explored which type of receptor this sensitization is mediated through.

6.2.2 Methods

A. Cell culture

Human hepatocellular carcinoma cells (HEPG2, ATCC) will be cultured in Corning™ Eagle’s MEM (Fisher Scientific) supplemented with 10% FBS and maintained in the presence of 5% CO2 at 37°C.

B. Insulin resistant HEPG2 cell model

HEPG2 cells will be plated in 6-well plates at a density of 1 million cells per well and grown to 65-70% confluency. Once cells reach confluency, the medium will be replaced with serum free medium and incubated overnight (~16 hrs) in the presence of 5% CO2 at 37°C. On the next day, the medium will be replaced with glucose and serum free Gibco™ DMEM (Fisher Scientific) supplemented with either a low glucose concentration (5.5 mM), a high glucose concentration (25 mM), or a high concentration of glucose (25 mM) and insulin (1
µM) for 24 hours. After 24 hrs, cells will be serum starved for 2 hrs. and subsequently dosed with insulin (100 nM) or various concentrations of IMZ (1 pM-1 µM) for 10 minutes. Another group of cells will be pretreated with the I₁R and α₂-AR antagonists, efaroxan, rauwolscine, and SK&F86466 for 30 minutes and then treated as previously mentioned in the presence of the inhibitors for 10 minutes. After treatment, cells will be lysed in CLB, centrifuged at 13,000 rpm for 10 min. at 4°C, and the supernatant stored at −80°C for western blot analysis.

D. Western blot analysis

Concentrations of protein in cell lysates will be determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). Protein (40-100 µg) will be separated on an 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA) for 2 hrs. at 50 volts in 4°C. PVDF membranes will be blocked in Odyssey® Blocking Buffer in tris-buffered saline (LI-COR, Lincoln, NE, USA) for 1 hr. at room temperature (RT), followed by incubation with specific primary antibodies, diluted 1:500 or 1:1000 in 5% bovine serum albumin (BSA), at 4°C overnight. Membranes will then be incubated with corresponding near-infrared fluorescent secondary antibodies (LI-COR), diluted in blocking buffer 1:15000, for 1 hr. at RT. Targeted proteins will be detected using an Odyssey® FC imaging system and band intensity was analyzed using Image Studio Lite Ver 5.2 (LI-COR). Primary antibodies to be used are as follows:
p44/42 MAPK (ERK1/2, 9107S), phospho-p44/42 (Thr202/Tyr204) MAPK (pERK1/2, 9101S), protein kinase B (AKT, 2920S), phospho-protein kinase B (S473, pAKT, 4060S) (Cell Signaling Technologies, Danvers, MA, USA), and GAPDH (Abcam, ab8245).

6.2.3 Expected results

The assessment of imidazoline compound activity in insulin resistant HEPG2 cells has not been previously studied. However, we expect that IMZ’s insulin sensitization in insulin resistant HEPG2 cells via AKT modulation will be blocked by the selective I1R antagonist efaroxan and not the selective α2-AR antagonists rauwolscine and SK&F86466. This study will provide some understanding as to whether IMZ has α2-AR activity in addition to IR activity.
6.3 Effect of IMZ on Activation of I$_2$Rs in Skeletal Muscle Cells

6.3.1 Introduction

Recent evidence indicates that activation of I$_2$Rs by MF can increase glucose uptake in skeletal muscle.$^{34,35}$ In this dissertation we demonstrated that IMZ may possibly be sensitizing cells to insulin and inducing insulin secretion through activation of I$_1$R and I$_3$R. However, we have not yet explored whether IMZ has an effect on I$_2$Rs.

6.3.2 Methods

A. Cell culture

The mouse derived myoblast C2C12 cell line will be purchased from ATCC. The cells will be maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in humidified atmosphere containing 5% CO2. For maturation, the cells will be grown to 70% confluency and then exposed to DMEM supplemented with 10% horse serum. Cells will be fused into multinucleated myotubes in 7-10 days. Cells will be serum starved for 4-6 hrs., treated with varying concentrations of IMZ (1pM-1µM) with and without the presence of BU224 (1µM) and LY294002 (1µM; I$_2$R and PI3K inhibitor, respectively) for 10 minutes, and then glucose uptake will be determined.
Inhibitors will be pretreated for 30 minutes and MF (2 mM, 1 hr.) will be used as a positive control.

**B. Glucose uptake**

At the end of the experiment glucose concentrations in the cell culture medium will be determined using the glucose oxidase method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). In addition, glucose uptake will be measured in another group of cells treated as previously mentioned using 2-[^14]C-deoxy-D-glucose (2-[^14]C-DG) (New England Nuclear, Boston, MA, USA) by the following procedure. At the end of the experiment the cells will be incubated further with 2-[^14]C-DG (1 µCi/mL) for 5 minutes at 37°C. Uptake will be terminated with aspiration of the radioactive culture medium, addition of ice-cold DMEM, and three rapid washes in cold PBS. Radioactivity associated with the cells will be disrupted with 1 M NaOH, neutralized with 1 M HCl, and then determined in cell lysis by scintillation counting. Aliquots of cell lysates will also be used for protein content determination by the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). The 2-[^14]C-DG uptake will be expressed as pmol of 2-[^14]C-DG per mg of protein in the cells. Nonspecific uptake will be obtained by parallel determinations in the presence of 20 µM cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA).
C. Western blot analysis

Concentrations of protein in cell lysates will be determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA). Protein (40-100 µg) will be separated on an 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon®-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA) for 2 hrs. at 50 volts in 4°C. PVDF membranes will be blocked in Odyssey® Blocking Buffer in tris-buffered saline (LI-COR, Lincoln, NE, USA) for 1 hr. at room temperature (RT), followed by incubation with specific primary antibodies, diluted 1:500 or 1:1000 in 5% bovine serum albumin (BSA), at 4°C overnight. Membranes will then be incubated with corresponding near-infrared fluorescent secondary antibodies (LI-COR), diluted in blocking buffer 1:15000, for 1 hr. at RT. Targeted proteins will be detected using an Odyssey® FC imaging system and band intensity was analyzed using Image Studio Lite Ver 5.2 (LI-COR). Primary antibodies to be used are as follows: GLUT4 (Santa Cruz Biotechnology, Inc., Dallas, TX), phosphor-PI3K p85 (Tyr458)/p55 (Tyr199) (4228S), PI3K p85 (4257S), PI3K p55 (11889S), p44/42 MAPK (ERK1/2, 9107S), phospho-p44/42 (Thr202/Tyr204) MAPK (pERK1/2, 9101S), protein kinase B (AKT, 2920S), phospho-protein kinase B (S473, pAKT, 4060S), phospho-AMPKα (Thr172, 2535S), AMPKα (2793S), and β-actin (4970S) (Cell Signaling Technologies, Danvers, MA, USA).
6.3.3 Expected results

From these studies, we expect to see similar results with IMZ that have been shown with MF and other known IR agonists in C2C12 cells. We expect that IMZ will show a significant induction in glucose uptake, which will be illustrated by an increase in 2-[\textsuperscript{14}C]-DG in C2C12 cells. In addition, we expect IMZ induced glucose uptake in C2C12 cells to be blocked by the I\textsubscript{2}R antagonist, BU224. We also expect that IMZ will increase phosphorylation of PI3K, AKT, ERK1/2, AMPK and increase GLUT4 expression in C2C12 cells. Moreover, these effects are also expected to be blocked by BU224.
6.4 Evaluation of the Affinity of IMZ for IRs and α-ARs

6.4.1 Introduction

Interest in the pharmacological activity of imidazolines started with the finding of the α-AR properties of tolazoline. Subsequently, numerous similar structured drugs with α-AR activity were synthesized and used for therapeutic benefit of hypertension in the clinic. Since the awareness of imidazoline receptors and their benefit in disorders associated with T2DM, the design and synthesis of new molecules binding preferably to imidazoline receptors over α-ARs has been brought to practice.²⁰³

In this dissertation we demonstrated that IMZ may possibly be sensitizing cells to insulin and inducing insulin secretion through activation of imidazoline receptors. However, we have not yet explored whether IMZ directly binds to either α-ARs or imidazoline receptors.

6.4.2 Methods

A. Membrane preparation

Male Wistar rat brain membranes will be prepared from whole rat brain removed immediately following euthanization. The brains will be homogenised in ice-cold TEM buffer (50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM MgCl₂, pH 7.4, supplemented with 50 μM phenylmethyl sulphonylfluoride, 2 μg/mL aprotonin, and 2 μg/mL leupeptin) and then centrifuged at 3,000 x g for 10 min at 4°C. The
supernatant will be removed and then centrifuged at 40,000 × g for 20 min 4°C. The remaining pellet will be resuspended in TEM buffer and recentrifuged. The final crude membrane fraction will be resuspended in TEM buffer and aliquots will be snap frozen in liquid nitrogen and stored at -80°C until further analysis. Protein content will be measured using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

The medullas of bovine adrenal glands will be dissected from the cortex, minced, and homogenized in ice-cold 10 mM HEPES buffer containing 330 mM sucrose at pH 7.4 and centrifuged at 40,000 × g for 15 minutes at 4°C. The supernatant will be centrifuged again for 20 minutes at 40,000 × g and 4°C to achieve the crude membrane fraction. Separation of mitochondrial membranes from plasma membranes will be performed by differential centrifugation at 6,500 × g (15 min) for mitochondrial membranes and 30,000 × g (20 min) for plasma membranes. Pellets will then be washed three times, resuspended in 50 mM Tris HCl containing 5 mM EDTA buffer (pH 7.4), and aliquots will be snap frozen in liquid nitrogen and stored at -80°C until further analysis. Protein content will be measured using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

Male Sprague-Dawley rats will be euthanized, and kidneys will be immediately removed. Kidneys will then be homogenized in 50 mM Tris HCl and 5 mM EDTA, pH 8.0 and centrifuged at 40,000 × g for 15 minutes at 4°C. The supernatant will be discarded, and the pellet will be resuspended in the original volume of homogenization buffer and recentrifuged. The pellet will then be
washed twice by centrifugation in buffer at 4°C, and the final pellet will be resuspended in buffer and aliquots will be snap frozen in liquid nitrogen and stored at -80°C until further analysis. Protein content will be measured using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

MIN6 cell membranes will be prepared after 7 days of culture. The culture medium will be removed and cells will be detached from the surface of the plastic flasks by 30 mL of phosphate/EDTA buffer (in mM: NaCl 136, KCl 27, Na2HPO4 8.1, EDTA 0.7) pH 7.5 at 37°C, and centrifuged at 4°C for 5 minutes at 500 x g. Cells will then be disrupted in 15 mL of Tris-HCl homogenization buffer (Tris 10 mM, NaCl 30 mM, dithiotreitol 1 mM, PMSF 5 mM) pH 7.5 at 4°C, using a glass homogenizer. The homogenate will then be layered over 15 ml of a 41% (wt/v) sucrose solution at 4°C and centrifuged at 95,000 x g for 60 minutes at 4°C. The band at the interface of the layers will be collected, diluted with 30 ml of Tris-HCl buffer and centrifuged at 46,000 x g for 10 minutes at 4°C. The final pellet will be resuspended in buffer and aliquots will be snap frozen in liquid nitrogen and stored at -80°C until further analysis. Protein content will be measured using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA).

B. Binding assays

The affinity of IMZ (1 nM-1 mM) for α₁- and α₂-ARs will be determined by the measurement of [³H]-Prazosin (4 nM, selective α₁-AR antagonist) and [³H]-RX821001 (3 nM, selective α₂-AR antagonist) displacement from membrane
binding sites of rat brain aliquots (100 µg). Non-specific binding will be
determined in the presence of phentolamine (10 µM) for α₁-ARs and [³H]-
RX821001 binding in the presence of yohimbine (10 µM) for α₂-ARs. After 90
minutes of incubation at 25°C, bound and free ligand will be separated, and the
reaction will be stopped by prompt vacuum filtration through Whatman GF/C
filters. The filters will be rapidly washed with ice-cold TEM buffer and their
radioactivity will be determined by liquid scintillation counting.

The affinity of IMZ (1 nM-1 mM) for I₁R will be determined by the
measurement of [³H]-moxonidine (5 nM) displacement from the membrane
binding sites of bovine adrenal glands (0.6 mg protein/mL) in the presence of
RX821002 (1 µM) to mask α₂-ARs. Non-specific binding will be determined in the
presence of S22687 (10 µM). After 40 minutes of incubation at 25°C, bound and
free ligand will be separated, and the reaction will be stopped by prompt vacuum
filtration through Whatman GF/C filters. The filters will be rapidly washed with
ice-cold TEM buffer and their radioactivity will be determined by liquid
scintillation counting.

The affinity of IMZ (1 nM-1 mM) for I₂R will be determined by the
measurement of [³H]-idazoxan (5 nM) displacement from the membrane binding
sites of rat kidney cortex (0.5 mg protein/mL) in the presence of rauwolscine (5
µM) to mask α₂-ARs. Non-specific binding will be defined as [³H]-idazoxan binding
in the presence of 2-BFI (10 µM). After 30 minutes of incubation at 25°C, bound
and free ligand will be separated, and the reaction will be stopped by prompt
vacuum filtration through Whatman GF/C filters. The filters will be rapidly washed with ice-cold TEM buffer and their radioactivity will be determined by liquid scintillation counting.

The affinity of IMZ (1 nM-1 mM) for I₃R will be determined by the measurement of [³H]-efaroxan (2 nM) displacement from the membrane binding sites of MIN6 pancreatic β-cells in the presence of yohimbine (10 mM) to mask α₂-ARs. Non-specific binding will be determined in the presence of KU14R (10 µM). After 60 minutes of incubation at 25°C, bound and free ligand will be separated, and the reaction will be stopped by prompt vacuum filtration through Whatman GF/C filters. The filters will be rapidly washed with ice-cold TEM buffer and their radioactivity will be determined by liquid scintillation counting.

6.4.3 Expected results

From these studies, we generally expect that IMZ will have preferential binding to imidazoline receptor subtypes over α-adrenergic receptor subtypes. More specifically, we expect that there will be no displacement of [³H]-Prazosin or [³H]-RX821001 binding in rat brain membranes to α₁-AR of α₂-AR sites respectively. In addition, we expect that IMZ will displace [³H]-moxonidine, [³H]-idazoxan, and [³H]-efaroxan binding to I₁R, I₂R, and I₃R sites, respectively.
6.5 Evaluation of the Metformin Effect on Methylglyoxal in Patients with Type 2 Diabetes (MET)

6.5.1 Introduction

This study is designed to investigate the effects of metformin on the levels of the potentially tissue damaging reactive product (MGO) in the blood, and its renally cleared counterpart in the urine (IMZ). The study will involve patients with elevated HbA1c randomized to receive either MF or a similar inactive placebo pill and a group of sex and age matched volunteers without diabetes. Subjects will be recruited from IBio at Wayne State University. Eighty patients with HbA1c > 6.0% will be randomized to receive either MF or a similar inactive placebo for 12 weeks. The response of these patients will be compared to 40 patients with normal HbA1c <5.6% who will not receive either study medication. Investigators will compare the plasma and urine MGO and IMZ concentration/content between the 3 groups before and after the 12-week intervention. It is hypothesized that plasma MGO levels in the MF treatment group will be less than in the group taking the placebo pill, while there will be no significant difference in MGO levels between the MF treatment group and the healthy volunteers without diabetes.

While HbA1c has long been used to assess long-term glucose exposure in diabetic patients it remains an imperfect predictor of diabetic complications. However, MF may benefit diabetic complications and outcomes independent of its effects on glycemic control, and its use might be encouraged even when
target HbA1c levels are no longer achievable with MF therapy alone or in pre-diabetic patients.

Study participants may benefit from the extra attention given to glycemic control and adjustment of diabetic medications. Some patients might benefit from continued use of MF at the conclusion of the study and would be able to work with their own physician/endocrinologist with the added knowledge of their response and toleration to the medication if MF were assigned.

The study proposed will better elucidate the scavenging mechanism of MF in lowering MGO concentration and formation of IMZ in T2DM patients on MF therapy. Moreover, the study may help establish the MGO-adducted product as a useful tool for monitoring the progression of T2DM and its complications.

6.5.2 Study aims

This study will evaluate the effect of MF, at the usual target dose for T2DM patients, on plasma and urine MGO and IMZ levels. The primary outcome is measurement of plasma MGO concentration after 12 weeks of MF administration. We hypothesize that MF treatment will significantly reduce MGO concentration from baseline after 12 weeks of therapy in patients with an elevated HbA1c (T2DM and pre-diabetes) when compared to placebo treatment. Also, it is hypothesized that there will be no significant difference in the plasma MGO levels between the matched non-diabetic group and the MF treatment group. Our secondary outcome is measuring the plasma and urinary IMZ concentration/content after 12 weeks of MF administration. We hypothesized
that a concomitant elevation in plasma and urinary IMZ concentration/content is associated with MF administration. In addition, measuring MGO derived plasma protein modifications after 12 weeks of MF administration is another secondary outcome. We hypothesized that MF will reduce MGO derived plasma protein modifications that will be observed in the placebo group. Protein modifications will not be significantly different between the MF-treated group and the healthy subjects.

6.5.3 Inclusion criteria

Elevated HbA1c > 6.0% (patients with type 2 diabetes mellitus or prediabetes), HbA1C < 9.0%; For matched non-diabetic patients: HbA1C ≤ 5.6%; Serum creatinine (SCr) < 1.5 mg/dL in males or < 1.4 mg/dL in females; Calculated glomerular filtration rate (GFR) > 60 ml/min/1.73m2; Body mass index (BMI) 25-40 kg/m2; Glycosylated hemoglobin A1C (HA1C) < 9%; No known intolerance to metformin; Has not taken metformin previous 3 months.

6.5.4 Exclusion criteria

Patients on thiazolidinediones (TZD); Patients with history of MF intolerance (gastrointestinal side effects or poor renal function; Serum Cr > 1.5mg/dL in males or > 1.4 mg/dL in females or calculated GFR < 60 mL/min/1.73m2); Patients with active (symptomatic or unstable) cardiovascular disease; Patients requiring home oxygen; Patients with end-stage liver disease (cirrhosis); Patients on oral glucocorticoids within the past 30 days (equivalent to prednisone > 5mg/day); Excessive alcohol intake (The Substance Abuse and
Mental Health Services Administration (SAMHSA) defines heavy drinking as
drinking 5 or more drinks on the same occasion on each of 5 or more days in the
past 30 days) 29; Pregnancy

6.5.5 Methods

Subjects will be recruited from IBio at Wayne State University. Eighty
patients with HbA1c > 6.0% will be randomized to receive either metformin or a
similar inactive placebo for 12 weeks. The response of these patients will be
compared to forty patients with normal HbA1c (< 5.6%) who will not receive
either study medication.

Twenty four-hour urine samples will be analyzed to measure the MF-MGO
reaction product (IMZ) at baseline (D0) and at the end of the study period. Blood
samples will be analyzed at the baseline to measure the plasma MGO
concentration (baseline MGO concentration) and re-measured at the end of the
study period. Mass spectrometry will be used for sample analysis to detect the
plasma MGO levels, the MF-MG reaction product (IMZ), and plasma protein
modifications at baseline and at the end of the study period (after 12 weeks).

A logbook will be provided to each participant to record their blood sugar
measurements at home. Investigators will provide a glucometer (One Touch
glucometer), glucometer strips, and lancets to patients who have never had a
glucometer before. Participants taking MF or placebo will be asked to perform a
4-point self-measured blood sugar profile with measurements taken once a week.
The results of the blood sugar measurements will help the study physician to adjust diabetic therapies in order for the study subjects to maintain appropriate blood glucose control (target pre-meal and bedtime glucose between 90-180 mg/dL).

The participants in the non-diabetic group will not receive MF hydrochloride or placebo. Their fasting blood glucose, plasma MG level, and urine IMZ level will be measured during the study; they will not be required to perform 4-point testing; they will have all the same clinic visits and laboratory orders as the diabetic patients, except for receiving the study medication.

6.5.6 Expected results

In this clinical trial it is expected that MF will reduce plasma MGO concentrations after 12 weeks of administration in T2DM patients when compared to the placebo treatment group. In addition, a concomitant elevation in plasma IMZ concentrations is expected. We also anticipate there will be no significant difference in the plasma MGO levels between the matched non-diabetic group and the MF treatment group. Moreover, it is expected that there will be elevations in MGO-modification on plasma proteins in the T2DM placebo group in parallel with lower levels of plasma protein modifications in the MF treatment group which is indistinguishable from the matched non-diabetic patients.
References
16 Hammes, H. P., Martin, S., Federlin, K., Geisen, K. & Brownlee, M. Aminoguanidine treatment inhibits the development of experimental


45 Soulis-Liparota, T., Cooper, M., Papazoglou, D., Clarke, B. & Jerums, G. Retardation by aminoguanidine of development of albuminuria, mesangial


60 Lo, T. W., Westwood, M. E., McLellan, A. C., Selwood, T. & Thornalley, P. J. Binding and modification of proteins by methylglyoxal under


118  Kamisaki, Y. et al. Binding of [3H]p-aminoclonidine to two sites, alpha 2-adrenoceptors and imidazoline binding sites: distribution of imidazoline.


131 Bhalla, S., Andurkar, S. V. & Gulati, A. Involvement of alpha(2)-adrenoceptors, imidazoline, and endothelin-A receptors in the effect of agmatine on morphine and oxycodone-induced hypothermia in mice.

170


Zhang, J. *et al.* Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study. *Scientific reports* 4, 5832 (2014).


