Therapeutic potential and physiological roles of Insulin-Degrading Enzyme illuminated by a DNA-templated macrocyclic inhibitor

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Accessibility
Therapeutic potential and physiological roles of Insulin-Degrading Enzyme illuminated by a DNA-templated macrocyclic inhibitor

A dissertation presented

by

Juan Pablo Maianti

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemistry and Chemical Biology

Harvard University
Cambridge, Massachusetts
May 2015
Therapeutic potential and physiological roles of Insulin-Degrading Enzyme illuminated by a DNA-templated macrocyclic inhibitor

Abstract

Insulin-Degrading Enzyme (IDE) is a zinc-metalloprotease responsible for the clearance of insulin in peripheral tissues. Despite decades of speculation that inhibiting endogenous insulin degradation might treat Type-2 Diabetes, the functional relationship between IDE and glucose homeostasis remains unclear. IDE inhibitors that are active in vivo are therefore needed to elucidate IDE’s physiological roles and to determine its potential to serve as a target for the treatment of diabetes.

In this thesis I describe the development of the first highly specific IDE in vivo probe, identified from a DNA-templated library of macrocycles, which enabled the first study of the physiological consequences of IDE inhibition. An X-ray structure of the macrocycle bound to IDE reveals that it engages a novel binding pocket away from the catalytic site, which explains its remarkable specificity and its suitability to study IDE in vivo. Treatment of lean and obese mice with this inhibitor revealed that IDE regulates multiple metabolic hormones, including glucagon and amylin, in addition to insulin. Under physiological conditions that mimic a meal, such as oral glucose administration, acute IDE
inhibition leads to substantial improvement in glucose tolerance, owing to the potentiation of endogenous insulin and amylin levels over glucagon signaling. These studies demonstrated the feasibility of modulating IDE activity as a therapeutic strategy to treat diabetes and expanded our understanding of the roles of IDE in glucose and hormone regulation.

Based on these studies we sought to develop substrate-selective inhibitors that block IDE’s ability to degrade insulin but not its ability to degrade glucagon, which would represent a major step forward towards IDE-targeted therapeutics. The first-generation DNA-templated inhibitor was retailored into a fluorescent anisotropy tool for high-throughput screening of diverse small-molecule libraries. We discovered and characterized a family of IDE inhibitors with sub-micromolar potencies that inherited the remarkable specificity for IDE over other metalloproteases, and selectively obstruct IDE-mediated insulin degradation in a way that accommodates for glucagon cleavage.

In conclusion, these findings offer new insights into the biological roles of IDE and establish a novel strategy to selectively potentiate the physiological insulin response in order to improve blood sugar control in Type-2 Diabetes.
Dedicated to Nicole.
“Study enzymes under the most natural conditions of action, in the living cell itself. From the standpoint of preparative chemistry they may be looked upon as being of the utmost impurity. However, if one finds molecules that selectively interact with the enzymes, the rest of the cell interferes as little as does the glass wall of a test tube in which a chemical reaction is carried out.”


“But Natural Selection, as we shall hereafter see, is a power incessantly ready for action, and is immeasurably superior to man's feeble efforts, as the works of Nature are to those of Art.”

― Charles Darwin, The Origin of Species, Chapter 3.
Acknowledgements

I acknowledge the financial support from Fonds de la Recherche en Santé du Québec (FRSQ) for a three year PhD fellowship (#20318). I also acknowledge the support from the Alfred Bader Fellowship during the academic year 2010-2011, and the GSAS Merit Fellowship from the John Parker Bequest fund during the academic year 2014-2015.

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I am very thankful to Prof. Nathanael Gray for taking part in my advising committee, for his continued encouragement, and his numerous insightful recommendations in small molecule development.

The research I described in this dissertation took our team in a number of particularly interdisciplinary directions, and undoubtedly, the bulk of this work would not have been achieved without the support of numerous talented student colleagues, our collaborators from other universities, and Harvard staff members.

I would like to thank Prof. Markus Seeliger and Zack Foda (Stony Brook University) for their excellent work in solving the IDE:6b co-crystal X-ray structure, which helped us understand the molecular basis of IDE inhibition. I
learnt a lot from Markus about protein structure when we mutated the inhibitor distal binding site to corroborate the placement of the 6b building blocks.

Dr. Maureen Charron (Albert Einstein College of Medicine) generously provided us with a large cohort of glucagon-receptor knockout mice. I thank her and Sally Du for welcoming me into her lab in New York on multiple occasions to carry out the experiments in collaboration with Sally. We are also indebted to Prof. Malcolm Leissring for providing the cohort of IDE knockout mice and controls that enabled us to corroborate the lack effects of 6bK in mice lacking the target.

I am very thankful to the contemporary colleagues of the DTS team for training and enriching exchange of ideas and protocols: Dr. Ralph Kleiner, Dr. Christoph Dumelin, Dr. Lynn McGregor, Dr. Jia Niu, Prof. Ryan Hilli, Dr. Rick McDonald, Dr. Dmitry Usanov, Alix Chan, Zhen Chen, Dennis Dobrovolsky, Dr. Phillip Lichtor, Dr. Yevgeny Brudno, Prof. David Gorin, Dr. Adam Kamlet, and Dr. Yu He. Ralph was an outstanding mentor during my early training.

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underscore the helpful molecular biology contributions of Ahmed. Thanks are also due to Alekandar Markovic for keeping the lab running smoothly year after year.

The Saghatelian group was my lab away from lab, and I thank all the members including Dr. Amanda McFedries, Dr. Edwin Homan, and Dr. Anna Mari Lone for their help. Edwin and Anna Mari taught me the first glucose tolerance test procedures, and Amanda worked diligently with me throughout 2013-2014 to characterize the effects of acute IDE inhibition in mice.

I would like to thank Dr. Chuck Vanderburg for enabling the readout of multiplexed magnetic bead hormone panels at the Advanced Tissue Resource Center of the MassGeneral Institute for Neurodegenerative Disease.

I acknowledge the generous contributions of Dr. Bridget Wagner and Dr. Amedeo Vetere when I approached them with the wild idea of screening for new IDE inhibitors using fluorescence polarization. Thank you so much for welcoming me into the Broad Institute and enabling the small molecule screens.

More recently we begun working in closer collaboration with the Broad Institute to further develop the substrate-selective IDE inhibitor hits. I would like to thank Prof. Schreiber for his advice and generously making available a number of resources. I thank Dr. Marshall Morningstar for helping us establish a contractor to produce the azetidine core, as well as Dr. Sivaraman Dandapani and Dr. Mark Fitzgerald for providing me with the synthetic starting material to make the first batch of BRD-297. Thanks are due to Dr. Stephen Johnston for the microsomal stability characterization of 6bK and azetidine hits, and to Dr. Josh Bittker for printing the azetidine library plates.
All the *in vivo* work discussed in this thesis would not have been possible without the excellent support of the veterinary staff of the Biology Infrastructure Facility, in particular Dawn Hidenfelter, Jaime Moreno, Mimi Crowley, Dr. Mimi Lam, and Dr. Steven Niemi.

I would also like to acknowledge our most recent collaborators Prof. Peter Kang and Dr. Qingen Ke at the Beth Israel Deaconess Medical Center for welcoming me into their lab to explore the effects of IDE inhibition on blood pressure.

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Finally, I would like to thank my family and friends, near and far, for their support and constant encouragement.

This thesis is dedicated to my loving wife Dr. Nicole Darricarrère in acknowledgement of her unconditional support, kindhearted advice, and admirable dedication, throughout the past 10 years doing science together around the globe.

Thank you all most sincerely.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B (PKB)</td>
</tr>
<tr>
<td>Alloc</td>
<td>Allyloxyacarbonyl protecting group</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP</td>
<td>Area Postrema of the brainstem</td>
</tr>
<tr>
<td>AR</td>
<td>Amylin receptor (calcitonin receptor plus RAMP1-3)</td>
</tr>
<tr>
<td>Ar</td>
<td>Aromatic</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>bisepi</td>
<td>Epimer differing in the configuration of two chiral centers</td>
</tr>
<tr>
<td>BRENDA</td>
<td>BRaunschweig ENzyme DAtabase</td>
</tr>
<tr>
<td>BRI</td>
<td>Biology Research Infrastructure facility at Harvard</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C-peptide</td>
<td>C-terminal peptide from insulin maturation</td>
</tr>
<tr>
<td>C-term</td>
<td>Carboxy terminal</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA, synthesized from RNA template</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-Gene Related Peptide</td>
</tr>
<tr>
<td>cLogP</td>
<td>Calculated octanol-water partition coefficient</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450 enzymes</td>
</tr>
<tr>
<td>DIBAL</td>
<td>Diisobutylaluminium hydride</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-Induced Obese</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Enzyme that catalyzes the hydrolysis of deoxyribonucleic acids</td>
</tr>
<tr>
<td>Dnp</td>
<td>2,4-Dinitrophenyl quencher</td>
</tr>
<tr>
<td>DOS</td>
<td>Diversity-oriented synthesis</td>
</tr>
<tr>
<td>Dpa</td>
<td>N-3-(2,4-Dinitrophenyl)-L-2,3-diaminopropionyl</td>
</tr>
<tr>
<td>DPP</td>
<td>Dipeptidyl peptidase</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase 4</td>
</tr>
<tr>
<td>DTS</td>
<td>DNA-templated synthesis</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>[E]</td>
<td>Enzyme concentration</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme code</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin-converting enzyme 1</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF-1</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>epi</td>
<td>Epimer</td>
</tr>
<tr>
<td>ESAC</td>
<td>Encoded self-assembling chemical (libraries)</td>
</tr>
<tr>
<td>ETC</td>
<td>Et cetera</td>
</tr>
<tr>
<td>ETH</td>
<td>Eidgenössische Technische Hochschule, Zürich, Switzerland</td>
</tr>
<tr>
<td>F</td>
<td>Faculty of Arts and Sciences</td>
</tr>
<tr>
<td>FMOC</td>
<td>Fluorenylmethyloxycarbonyl protecting group</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1 transcription factor</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence polarization</td>
</tr>
<tr>
<td>G</td>
<td>Glucose 6-phosphatase</td>
</tr>
<tr>
<td>GCGR</td>
<td>G-coupled glucagon receptor</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth-hormone-releasing hormone</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>Grb2/SOS</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GS</td>
<td>Glycogen synthase</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>H</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High precision liquid chromatography</td>
</tr>
<tr>
<td>HTRF</td>
<td>Homogeneous Time Resolved Förster Resonance Energy Transfer</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IC\textsubscript{50}\text{app}</td>
<td>Apparent half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin-Degrading Enzyme</td>
</tr>
<tr>
<td>IDE-CF</td>
<td>Cysteine-free insulin-degrading enzyme</td>
</tr>
<tr>
<td>IDMS</td>
<td>Isotope-dilution mass spectrometry</td>
</tr>
<tr>
<td>IGF-1/2</td>
<td>Insulin-like growth factors 1 and 2</td>
</tr>
<tr>
<td>II1</td>
<td>IDE inhibitor 1</td>
</tr>
<tr>
<td>IKKb</td>
<td>Inhibitor of nuclear factor kappa-B kinase subunit beta</td>
</tr>
<tr>
<td>I\textsubscript{MAX}</td>
<td>Inhibition maximum</td>
</tr>
<tr>
<td>INSL-3</td>
<td>Insulin-like 3</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
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<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
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<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
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<tr>
<td>J</td>
<td>c-Jun N-terminal kinases</td>
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<td>K</td>
<td>$K_M$</td>
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<td>$K_M^{app}$</td>
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<td>LogP</td>
<td>Octanol-water partition coefficient</td>
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<td>M</td>
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<td>mTORC1</td>
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<td>N</td>
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<td>NPH insulin</td>
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<td>Nu</td>
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<td>O</td>
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<td>PDI</td>
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<td>PDK1</td>
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<td>$P_e$</td>
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<td>PEG</td>
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<td>PGP</td>
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<td>PI3K</td>
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<td>pIR</td>
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<td>PKB</td>
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<td>PKC0</td>
</tr>
</tbody>
</table>
PMSF  Phenylmethylsulfonyl fluoride
PPAR-γ  Peroxisome proliferator-activated receptor gamma
PreP  Prolyl endopeptidase
PtdIns(4,5)P₂  Phosphatidylinositol (3,4)-bisphosphate
PTEN  Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
PTP1B  Protein-tyrosine phosphatase 1B
PTPL1  Protein tyrosine phosphatase non-receptor 13 (PTPN13)
PTT  Pyruvate tolerance test
PVDF  Polyvinylidene fluoride

Q  Q-TOF  Quadrupole time-of-flight

R  RCM  Ring-closing metathesis
RMSD  Root-mean-square deviation
RNA  Ribonucleic acid
RNAsi  Ribonucleic acid interference
RT-PCR  Reverse-transcriptase polymerase chain reaction
RTK  Receptor tyrosine kinase

S  [S]  Substrate concentration
s.c.  Subcutaneous
SAR  Structure-activity relationship
SEM  Standard error of the mean
SGLT-2  Sodium/glucose cotransporter 2
SHIP2  Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase
sNHS  Sulfo N-Hydroxysuccinimide
SOS  Son of Sevenless, guanine nucleotide exchange factors
Src  Proto-oncogene tyrosine-protein kinase c-Src
SSAR  Stereochemical structure-activity relationship

T  TAPP1/2  Pleckstrin homology domain-containing family A1/2
TBST  Tris-Buffered Saline with Tween 20
TCEP  Tris(2-carboxyethyl)phosphine
TFA  Trifluoroacetic acid
TGFα  Tissue growth factor alpha
THF  Tetrahydrofuran
THOP  Thimet oligopeptidase
Trt  Triphenylmethane protecting group
TSC1/2  Tuberous sclerosis proteins 1 and 2 complex, hamartin and tuberin

U  U/kg  Units per kilogram
USD  United States Dollar
USER  Uracil-Specific Excision Reagent
UV  Ultraviolet
Vₘₐₓ  Maximum velocity at saturating substrate concentration

W  WT  Wild-type

X  XPhos-Pd G3  (2-Dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl)[2-(2'-amino-1,1'-biphenyl)]palladium(II) methanesulfonate
Chapter 1

Introduction to Insulin-Degrading Enzyme and DNA-encoded platforms for small molecule discovery

1.1 – **Introduction: small molecule discovery using DNA-encoded libraries**

The discovery of small molecules capable of selectively modulating the activity of biological targets is a major focus of research endeavors in academic and pharmaceutical laboratories worldwide. Because this form of biological intervention has traditionally provided the most widely applicable route towards therapeutics, our society invests heavily on bioactive small molecule research and development, totaling over USD ~100 billion per year globally\(^1\)\(^2\). Furthermore, there is a continuous push to complement the limitations of small molecules with emerging therapeutic approaches based on biologic macromolecules (hormones, vaccines, antibodies, nucleic acids, genome editing enzymes, protein delivery, *etc.*\(^3\)\(^4\)) and cell-based treatments (transplantation, encapsulation, induced-pluripotent stem cells, *etc.*\(^5\)). Small molecule probe compounds that are active *in vivo* have also proven valuable to study biological processes that are inaccessible using other techniques, such as the validation of putative therapeutic targets through proof-of-concept experiments\(^6\)\(^7\)\(^8\). Biological interrogation with small molecules is especially powerful in combination with genome-wide association studies\(^9\), animal models of disease\(^10\), genetic modification tools\(^11\), modern analytical techniques\(^12\)\(^13\) and high-throughput sequencing\(^14\)\(^15\)\(^16\).

DNA-encoded libraries are an emerging technology that is being increasingly implemented to meet the continuous demand to discover novel small molecules\(^17\). Over the course of the last decade, these technologies have gained recognition as useful discovery platforms complementary to conventional high-throughput
screening\textsuperscript{18,19}, as well as fragment-based screening\textsuperscript{20,21}, transition state-based design\textsuperscript{22}, unbiased phenotypic screening\textsuperscript{23,24}, and natural product prospecting\textsuperscript{25-28}.

The inherent features of DNA as a high-density information polymer, which can be replicated and sequenced, make it an excellent medium for molecular barcoding of synthetic molecules\textsuperscript{17}. The theoretical diversity of a 20-mer oligonucleotide can be used to encode billions of synthetic molecules (4\textsuperscript{20}), yet the decoding and deconvolution of this mixture does not scale linearly with library size\textsuperscript{17}. The sub-femtomol sensitivity of PCR amplification enables the routine evaluation of minute quantities of DNA-barcodes, coupled with inexpensive and high-coverage DNA sequencing methods that provide the statistical power to detect even modest changes.

\textbf{Figure 1.1} | Scheme of an \textit{in vitro} selection using a DNA-encoded library, followed by high-throughput sequencing decoding and deconvolution.
in the relative composition of a DNA-encoded library\textsuperscript{17,29}. Therefore, large
collections of small molecules covalently tagged with DNA barcodes are amenable to
selection-based methods (Figure 1.1), in which all the library members are exposed
at the same time to a target of interest. This “one-pot” experiment is subsequently
manipulated, washed, or separated, in order to enrich the library members that
interact with the target, over the vast majority of irrelevant molecules. This
enrichment causes a telling shift in the library composition that is detected by high-
throughput sequencing pre- and post-selection (Figure 1.1)\textsuperscript{17,30,31}.

\textbf{Figure 1.2} Complementary methods to produce DNA-encoded libraries\textsuperscript{17}. 
In practice, many dozen targets of interest are evaluated in parallel selection experiments, then each PCR amplification step is carried out with indexing primers that track the target identity, and allow pooling for high-throughput DNA sequencing *en masse* (Figure 1.1)\(^{32}\). Only a minute fraction of a DNA-encoded library is exposed to the target (<1 pmol), and the eluate can be recycled multiple times. Hence, *in vitro* selection is a simple but powerful and efficient technology to discover small molecules, complementary to traditional assay-based screening (Table 1.1)\(^{18,19}\). The use of DNA-encoded libraries is not limited to affinity-based enrichment with immobilized targets\(^{30}\), since other forms of selective pressure can be the basis of selection\(^{33,34}\), including proximity-based PCR priming (reaction- or interaction-dependent)\(^{31,35}\), and potentially even gel-extraction of large circularized DNA could serve as the basis of *in vitro* selection\(^{36}\). The hits identified from high-throughput screening are resynthesized in scale, and subjected to a “counter-screen” or activity-based assay, which is not subject to the limitations of scale-up and optimization necessary for high-throughput screening\(^{18,19}\).

The predecessors of DNA-encoded libraries were biological materials that inherently link a phenotype to a genotype. For example, technologies such as RNA/DNA aptamers\(^{37}\), phage-display\(^{38}\), and mRNA-display\(^{39,40}\), share this quality and are amenable to *in vitro* selection methodologies. However, Nature does not provide equivalent machinery for the translation of DNA into synthetic molecules\(^{41-46}\) nor sequence-defined synthetic polymers\(^{47,48}\). The first theoretical framework to
Table 1.1 | Advantages and limitations of DNA-encoded libraries and *in vitro* selection for small molecule discovery.

<table>
<thead>
<tr>
<th><strong>In vitro selection, DNA-encoded libraries</strong></th>
<th><strong>Target-based high-throughput screening</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 pmol library aliquots stored at -80 °C, which can be recycled multiple times</td>
<td>plate collection requires upkeep and chemical resynthesis</td>
</tr>
<tr>
<td><em>en masse</em> one-pot binding interrogation, only hits validated by activity-based assays</td>
<td>discrete interrogation of each library member in activity-based assays</td>
</tr>
<tr>
<td>routine off-the-shelf activity-based assays can be used to counter-screen hits</td>
<td>extensively optimized activity-based assays are required (Z-factor &gt; 0.5)</td>
</tr>
<tr>
<td>requires low-tech equipment (pipettes, magnets, etc.) and access to inexpensive high-throughput DNA-sequencing facility</td>
<td>requires automated screening infrastructure, specialized instruments for reading each assay, and compound management system</td>
</tr>
<tr>
<td>library size can be increased with a complementary DNA barcode set</td>
<td>plate collection requires upkeep and resynthesis</td>
</tr>
<tr>
<td>synthesis is technically more challenging but effort does not scale linearly with library size</td>
<td>combinatorial or diversity-oriented synthesis required for each library member</td>
</tr>
<tr>
<td>library of sizes &gt;10⁶–10⁷ have been routinely evaluated with targets of interest, libraries as large as 10⁸ have been reported</td>
<td>individual screening campaigns of 10⁵–10⁶ compounds from collections comprising &lt;10⁷ compounds</td>
</tr>
<tr>
<td>multiple replicates and variations in selection conditions can be evaluated (washing stringency, ligand elution, additives, etc.)</td>
<td>usually limited in resources to run one or two replicates, using one assay condition</td>
</tr>
<tr>
<td>repertoire of building block coupling reactions is limited to DNA- and water compatible reactions (needs improvement)</td>
<td>extensive repertoire of combinatorial building block coupling reactions</td>
</tr>
<tr>
<td>limited chemotypes in each library, building blocks and appendages provide diversity</td>
<td>small collections with multiple chemotypes effectively explore ‘chemical space’</td>
</tr>
<tr>
<td>incomplete building block coupling may difficult barcode interpretation, only the Liu macrocycle library has a built-in purification</td>
<td>the purity and identity of the hit compounds is corroborated afterwards</td>
</tr>
</tbody>
</table>

encode synthetic peptides using DNA was first provided by Brenner and Lerner⁴⁹. They proposed the “recording” of a sequence of split-and-pool synthetic steps by
applying consequent steps of DNA synthesis. While visionary, this strategy posed significant restrictions and technical challenges\textsuperscript{50,51}. Therefore, multiple groups have developed novel and improved strategies for the production of DNA-encoded libraries, which offer complementary solutions for bringing together combinatorial chemistry or “split-and-pool” chemical synthesis and DNA barcodes (Figure 1.2)\textsuperscript{17}.

The three conceptually distinct strategies that emerged over the past decade are i) “DNA-templated synthesis”, developed by the Liu group\textsuperscript{41-46,52,53}, ii) “DNA-recording” of chemical steps using several methods that improve on the original technique\textsuperscript{51,54-57}; and more recently iii) “DNA-routing” of reagents into spatial segregation for split-and-pool chemical synthesis\textsuperscript{58-61} (Figure 1.2). In addition, the approach known as encoded self-assembling combinatorial (ESAC) libraries\textsuperscript{62}, is a variation on the use of base-pairing to display synthetic fragments in close proximity, under the assumption that they may bind a target as one entity (Figure 1.2).

The general approach to “DNA-recording” requires iterative extension steps of the oligonucleotide barcode catalyzed by a DNA polymerase or DNA ligase enzyme to record each chemical reaction\textsuperscript{17}. Hence, the growing barcode records the history of the reagents as they are subjected to split-and-pool chemical synthesis. Similarly, “DNA-routing” involves using solid supports tagged with DNA sequences that use base-pairing hybridization to enforce the spatial segregation of complementary DNA-barcoded materials into reaction vessels\textsuperscript{58}, which is essentially a DNA-directed
Representative reactions compatible with DNA-encoded libraries:

- amide/ester coupling
- Diels-Alder [4+2]
- Huisgen “click” [3+2]
- Nu-Ar substitution
- 1,4-addition
- Wittig olefination
- imine/hydrazone condensations
- reductive amination
- S- or N-alkylation
- mild acid/base treatment
- periodate diol cleavage
- thiol redox

**Figure 1.3** | (A) Representative DNA-encoded libraries that resulted from DNA-recorded and DNA-routed methods. (B) Chemical reactions that have been carried out in the context of DNA-encoded libraries or that are predicted to be compatible.

These technical advances have enabled the production of libraries comprised of $10^6$ to $10^8$ compounds suitable for *in vitro* selection experiments (**Figure 1.3**). Each of these libraries shows ingenuity in solving to some degree the limitations discussed in Table 1.1, including the repertoire of compatible chemistry, and the diversity of chemotypes and appendages, to promote an effective exploration of the ligands ‘chemical space’ beyond relying on the massive size of the DNA-encoded library (**Figure 1.3**).

David Liu and co-workers discovered that nucleic acid hybridization is a versatile and generally effective method to promote bond-forming reactions by
increasing the effective molarity of chemical reactants tagged with DNA. The DNA templates and DNA-reagents share a codon-anticodon relationship reminiscent of ribosomal protein synthesis. The design of a “codeset” of mutually orthogonal codons enabled ordered-multistep DNA-guided translation of libraries of templates with multiple reagents in a single solution with high sequence fidelity. Gartner, Tse, and Liu described the first DNA-templated small molecule library by sequentially applying three amide condensation steps and one Wittig macrocyclization (Figure 1.4), a technology commercialized by Ensemble Therapeutics\textsuperscript{17}.

**Figure 1.4** | Ordered multistep DNA-templated translation of a mixture of DNA barcodes into a pilot 13,824 membered DNA-encoded library of macrocycles. Adapted from ref. 63.
Figure 1.5 | Building blocks used in the 13,824 library of macrocycles, adapted from ref. 63.

Subsequent advances in DNA-templated synthesis enabled the production of a 13,824-membered macrocycle library comprising non-proteinogenic amino acid building blocks (Figure 1.5)\(^63\) suitable for *in vitro* selection and small molecule discovery\(^32\), which was used in this dissertation (Chapter 2)\(^64\). The promising results from this prototype library have prompted the Liu group to expand the codeset of DNA templates to produce a 256,000-membered library of macrocycles\(^63\).

Whereas the DNA-encoded library efforts shown in Figure 1.3 have focused on maximizing library size\(^57,61\), the pilot size 13,824-membered DTS macrocycle library\(^63\) has proven very competitive in the number of hit reported and target
versatility\textsuperscript{32,64}. These medium-sized peptidic macrocycles occupy an intermediate chemical space between small molecule and small proteins\textsuperscript{65-67} (e.g. peptidic hormones, toxins, venoms, and stapled peptides), and may result in lower per-atom ligand efficiency than in traditional small molecules. Furthermore, macrocycles are generally thought as privileged structures due to their biophysical and pharmacological properties discussed in detail in Chapter 3 (Section 3.1)\textsuperscript{65-67}.

Demonstrating the generality of the concept of DNA-templated synthesis (DTS) the Liu lab has applied it to the synthesis of DNA-encoded libraries\textsuperscript{63}, a system for discovery of bond-forming reactions\textsuperscript{34}, and a system for enzyme-free translation of sequence-defined synthetic polymers\textsuperscript{48}. Furthermore, over the course of just a few years \textit{in vitro} selections using the DNA-templated macrocycle library has led to the identification of potent and selective \textit{Src} kinase inhibitors developed by Ralph Kleiner\textsuperscript{17,32}, and the IDE inhibitors described in this work.

\textbf{1.2 – The need for therapeutic validation of Insulin-Degrading Enzyme}

The controversial term “undruggable” has surfaced as a descriptor for targets displaying biophysical properties that repeatedly frustrate small molecule development and/or screening efforts\textsuperscript{68,69}, however, with continued effort many targets once considered “undruggable” have been conquered\textsuperscript{70,71}. An important amount of resources in academia and industry are devoted to identifying small molecules for these targets, because they have been strongly implicated in disease mechanisms based on evidence from multiple complementary techniques and proof-
of-principle experiments\textsuperscript{69,71} (e.g. knock-out, knock-down, mutagenesis, overexpression, bump-hole, targeted degradation, \textit{etc.})\textsuperscript{72}. Meanwhile, another class of untapped targets have proven intractable for proof-of-concept validation, or attempts to evaluate their physiological roles in disease mechanisms have led to obscure or unexplainable \textit{in vivo} phenotypic observations, which further discouraged researchers from developing bioactive small molecule probes\textsuperscript{6,73}. Insulin-degrading enzyme (IDE) is a long-standing member of this class of “neglected” putative therapeutic targets\textsuperscript{11,71,74}.

The discovery of insulin and its use to treat diabetes was a turning point in the history of medicine, for which Frederick Banting and John Macleod were awarded the Nobel Prize in 1923\textsuperscript{75}. Long before we understood the structure of insulin\textsuperscript{76} or molecular details of hormone signaling, researchers reasoned that insulin should be subject to endogenous inactivation\textsuperscript{77}, and that blocking this process could mimic the supplementation of insulin. Advances in methods for the detection and measurement insulin\textsuperscript{78}, enabled the search for the degradation pathway\textsuperscript{79}. In a seminal 1949 publication, the English physician I. Arthur Mirsky\textsuperscript{80} reported the biochemical fractionation of rat and cattle livers to identify the first enzyme capable of degradation of radiolabeled insulin\textsuperscript{81}. Mirsky gave this activity the name \textit{insulinase}, and over the decades it has been referred to as \textit{neutral thiol peptidase}, \textit{insulysin}, and more recently as Insulin-Degrading Enzyme, or IDE (EC 3.4.24.56, GO:0004231)\textsuperscript{82}.

We currently have a sophisticated level of understanding of the structural and biochemical features of IDE (\textbf{Figure 1.6}). IDE is a 110 KDa globular zinc-dependent
metalloprotease of an evolutionary distinct origin, evidenced by an inverted set of zinc-binding residues (HxxEH motif)\textsuperscript{83}, which is an example of convergent evolution relative to the HExxH motif of other metalloprotease classes. Orthologs of IDE have been found throughout the tree of life, including bacteria and fungi\textsuperscript{84}, and it is highly conserved in mammals (>95% amino acid identity between human and mouse

![Image](image.png)

**Figure 1.6** | Unique structural and biochemical features of IDE. (A) Organization of IDE domains (domains 1, 2, 3, and 4 are colored green, blue, yellow, and red, respectively). (B) Comparison of the internal chambers (red volumes) of IDE, preprolyl peptidase PreP and neprilysin. Other colors highlight the hinges regions (yellow) connecting the N-terminal (blue) and C-terminal (grey) halves. (C) Location of the catalytic site and exo-site on the N-terminal half of IDE. (D) Network of hydrogen-bonds between the distal domains 1 and 4 that activate the deprotonation of water by Zn\textsuperscript{2+} when IDE is in the closed state. Adapted from reference 86.
orthologs, Chapter 2). The main isoform of human IDE\textsubscript{(42-1019)} makes a cooperative homodimer\textsuperscript{85}, and each monomer is comprised of four structurally homologous \(\alpha\beta\) roll domains that together define a massive internal chamber (Figure 1.6)\textsuperscript{86}. Only domain 1 has a zinc-dependent catalytic site, and the other domains appear to be ancient remnants of gene duplication events that led to the evolution of the cavity (<25\% sequence similarity).

The IDE chamber has an approximately triangular prism shape\textsuperscript{86} (base dimensions 35\(\times\)34\(\times\)30 Å and a height of 36 Å) with a total cavity volume of \(~13,000\) Å\(^3\) (Figure 1.6A). This cavity perfectly encapsulates the entire insulin hormone (51 residues) and is the largest chamber defined by a single polypeptide encoded in our genome, over twice the volume of the prolyl endopeptidase (PreP) or neprilysin (NEP) active sites (Figure 1.6B). An extended loop “hinge” of 28 residues connects domains 2 and 3 to allow the N- and C-terminal halves to open (Figure 1.6B)\textsuperscript{86,87}. However, the catalytic site of domain 1 is only activated in the closed state, owing to hydrogen-bond contributions from domain 4 (Figure 1.6D)\textsuperscript{86}.

Unlike most proteases, the peptide substrates cleaved by IDE lack a consensus sequence. Instead, the biophysical features of IDE dictate whether a substrate can interact and become entrapped in the chamber\textsuperscript{86,88}. These features are: \(i\) overall size and shape (<51 residues); \(ii\) positive charge repulsion at the substrate C-terminus, \(iii\) binding of hydrophobic patches along the catalytic cleft; and \(iv\) the ability of the substrate to make cross-\(\beta\)-sheet interactions with an exposed region of the IDE chamber known as the \textit{exo}-site (Figure 1.6C)\textsuperscript{86-88}. Notably, this is a characteristic of
amyloidogenic peptides that are effectively degraded by IDE (Aβ, amylin, and insulin)\textsuperscript{89} suggesting this aggregating behavior may have evolved to promote their degradation.

The biochemical properties of IDE chamber and the unique substrate recognition mechanism\textsuperscript{86-90} has posed a significant challenge to understanding the repertoire of \textit{in vivo} substrates beyond insulin\textsuperscript{82}. In fact, IDE was identified before most hormones had been discovered, and over the past three decades multiple research groups have found that IDE is capable of cleaving a wide range of peptide substrates \textit{in vitro} (Figure 1.7A) for which experimental validation \textit{in vivo} (or \textit{ex vivo}) has not previously been possible. The kinetic parameters for most putative substrates were measured by approximate methods (Table 1.2). Nevertheless, it is possible to recognize two broad classes: those substrates of high affinity for the IDE chamber (low $K_M$, e.g. insulin and homologs, Aβ peptides, CGRP group, ANP, \textit{etc.})\textsuperscript{86-90}, and substrates that interact with low affinity but that are rapidly cleaved ($K_M >1 \mu M$, e.g. glucagon, bradykinin, somatostatin, \textit{etc.})\textsuperscript{91}. Despite decades of research, these putative substrates have only been shown to be cleaved \textit{in vitro} by purified IDE (Table 1.2).
Figure 1.7 | (A) Classes of structurally distinct hormones cleaved by IDE in vitro assays. (B) Known non-substrates of IDE are labeled with a cross (✗), and substrates for which there is evidence in vivo are labeled with a tick (✔). (C) Conditions that need to be met for a substrate to be functionally relevant in vivo, thus making a cursory assignment invalid.

Steady-state kinetic parameters are not predictive of enzymatic physiological function in vivo⁹², given that encounters between enzyme and substrates often occur in equimolar amounts (<K_M), and compartmentalization can make these interactions greatly enhanced by co-localization⁹², or inherently impossible by separation⁸². Furthermore, alternative enzymatic degradation pathways may contribute to the clearance of most putative IDE substrates (Table 1.2), potentially limiting their physiological function in a redundant or epistatic manner that may overshadow a role of IDE activity. Hence, an assignment of candidate IDE substrates has not been
Literature survey of putative IDE substrates identified by in vitro assays.

<table>
<thead>
<tr>
<th>IDE substrates <em>in vitro</em> (reference)</th>
<th>Alternative degrading enzymes (Brenda db)</th>
<th>( k_{cat} ) (min(^{-1}))</th>
<th>( k_{m} ) (( \mu )M)</th>
<th>( k_{cat}/k_{m} )</th>
<th>IC(_{50}) (( \mu )M)</th>
<th>X-ray struct.</th>
<th>IDE substrate <em>in vivo</em> (ref.)</th>
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<td>insulin (^{81,82})</td>
<td>lysosomal proteases, disulfide reductase</td>
<td>1.52</td>
<td>&lt;0.03</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>KO study (^{93,94})</td>
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<tr>
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<td>NEP, cathepsin D</td>
<td>52</td>
<td>1.23</td>
<td>43</td>
<td>-</td>
<td>2G47</td>
<td>KO study (^{90,92,97})</td>
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<tr>
<td>calcitonin-gene related peptide (^{98})</td>
<td>ECE, PREP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>NEP, nardilysin, DPP-IV, cathepsins B and C</td>
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<td>3.46</td>
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<td>weak</td>
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<td>this thesis</td>
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<td>-</td>
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<td>n/a</td>
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<td>bradykinin (^{105})</td>
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<td>-</td>
<td>4.2</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>NEP, fibroblast activation protein-(\alpha)</td>
<td>-</td>
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<td>&gt;1</td>
<td>3N56</td>
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<td>DPP-IV, NEP</td>
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<td>11.9</td>
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<td>39.4</td>
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<td>0.33</td>
<td>60.5</td>
<td>0.01</td>
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</table>

(*) \( K_M \) estimated based on IC\(_{50}\) for inhibition of insulin degradation. Abbreviations: \(\alpha\beta\) = amyloid beta; TGF-alpha = transforming growth factor-alpha; IGF = insulin-like growth factor; NP = natriuretic peptide; GRF = gastrin release factor; NEP = nardilysin; PREP = prolyl endopeptidase; ECE = endothelin converting enzyme; THOP = thimet oligopeptidase; ACE = angiotensin-converting enzyme; DPP = dipeptidylpeptidase. Known non-substrates of IDE include glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), epithelial growth factor 1 (EGF-1), pro-insulin, and C-peptide.
possible. Only two substrates, beyond insulin, had been functionally linked to IDE activity in vivo (Figure 1.7A) based on the elevated levels measured in knockout IDE−/− mice, Aβ was reported by Selkoe and coworkers93, and CGRP was reported the Saghatelian group98. Recently, human genome-wide association studies (GWAS) have identified predisposing and protective variants of the IDE locus linked to Type-2 Diabetes110-114 and Alzheimer’s disease114-118. These studies suggest an evolutionarily conserved functional connection between IDE and glucose regulation.

Figure 1.8 | (A) Insulin signaling cascade, showing the potential of IDE-mediated degradation to modulate insulin signaling on the insulin receptor (IR). (B) Effects of insulin resistance on the insulin signaling cascade: IR gene down-regulation (↓), reduced tyrosine kinase activity (↓), increased IR-inhibitory serine phosphorylation (↑), and physiological compensation by elevated insulin levels (↑). The role of approved anti-diabetic treatments are shown, and hypothetical untapped drug targets are highlighted, including IDE. Adapted from ref. 110.
in humans, and also corroborate that IDE activity is one of the enzymatic mechanisms that contribute to Aβ clearance in the brain\textsuperscript{93}.

The biochemical characterization of IDE-mediated insulin degradation \textit{ex vivo} and \textit{in vivo} was carried out by Duckworth, Hamel, Bennett, and others over the decades 1980–2000\textsuperscript{82}. These studies support a role for IDE as the primary enzymatic mechanism for insulin clearance in the body, complemented by minor contribution from redundant non-specific pathways including protein-disulfide isomerase (PDI), and lysosomal acidic proteases (e.g. cathepsin D)\textsuperscript{82}. The insulin cleavage pattern of IDE \textit{in vitro} matched the cellular and endosomal degradation products, suggesting that IDE is the rate limiting step of the pathway of insulin degradation (\textbf{Figure 1.8})\textsuperscript{82}.

Although IDE is expressed ubiquitously in all cells, the liver and kidneys are the most important sites of insulin degradation\textsuperscript{82}. Insulin is secreted by the pancreas into portal circulation, and up to half may be removed by first-pass transit through the liver by receptor-mediated internalization\textsuperscript{119}. This process provides the first opportunity for IDE-mediated degradation. Subsequently, the kidney is responsible for removal of approximately 50\% of insulin from systemic circulation, followed by the bulk of muscle tissue that accounts the remaining insulin clearance\textsuperscript{120}. Although relatively little insulin is ultimately excreted in urine, the processes of endocytosis, pinocytosis, and transcytosis exposes insulin to IDE, as well as the lysosomal
Figure 1.9 | Phenotypic disconnect between (A) cell-based observations of IDE inhibition versus (B) studies using IDE−/− mice studies due to a metabolic compensatory phenotype. Figures adapted from references 109 and 111, respectively. The effect of i.p. glucose on IDE−/− versus IDE+/+ mice was previously reported, and the experiment shown here was repeated in collaboration with Edwin Homan in the Saghatelian group.

degradation pathway. The combination of these processes contributes to the short plasma half-life of insulin in vivo (4–6 min), that allows for swift responses to changes in blood sugar. Hence, researchers have speculated for at least six decades that endogenous insulin signaling could be potentiated to improve glucose tolerance
by inhibiting IDE-mediated degradation in the liver, kidneys, and insulin-responsive tissues (Figure 1.8)\textsuperscript{81,82,123}.

In cell culture, IDE overexpression leads to increased insulin degradation\textsuperscript{124}, and blocking IDE using a zinc-chelating inhibitor leads to elevated IR phosphorylation and downstream signaling\textsuperscript{121} (Figure 1.9). These studies strongly suggest that endosomal IDE-mediated degradation modulates insulin signaling activity at the cellular level\textsuperscript{125} (Figure 1.8). Based on the known biochemistry of IDE, inhibition of this enzyme is expected to elevate insulin levels and augment the response to glucose\textsuperscript{81,82}. To validate this hypothesis using a surrogate genetic model, the Selkoe group produced mice lacking a functional IDE gene (IDE\textsuperscript{−/−} mice). The IDE\textsuperscript{−/−} mice were shown to have elevated insulin levels, but counterintuitively these animals exhibited impaired, rather than improved, glucose tolerance\textsuperscript{93,94}. Physiological studies with IDE\textsuperscript{−/−} mice concluded that chronic elevation of insulin in these animals may result in a compensatory lowering of insulin receptor expression levels, which leads to impaired glucose clearance following a glucose load\textsuperscript{93,94}. Hence, the insulin resistance phenotype may also explain the elevated insulin (hyperinsulinemia, Figure 1.8). This model raises the possibility that in the absence of such compensatory effects, acute inhibition of IDE may lead to improved physiological glucose tolerance by enhancing glucose uptake at multiple insulin-responsive tissues\textsuperscript{82,123}. These observations highlight the need for a selective small-molecule IDE inhibitor to characterize the biological functions and therapeutic relevance of this enzyme in
vivo⁶, uncoupled from confounding physiological adaptations that arise in IDE knock-out mice⁹³,⁹⁴.

1.3 – The search for the first physiologically active IDE inhibitor

Research into the biosynthesis, secretion, and signaling of insulin has led to the development of multiple treatments for diabetes (Figure 1.10). In contrast, there is no therapeutic strategy that deals with the stabilization of endogenous insulin elicited in response to blood sugar. The dynamic interplay between the production and proteolytic degradation of peptide hormones is a key mechanism underlying the regulation of human metabolism. Inhibition of the peptidases and proteases that degrade these hormones can elevate their effective concentrations and augment signaling. The resulting insights can lead to the development of novel therapeutics¹²⁶, for example dipeptidyl peptidase 4 (DPP4) inhibitors are anti-diabetic.

<table>
<thead>
<tr>
<th>insulin supplementation</th>
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<th>insulin sensitizers</th>
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<tr>
<td>insulin (humulin-R)</td>
<td>sulfonylureas</td>
<td>metformin</td>
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<td>fast-acting (lispro)</td>
<td>glibenclamide</td>
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<td>long-acting (NPH insulin)</td>
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<tr>
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<tr>
<td></td>
<td>triamteren</td>
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</table>

**Figure 1.10** | Anti-diabetic treatments classified based on their impact on insulin signaling.
drugs that increase the concentration of the insulin-stimulating hormone glucagon-like peptide 1 (GLP-1), resulting in elevated insulin concentrations and lower blood glucose levels\textsuperscript{127}. Despite 60 years of speculation that inhibiting the degradation of insulin could treat diabetes, the relationship between IDE and glucose homeostasis remains unclear due to the lack of IDE inhibitors that are active \textit{in vivo}\textsuperscript{81,82}. Indeed, the blockbuster DPP4 inhibitor drugs provide a template for the development of IDE-based treatments, in particular because DPP4 also cleaves a complex repertoire of substrates \textit{in vitro}\textsuperscript{128,129}. This is also the case for the peptidases angiotensin-converting enzyme\textsuperscript{130} (ACE) and neprilysin\textsuperscript{131,132} (NEP) that are targets of anti-hypertensive drugs.

In contrast to genetic approaches, small molecules can probe a target’s therapeutic potential in a manner uncoupled from compensatory developmental mechanisms, with dose and temporal control.\textsuperscript{133,134} A selective and physiologically active small-molecule IDE inhibitor would therefore enable the validation of IDE as a potential anti-diabetes target and provide a starting point for the development of a new class of diabetes therapeutics (\textbf{Figure 1.8}). There is ample precedent for metalloprotease inhibitors that target the catalytic site\textsuperscript{135}. However, a central challenge is specific inhibition of one homolog over >185 metalloproteases encoded in the human and mouse genomes\textsuperscript{136}. Three classes of weak or non-specific IDE inhibitors have been previously identified \textit{ad hoc} and by high-throughput screening (\textbf{Figure 1.11A-C})\textsuperscript{121}. These comprised multiple metal-binding chemotypes\textsuperscript{121,137,138},
thiol reactive analogs\textsuperscript{121,139}, and the depsipeptide antibiotic bacitracin that acts as a weak competitive inhibitor\textsuperscript{82}. More recently, the research groups of Professors

![Diagram of inhibitors](image)

**Figure 1.11** | Known inhibitors of IDE are weak or non-selective. (A) Multiple metal-binding chemotypes that inhibit IDE identified \textit{ad hoc} and by high-throughput screening\textsuperscript{121,137,138}. (B) Non-specific thiol reactive analogs identified by high-throughput screening\textsuperscript{121,139}. (C) Structure of the major component of the depsipeptide antibiotic bacitracin that acts as a weak competitive inhibitor of IDE\textsuperscript{82}. (D) Zinc-chelating IDE inhibitor \textit{II1} comprising a hydroxamic acid (red) connected to a linear peptide\textsuperscript{121}. (E) Recently reported thiol-reactive benzoisothiazolidone covalent IDE inhibitor \textit{ML345} identified from a high-throughput screening effort\textsuperscript{139} (see the analog in entry 3 of panel B).
Leissring and Selkoe reported the design of zinc-chelating inhibitors of IDE comprising a hydroxamic acid connected to a linear peptide or retro-inverso peptide scaffold (Figure 1.11D)\textsuperscript{121,137}. The inhibitor II1 was reportedly unstable \textit{in vivo}\textsuperscript{94,139}, and displays modest specificity for IDE (Chapter 2), given that such compounds derive potency from the zinc-chelating hydroxamic acid group, which has the potential to interact strongly with other metalloproteases and metal-binding proteins\textsuperscript{140}. More recently, a thiol-reactive covalent inhibitor ML345 was identified from a high-throughput screening effort\textsuperscript{139} (Figure 1.11E, this is a benzoisothiazolidone analog of entry 3 in panel B). This compound is modestly selective for IDE Cys819 over other thiols, however, the specificity profile was only tentatively determined <2 µM, and \textit{in vivo} activity was not reported\textsuperscript{139}.

IDE inhibitors that are active \textit{in vivo} are therefore needed to elucidate IDE’s physiological roles and to determine its potential to serve as a target for the treatment of diabetes. The Liu and Saghatelian labs therefore initiated a multidisciplinary collaboration integrating synthetic chemistry, chemical biology, structural biology, and animal physiology to discover and characterize small molecules that inhibit IDE.

1.4 – Outline of this thesis

In this thesis I describe the development of a new small-molecule IDE inhibitor identified from a DNA-templated library (Chapter 2) that is potent and inhibits IDE with high specificity through a novel binding pocket outside the catalytic site. We demonstrated this IDE inhibitor is active \textit{in vivo} and validated it as a probe tool
(Chapter 3), which was the key to perform the first therapeutic validation experiments of acute IDE inhibition in mice (Chapter 4). Following up on the observations of the physiological consequences of inhibiting IDE on multiple hormone substrates modulated in vivo, we developed a second new class of substrate-selective IDE inhibitors that obstruct insulin degradation by IDE, but accommodate for IDE-mediated cleavage of other substrates, especially glucagon (Chapter 5).

The findings reported in this thesis offer new insights into the physiological roles of IDE, indicate specific strategies for modulating metabolic hormone physiology through IDE inhibition in combination with synergistic drugs, and provide an unprecedented approach to selectively inhibit IDE-mediated degradation of insulin over other substrates, rather than merely blocking the enzyme. More broadly, this work heralds the development of IDE-based therapeutics for the treatment of high blood sugar by selectively potentiating insulin, and favorably shifting the insulin-glucagon ratio, which has broad implications for glucoregulation in multiple stages of Type-2 Diabetes.

1.5 – References


Chapter 2

Identification of potent and highly specific IDE inhibitors from a DNA-templated library of macrocycles


Contributions: I synthesized analogs, performed the specificity studies, and determined the mode of inhibition of the inhibitors described in this chapter. Dr. Ralph E. Kleiner performed the in vitro selection and validated the hits. Zachariah H. Foda solved the X-ray structure of IDE-6b. Help from other colleagues is acknowledged in the text.
2.1 – \textit{In vitro} selection reveals a series of potent macrocyclic IDE inhibitors

To discover small-molecule binders of IDE, the Liu and Saghatelian labs undertook a collaboration to perform \textit{in vitro} selections on the previously described DNA-templated library of 13,824 synthetic macrocycles\textsuperscript{1,2} for the ability to bind immobilized mouse IDE (\textbf{Figure 2.1}). The protein N-His\textsubscript{6}-mIDE\textsubscript{(49-1019)} for this experiment was provided by Whitney Nolte in Alan Saghatelian’s group.

This library of 13,824 DNA-templated macrocycles\textsuperscript{1,2} had previously yielded highly selective \textit{Src} kinase inhibitors\textsuperscript{3,4}. The unbiased design features and structural diversity of the macrocycle library suggested it may also contain ligands for other classes of proteins. The two independent IDE selection experiments performed by Ralph Kleiner resulted in the identification of six putative IDE-binding macrocycles that share common structural features (\textbf{Figure 2.2}).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{enrichment.png}
\caption{Enrichment results from two independent \textit{in vitro} selections against mouse \textit{N}-His\textsubscript{6}-IDE using the DNA-templated macrocycle library\textsuperscript{18}. The numbers highlight compounds enriched at least 2-fold in both selections.}
\end{figure}
These six macrocycles were synthesized without their oligonucleotide templates and without the 5-atom macrocycle-DNA linker using a combination of solid- and solution-phase synthesis as either of two possible cis- or trans-alkene stereoisomers. Biochemical assays revealed that four of the six trans-macrocycles assayed were bona fide inhibitors of IDE with IC$_{50}$ $\leq$ 1.5 $\mu$M (Figure 2.2) using the commercially available fluorogenic peptide Mca-RPPGFSAFK(Dnp)-OH (R&D).

I corroborated these results by performing an in vitro selection against human IDE using the same DNA-templated library, which revealed enrichment of the barcodes for hits 1, 2, 5, and 6 previously identified as binders of mouse IDE, as well as structurally related macrocycles, which is to be expected given that mouse and human share IDE 95% amino acid identity (see Methods, Figure 2.15). The results of the second in vitro selection using human IDE (Figure 2.3) are consistent with the low to sub-micromolar IC$_{50}$ values of the trans-isomers 1b, 2b, 5b and 6b, however, human IDE did not produce enrichment of the structures 3 or 4, consistent with the relatively modest inhibitory activity of these macrocycles of either cis- or trans-alkene configuration against the human ortholog of IDE. The most active inhibitor among the library members enriched in all three selections, the 20-membered macrocycle 6b, potently inhibits human IDE (IC$_{50}$ = 60 nM).
Figure 2.2 | Structures of IDE-binding macrocycles 1–6 decoded from DNA library barcodes corresponding to building blocks A, B, C and D (see Chapter 1, Figure 1.5). The cis- and trans-alkene isomers are labeled ‘a’ and ‘b’, respectively.

2.2 – Complementary biochemical assays for IDE inhibition

The inhibitory activity of 6b to on IDE proteolytic activity was confirmed by four complementary in vitro assays, which reveal the cleavage of multiple substrates and use different sources of IDE. The most useful method for routine IC₅₀ determination and comparison among 6b analogs is the known fluorogenic peptide assay, using the decapeptide Mca-RPPGFSAFK(Dnp)-OH. This is a weak affinity
Enrichment results from a third independent *in vitro* selection against human N-His$_6$-IDE$_{(49-1019)}$ using the DNA-templated macrocycle library$^{18}$. The labeled red points represent the barcodes for the structures 1 – 6 observed in previous selections, and the green points highlight barcodes related to the sequences A12-B8-Cx-Dx or Ax-B8-C6-Dx.

substrate for IDE ($K_M \approx 5$ µM) derived from the sequence of Bradykinin (RPPGFSPFR), which is also a low affinity *in vitro* substrate of IDE (Chapter 1, Table 1.2) and multiple other enzymes (ECE-1, ACE, Neprilysin, and Cathepsins). The fluorogenic peptide assay revealed the IC$_{50}$ potency of 6b was comparable for the His$_6$-tagged main isoforms of mouse and human IDE$_{(49-1019)}$ (*Figure 2.4*), using protein expressed from *E. coli* in house, or purchased from R&D produced in insect cells (*Spodoptera frugiperda* Sf 21).
Figure 2.4 | Cleavage of the fluorogenic substrate peptide Mca-RPPGFSAFK(Dnp)-OH by human and mouse IDE in the presence of inhibitors (A) 6b and (B) 6bK (C = Lys).

Figure 2.5 | (A) Homogeneous time-resolved FRET (HTRF) assay for IDE-mediated insulin degradation in the presence of 6b analogs. (B) Assays comparing the EC$_{50}$ of 6bK inhibition of IDE cleavage of insulin, glucagon, and a fluorogenic amylin analog (see Chapter 5).

To confirm inhibition of IDE cleavage activity on relevant metabolic hormone substrates, I optimized dual-antibody Homogeneous Time-Resolved FRET (HTRF) assays against insulin and glucagon. This endpoint assays reveal the extent of IDE-
mediated cleavage following a short incubation and quenching with excess Ii1 to stop the reaction (Figure 2.5). I investigated 6b and the analog 6bK, which has the glutamine residue substituted by lysine. The inhibitors 6b and 6bK achieved complete inhibition of IDE cleavage of insulin at concentrations above 10 µM with \( EC_{50} = 0.1 \) µM, which is consistent with the data derived using the fluorogenic decapeptide. Furthermore, 6bK inhibited IDE-mediated degradation of glucagon and amylin, with similar \( EC_{50} \) as insulin, suggesting that the macrocycles obstruct IDE in

Dr. Yun-Gon Kim in Alan Saghatelian’s group corroborated that 6b inhibits the cleavage of calcitonin-gene related peptide (\( \alpha \)-CGRP\(_{1-37} \)) added to diluted mouse plasma harboring endogenous IDE, detected by LC-MS assay\(^5\) (Figure 2.6).

**Figure 2.6** | LC-MS assay for *ex vivo* degradation of \( \alpha \)-CGRP (10 µM) by endogenous IDE in mouse plasma in the presence of 6b. Plasma from IDE\(^{-/-}\) knockout mice and WT plasma treated with inhibitor Ii1 were used as controls.
2.3 – Structure-activity relationship of macrocyclic IDE inhibitors

We devised general synthetic routes towards cis- and trans-alkene macrocycles using on-resin amide macrocyclization (Figure 2.7). For convenience we replaced the Wittig reaction that produces unreliable yields of cis- and trans-isomers, which are often difficult to purify\(^3\). We synthesized and biochemically assayed 30 analogs of 6b in which each building block was systematically varied to elucidate structure-activity relationships of these inhibitors (Figure 2.8). Consistently with the results of in vitro selection using human IDE (Figure 2.3) these studies revealed the structural requirements required for potent IDE inhibition by this new class of molecules, including a trans-fused 20-membered macrocycle, the stereochemistry of the macrocycle substituents, and the size, shape, and hydrophobicity of the A and B building blocks (Figure 2.8).

The IDE chamber harbors eight exposed cysteine residues, therefore non-selective covalent mode of inhibition has been observed in multiple small molecule development efforts (Chapter 1, Figure 1.11). Using two 6b analogs, I was able to rule out a covalent-adduct inhibitory mechanism through either Michael addition or a Schiff base by synthesizing the analogs 6c (reduced linker, IC\(_{50} = 570\) nM) and 7 (lacking the ketone, IC\(_{50} = 60\) nM). The fact that IDE is subject to inhibition by non-specific covalent modifiers as well as zinc chelation has hindered the identification of selective IDE inhibitors using high-throughput screening assays for over a decade. Recently, the first covalent IDE inhibitor ML345 (in vitro IC\(_{50} \sim 200\) nM) was
published by the NIH Molecular Libraries Program, but \textit{in vivo} data has not been reported to date.

In contrast to the strict requirements at positions A and B of the 6b structure, different building blocks were tolerated at position C (Figure 2.7). Based on these results, we identified the inhibitor 6bK (IC\textsubscript{50} = 50 nM) as an ideal candidate for \textit{in vivo} studies because it exhibits enhanced water solubility relative to 6b, and can be readily synthesized on gram scale (Chapter 3, Figure 3.1\textsuperscript{6}).

The original point of attachment of 6b to the oligonucleotide DNA template and 5-atom linker tolerated appendages such as a biotin handle (30, IC\textsubscript{50} = 80 nM) or fluorescein (31, IC\textsubscript{50} = 100 nM). I demonstrated the benzophenone groups of the tagged inhibitors 30 and 31 can generate adducts by exposure to UV light (360 nm, 1 min) specifically with IDE, but not BSA, and this interaction was outcompeted by 10 \textmu M 6b. This UV-induced tagging method may prove useful as a tool to characterize IDE in cell culture.
Figure 2.7 | Solid supported synthesis routes to cis- and trans-alkene isomers of macrocycles based on a common intermediate.
Figure 2.8 | IDE inhibition potency of selection hits 1b to 6b and 30 structurally related analogs in which the linker, scaffold, and three building blocks were systematically varied.
2.4 – Metalloprotease selectivity of macrocyclic IDE inhibitors

Selectivity is a crucial feature of effective probes to elucidate physiological functions. In particular, the active sites of metalloproteases share multiple features and are not easily discriminated by competitive inhibitors. It is arguably a simple approach to inhibit IDE by chelation of the catalytic site zinc atom, however, there is an inherent concern in achieving selectivity for IDE over the >185 metalloproteases encoded in the human genome, as well as the other zinc-binding proteins in vivo (e.g. the insulin hexamers that are stabilized by a zinc atom).

For comparison in specificity studies, I synthesized the hydroxamic acid inhibitor Ii1 using the reported procedures, and purified it by HPLC. Next, I characterized the protease inhibition selectivity of 6bK; this inhibitor exhibited ≥ 1,000-fold specificity in vitro for inhibition of IDE over all other metalloproteases tested: thimet oligopeptidase (THOP), neurolysin (NLN), neprilysin (NEP), matrix metalloprotease 1 (MMP-1), and angiotensin converting-enzyme (ACE) (Figure 2.9). In contrast, the substrate mimetic hydroxamic acid inhibitor Ii1 was not as selective and it potently inhibited IDE (IC_{50} = 0.6 nM), THOP (IC_{50} = 6 nM), and NLN (IC_{50} = 185 nM) (Figure 2.9). More recently, a smaller variant of Ii1 called 14a, comprising the hydroxamic acid and the first two residues, was reported to have similar potency and selectivity profile as Ii1. Therefore, the 6bK inhibitor is vastly superior in IDE specificity to the literature standards.
The remarkable selectivity of 6bK, in contrast with the known promiscuity of some active site-directed metalloprotease inhibitors\(^9\), led us to speculate that the macrocycle engages a binding site distinct from the enzyme’s catalytic site.

**Figure 2.9** | Selectivity analysis of macrocycle 6bK reveals > 1,000-fold selectivity for IDE (■, IC\(_{50} = 50\) nM) over all other metalloproteases tested. Inhibitor Ii1\(^7\) inhibits IDE (■ IDE, IC\(_{50} = 0.6\) nM), and also thimet oligopeptidase (■ THOP, IC\(_{50} = 6\) nM) and neurolysin (■ NLN, IC\(_{50} = 185\) nM), but not neprilysin (■ NEP), matrix metalloprotease 1 (■ MMP1), or angiotensin converting enzyme (■ ACE).

**2.5 – Biochemical studies and mode of inhibition of macrocycle 6b**

In solution IDE exists predominantly as a homodimer, in equilibrium with monomer and tetramer species\(^10\). Cooperativity and structural cross-talk due to the dimeric nature of IDE deviate its biochemical parameters from the Michaelis–Menten model, in particular for proteolysis of small substrates\(^11\) (**Figure 2.10**). For such
cases, the mode of inhibition of IDE inhibitors should not be interpreted using the
linearization methods of Lineweaver–Burk (double-reciprocal plots) or Eadie–
Hofstee because the lines are curved in these diagrams. The substrate saturation plots
for IDE display changes in both $K_{M^{\text{app}}}$ and $V_{\text{max}}$ with increasing 6b due to the dimer
cooperativity, and the lines are of sigmoidal shape with a Hill coefficient $n = 2.1$ to
1.7 (Table 2.1), a level of homodimer cooperativity that is consistent with literature
reports. The extrapolation of the plot of $K_{M^{\text{app}}}/V_{\text{max}}$ versus 6b concentration
suggests a competitive $K_i = 55$ nM (Figure 2.10), a parameter that approximately
predicts the IC$_{50} = 85$ nM for 6b using 2.5 μM fluorogenic substrate (observed IC$_{50} =$
60 nM, Figure 2.1).

Next, I studied the mode of inhibition of 6b using Dixon and Cornish-Bowden
plots, which combined provide a simple graphical method for determining inhibition
constants and mode of inhibition for competitive, mixed, uncompetitive, and non-
competitive inhibitors. The plot profiles that arise from increasing substrate
concentration are predictive of a competitive mode of inhibition for 6b (Figure
2.11). The intersecting lines extrapolate to $K_i = 35$ nM, a parameter that accurately
predicts the IC$_{50} = 57$ nM for 6b at 2.5 μM fluorogenic substrate (observed IC$_{50} = 60$
 nM, Figure 2.1).
Figure 2.10 | Saturation plot for 6b inhibition of IDE proteolysis of the fluorogenic peptide substrate. In this biochemical assay both $K_M^{\text{app}}$ and $V_{\text{max}}$ change with increasing 6b due to the known dimer cooperativity in IDE (Hill coefficient $n = 2.1$ to 1.7)$^{12}$. The $K_M^{\text{app}}$ and $V_{\text{max}}$ values obtained from non-linear fitting (Table 2.1) predict $K_I = 55 \text{ nM}$ for 6b by extrapolation.

Table 2.1 | $K_M^{\text{app}}$ and $V_{\text{max}}$ parameters of IDE calculated by non-linear regression.

<table>
<thead>
<tr>
<th>Value ± SE</th>
<th>DMSO alone</th>
<th>35 nM 6b</th>
<th>70 nM 6b</th>
<th>140 nM 6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (pmol.min$^{-1}$)</td>
<td>2396 ± 46</td>
<td>2262 ± 87</td>
<td>2092 ± 27</td>
<td>1651 ± 24</td>
</tr>
<tr>
<td>$K_M^{\text{app}}$ (µM)</td>
<td>4.6 ± 0.3</td>
<td>8.2 ± 0.6</td>
<td>9.4 ± 0.3</td>
<td>10.8 ± 0.3</td>
</tr>
<tr>
<td>Hill coef. $n$</td>
<td>2.1 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

These preliminary biochemical studies suggested that 6b blocks substrate binding competitively. To investigate if 6b interacts within the large IDE cavity I used Yonnetani–Theorell double inhibitor plots that reveal if two ligands bind mutually exclusively within the enzyme, or if their binding sites are not overlapping$^{14}$. The antibiotic bacitracin, which displays weak affinity for the IDE
Dixon and Cornish-Bowden plots generated for 6b inhibition of IDE using the fluorogenic peptide substrate. This specific combination of plot profiles that arises from increasing substrate concentration is predictive of a competitive mode of inhibition for 6b. The intersecting lines extrapolate to $K_i = 37$ nM for 6b.

cavity ($IC_{50} = 10$ µM) displayed mutually exclusive activity in the presence 6b (Figure 2.12). However, the Yonetani–Theorell double inhibitor kinetic assay revealed synergistic, rather than competitive, inhibition by macrocycle 6b and the substrate mimetic Ii1 (Figure 2.12). The X-ray co-crystal structure of Ii1 within IDE shows the inhibitor interacting with the zinc atom in the catalytic domain as well as the groove where the substrate backbone is held for proteolysis. Therefore, the biochemical data strongly suggests that 6b inhibits IDE competitively within the cavity from a site that is distal to the catalytic domain, which explains the remarkable level of selectivity for IDE versus other metalloproteases (Figure 2.8).
2.6 – Structural basis of IDE inhibition and mutagenesis study to corroborate the novel distal binding site

In collaboration with Prof. Markus Seeliger and Zachariah Foda (Stony Brook University) we determined the X-ray crystal structure of catalytically inactive cysteine-free human IDE-E111Q\textsuperscript{15} bound to 6b at 2.7 Å resolution (Figure 2.13, see Table 2.2 for refinement statistics). The enzyme adopted a closed conformation and its structure is essentially identical to that of apo-IDE (PDB 3QZ2, RMSD = 0.257 Å). Macrocycle 6b occupies a binding pocket at the interface of IDE domains 1 and 2 (Figure 2.13A), and is positioned more than 11 Å away from the zinc ion in the catalytic site (Figure 2.13B-C). This distal binding site is a unique structural feature
of IDE compared to related metalloproteases\textsuperscript{16}, and does not overlap with the binding site of the substrate-mimetic inhibitor Ii1\textsuperscript{7}. The structure suggests that by engaging this distal site, the macrocycle competes with substrate binding and abrogates key interactions that are necessary to unfold peptide substrates for cleavage\textsuperscript{17-19} (Figure 2.13D).

Because a section of the macrocycle was unresolved in the electronic density map, as observed with other ligands co-crystallized within the IDE cavity,\textsuperscript{20} I sought to test the relevance of our structural model of the macrocycle:IDE complex (Figure 2.13) to macrocycle:IDE binding in solution. I identified IDE mutations predicted by the structural model to impede 6b binding (Figure 2.13B), prepared the corresponding mutant IDE proteins, and measured their abilities to be inhibited by 6b and 6bK. In addition, I also synthesized 6b analogs designed to complement these

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**Figure 2.13 | Structural basis of IDE inhibition by macrocycle 6b.** (A) X-ray co-crystal structure of IDE bound to macrocyclic inhibitor 6b (2.7 Å resolution, pdb: 4LTE). IDE domains 1, 2, 3, and 4 are colored green, blue, yellow, and red, respectively. Macrocycle 6b is represented as a ball-and-stick model, and the catalytic zinc atom is represented as an orange sphere. (B) Relative position of macrocycle 6b bound 11 Å from the catalytic zinc atom. The glutamine residue and four atoms of the macrocycle were unresolved (spanning a distance of 6.9 Å corresponding to four unresolved bonds). (C) Electron density map (composite omit map contoured at 1σ) and model of IDE-bound macrocycle 6b interacting with a 10 Å-deep hydrophobic pocket. The p-benzoyl-phenylalanine is shown in red, the cyclohexylalanine in blue, the fumarate linker in grey, and the D-lysine backbone in purple. (D) Superposition of the 6b model (surface rendering) on the IDE:insulin co-crystal structure (pdb: 2WBY)\textsuperscript{19}. 

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Figure 2.13 | Structural basis of IDE inhibition by macrocycle 6b (full caption in the previous page)
mutations and rescue inhibitor potency (Figure 2.14). Building block A (p-benzoyl-phenylalanine in 6b) occupies a 10 Å-deep pocket in the crystal structure (Figure 2.13C), defined by residues Leu201, Gly205, Tyr302, Thr316, and Ala479. As predicted by the structural model, mutation of Ala479 to leucine decreased the potency of inhibitors 6b and 6bK more than 600-fold, consistent with a significant steric clash in the binding site between Leu479 and the distal benzoyl group in building block A (Figure 2.15). Replacement of the p-benzoyl-phenylalanine building block with the smaller tert-butyl-phenylalanine, macrocycle 9, inhibited Ala479Leu-IDE with equal potency as wild-type IDE, consistent with the ability of the smaller macrocycle 9 to accommodate the added bulk of the leucine side chain (Figure 2.15). Likewise, building block B (cyclohexylalanine in 6b) makes contacts in the structure with the peptide backbone and residues Ile374, Val360, Gly361, and Gly362 located on the lateral β-strand 13 of IDE domain 2. These residues are thought to assist in unfolding of large peptide substrates by promoting cross-β-sheet interactions. Mutation of Gly362 to glutamine decreased the inhibition potencies of 6b and 6bK at least 50-fold compared to wild-type IDE (Figure 2.15). A modified macrocycle (13) in which the cyclohexylalanine building block was replaced with a smaller leucine residue inhibited Gly362Gln-IDE and wild-type IDE comparably, consistent with a model in which the smaller B building block complemented the larger size of the glutamine side chain (Figure 2.15).
Figure 2.14 | Activity assays for wild-type or mutant human IDE variants in the presence of 6bK. (A) Mutagenesis of residues Ala479Leu (■) and Gly362Gln (■) hindered the inhibition potency of 6bK by > 600- and > 50-fold, respectively, compared to that of wild-type human IDE (■). (B) The mutations Ala198Tyr (■), Ile374Gln (■), Phe202Arg (■), and Trp199Phe (■) modestly impact the potency of 6bK by 3 to 11-fold. (C) View of the binding site of macrocycle 6b, with the mutated residues color-coded.
Figure 2.15 | Small molecule-enzyme mutant complementation study to confirm the macrocycle binding site and placement of the benzophenone and cyclohexyl building-block groups. (A) IDE mutant A479L is inhibited by 6b >600-fold less potently compared to wild-type IDE. (B) Analog 9, in which the p-benzoyl ring is substituted for a smaller tert-butyl group, inhibits A479L IDE and WT IDE comparably. (C) Similarly, IDE mutant G362Q is inhibited 77-fold less potently by 6b compared with WT IDE. (D) Analog 13, in which the L-cyclohexyl alanine side chain was substituted with a smaller L-leucine side chain, inhibits G362Q IDE and WT IDE comparably. The full list of IDE mutants investigated is shown in Table 2.6 (Methods section).
Taken together, these structural and biochemical studies provide strong evidence for the proposed distal binding site of 6b and demonstrate the ability of the DNA-templated macrocycle library to provide inhibitors that achieve unusual selectivity by targeting residues beyond the catalytic site. IDE is the only homolog of the M16 clade of metalloproteases, which is evolutionarily distinct from other zinc-dependent metalloprotease members and characterized by an inverted zinc-binding motif\(^6-19\). This distinct phylogenetic origin, together with the unusual binding mode of these macrocyclic inhibitors to IDE, may contribute to the unusual specificity of 6bK among proteases tested and this encouraged us to explore the properties of 6bK in vivo ( Chapters 3–4).

2.7 – Methods

**In vitro selection of a DNA-templated library with immobilized mouse IDE.** The *in vitro* selection used here was adapted from previously described protocols\(^1,3\) using a DNA-templated library of 13,824 macrocycles\(^2\). Recombinant N-His\(_6\)-tagged mouse IDE\(_{42-1019}\) (isoform containing the amino acids 42-1019 of the IDE sequence) was purified using immobilized cobalt magnetic beads (Dynabeads® His-Tag Isolation & Pulldown, Invitrogen®) according to the manufacturer’s instructions. This purified IDE was confirmed to be catalytically active using the peptide substrate assay described below. IDE protein (~20 µg) was loaded onto the solid support by
incubating the protein with beads (30 µL) at 25 °C for 30 min in 300 µL of pH 8.0 buffer containing 50 mM phosphate, 300 mM NaCl and 0.01 % Tween-20 (PBST buffer), and washed twice with the same buffer. Two individually prepared protein-bead suspensions were incubated for 30 min with 5 pmol of the DNA-templated macrocycle library at RT, in pH 7.4 buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween-20 (TBST buffer) supplemented with 0.01 % BSA and 3 mg/mL yeast RNA (Ambion®). The beads were washed three times with 200 µL TBST buffer. The enriched library fraction was eluted by treatment with 200 mM imidazole in PBST buffer (50 µL) for 5 min.

The eluate solution was isolated and purified by buffer exchange using Sephadex spin-columns (Centrisep, Princeton Separation), according to the manufacturer’s instructions. PCR amplification of the enriched pool of library barcodes was performed as previously reported\(^3\), using primers that append adaptors for Illumina sequencing and a 7-base identifier. The long adaptor primer was

\[
5'\text{-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT}
\]

ACACGACGCTCTTCCGATCTXXXXXCCCTGTACAC and the short adaptor primer was

\[
5'\text{-CAAGCAGAAGACGGCATACGAGCTCTTCCGATC}
\]

tGAGTGAGTGATG (the 7-base identifier was XXXXX). The PCR amplicons were purified by polyacrylamide gel electrophoresis, extracted, and quantified using qPCR and Picogreen assays (Invitrogen).

High-throughput DNA sequencing was performed on an Illumina Genome Analyzer instrument at the Harvard FAS Center for Systems Biology, Cambridge,

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MA, to yield an average of ~3.8 million sequence reads for each selection, untreated bead control and pre-selection library. Deconvolution of library barcodes and enrichment calculations were performed with custom software as described previously\textsuperscript{3}. Variations in library member abundance as a result of binding to immobilized IDE was revealed by calculating fold-enrichment over the pre-selection library for the two independent selection experiments.

**Protease assays with fluorogenic peptide substrates.** The proteases IDE\textsubscript{42-1019}, recombinant human IDE\textsubscript{42-1019} (R&D Systems), neprilysin (R&D), and angiotensin-converting enzyme (R&D) were assayed using the fluorophore/quencher-tagged peptide substrate Mca-RPPGFSAFK(Dnp)-OH (R&D) according to the manufacturer’s instructions and recommended buffers. For IDE the recommended buffer is 50 mM Tris pH 7.5, 1 M NaCl. The enzyme mixtures (48 \(\mu\)L) were transferred to a 96-well plate and combined with 2 \(\mu\)L of inhibitor in DMSO solutions, in 3-fold dilution series. The mixtures were allowed to equilibrate for 10 min and the enzymatic reaction was started by addition of substrate peptide in assay buffer (50 \(\mu\)L), mixed, and monitored on a fluorescence plate reader (excitation at 320 nm, emission at 405 nm). Similarly, thimet oligopeptidase (R&D) and neurolysin (R&D) were assayed using substrate Mca-PLGPK(Dnp)-OH (R&D) according to the manufacturer’s instructions and recommended buffers. Matrix metalloproteinase-1 (R&D) was activated and assayed according to the manufacturer’s instructions with substrate Mca-KPLGL-Dpa-AR-NH\(_2\) (R&D). All
assay data points were obtained in duplicate. Data for the Yonetani–Theorell double inhibition plot was generated using 1 µL of each inhibitor (6b, and Ii1 or bacitracin) under otherwise identical conditions as above, at concentrations corresponding to ⅓x, 1x, 3x and 9x of respective IC₅₀ values against hIDE (R&D).¹⁴,²¹

**IDE degradation assays for insulin (HTRF).** A solution of 0.8 µg/mL IDE (R&D) in pH 7.5 buffer containing 20 mM HEPES, 135 mM NaCl (24 µL) was transferred to a PCR strip, and combined with 1 µL of each inhibitor (10 mM in DMSO, or as a 3-fold dilution series). A solution of insulin in “Assay Diluent” (25 µL) was added to a final concentration of 20 ng/mL, and incubated at 30 °C for 15 min. This procedure was optimized to result in ~75 % degradation of insulin. The reaction was terminated by 1:1 dilution into “Assay Diluent” containing inhibitor Ii1 at 200 nM (or 6bk, 10 µM) and chilled on ice. The remaining insulin in the sample was quantified using 10 µL of the quenched enzymatic reaction using the sensitive-range protocol Homogeneous Time-Resolved FRET Insulin assay (CisBio® 62INSPEB) in 20 µL total volume according to the manufacturer’s instructions (384 well-plate Greiner 784904 non-binding). Fluorescence was measured using a Tecan M1000Pro plate reader (excitation = 320 nm, emission = 665 and 620 nm, lag time = 60 µs) according the assay manufacturer’s recommendations. A blank well and a standard curve are required (non-linear).
**IDE degradation assays for glucagon (HTRF).** A solution of 0.05 µg/mL IDE (R&D) in 1x assay “Diluent #5” (24 µL) was transferred to a PCR strip, and combined with 1 µL of each inhibitor (10 mM in DMSO, or as a 3-fold dilution series). A solution of glucagon in the same buffer (25 µL) was added to a final concentration of 4 ng/mL, and incubated at RT for 10 min. This procedure was optimized to result in ~75 % degradation of glucagon. The reaction was terminated by 1 µL \textbf{Ii1} in 1x assay “Diluent #5” containing inhibitor \textbf{Ii1} at 5 µM (or \textbf{6bk}, 0.5 mM) and chilled on ice. The remaining glucagon in the sample was quantified using 10 µL of the quenched enzymatic reaction using the sensitive-range protocol Homogeneous Time-Resolved FRET Glucagon assay (CisBio® 62GLCPEF) in 20 µL total volume according to the manufacturer’s instructions (384 well-plate Greiner 784904 non-binding). Fluorescence was measured using a Tecan M1000Pro plate reader (excitation = 340 nm, emission = 665 and 620 nm, lag time = 60 µs) according the assay manufacturer’s recommendations. A blank well and a standard curve are required (linear).

**IDE degradation assay for calcitonin-gene related peptide (CGRP).**

Degradation of CGRP by endogenous IDE in mouse plasma was analyzed by LC-MS for the formation of CGRP1-17 and CGRP18-37 as previously reported\(^5\). The substrate was added to a final concentration of 10 µM, and \textbf{6b} was added to a final concentration of 0.1 to 10 µM.
General procedure for synthesis of macrocycle inhibitors. Rink amide resin (NovaPEG Novabiochem®, ~0.49 mmol/g, typically at a scale of 0.1 to 2 mmol) was swollen with ~10 volumes of anhydrous DMF for 1 h in a peptide synthesis vessel with mixing provided by dry nitrogen bubbling. In a separate flask, $N^\alpha$-allyloxycarbonyl-$N^\epsilon$-2-Fmoc-L-lysine (5 equiv.) and 2-($1H$-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 4.75 equiv.) were dissolved in anhydrous DMF (~10 vol.), then treated with $N,N'$-diisopropylethylamine (DIPEA, 10 equiv.) for 5 min at RT. The solution was combined with the pre-swollen Rink amide resin and mixed with nitrogen bubbling overnight. The vessel was eluted and the resin was washed three times with $N$-methyl-2-pyrrolidone (NMP, ~10 vol.). Following each coupling step, Fmoc deprotection was effected with 20 % piperidine in NMP (~20 vol.) for 20 min, repeated three times, followed by washing three times with NMP (~10 vol.) and twice with anhydrous DMF (~10 vol.). The general procedure for amide coupling of building blocks A, B and C was treatment of the resin with solutions of HATU-activated $N^\alpha$-Fmoc amino acids (5 equiv.) for 3-5 hours in anhydrous DMF, mixing with dry nitrogen bubbling. The general procedure for HATU-activation was treating a solution of $N^\alpha$-Fmoc amino acid (5 equiv.) and HATU (4.75 equiv.) in anhydrous DMF (10 vol.) with DIPEA (10 equiv.) for 5 min at RT.

Following the final Fmoc deprotection procedure, the $\alpha$-amine of building block C was coupled with allyl fumarate monoester (10 equiv.) using activation conditions as previously described with HATU (9.5 equiv.) and DIPEA (20 equiv.) in
anhydrous DMF (~10 vol.). Allyl fumarate coupling was accomplished by overnight mixing with dry nitrogen bubbling, followed by washing five times with NMP (~10 vol.) and three times with CHCl₃ (~10 vol.). Simultaneous allyl ester and N-allyloxycarbonyl group cleavage in solid support was effected with three consecutive treatments with a solution of tetrakis(triphenylphosphine)palladium(0) (0.5 equiv.) dissolved in degassed CHCl₃ containing acetic acid and N-methylmorpholine (40:2:1 ratio, ~20 vol.), mixing by nitrogen bubbling for 30 min. The resin was then washed twice subsequently with ~20 vol. of 5 % DIPEA in DMF, then twice with a 5 % solution of sodium diethylidithiocarbamate trihydrate in DMF (~20 vol.), twice with 5 % solution of hydroxybenzotriazole monohydrate in DMF, and finally washed with 50 % CH₂Cl₂ in DMF and re-equilibrated with anhydrous DMF (~10 vol.).

Treating the resin with pentafluorophenyl diphenylphosphinate (5 equiv.) and DIPEA (10 equiv.) in anhydrous DMF (~10 vol.) mixing by nitrogen bubbling overnight produced the macrocyclized products. The resin was washed with NMP (~20 vol.) and CH₂Cl₂ (~20 vol.) and dried by vacuum. The macrocyclized product was cleaved from the resin by two 15 min treatments of the macrocycle-bound resin with 95 % TFA containing 2.5 % water and 2.5 % triisopropylsilane (~20 vol.), followed by two TFA washes (~5 vol.). The TFA solution was dried to a residue under rotatory evaporation, and the peptide was precipitated by the addition of dry Et₂O. The ether was decanted and the remaining solid was dried and dissolved in a minimum volume of 3:1 DMF-water prior to purification by liquid chromatography. HPLC purification was performed on a C18 21.2x250 mm column (5 µm particle,
100 Å pore size, Kromasil®), using a gradient of 30 % to 80 % MeCN/water over 30 min, and solvents containing 0.1 % TFA. Fractions containing the desired macrocyclic peptide were combined and freeze-dried to produce a white powder. Typical yields were 5-15 % based on resin loading. Purity was determined by HPLC (Zorbax SB-C18 2.1x150 mm column, 5 µm particle) with UV detection at 230 nm, using a gradient of 30 % to 80 % MeCN/water over 30 min, and solvents containing 0.1 % TFA. The formula of final products was confirmed by accurate mass measurements using an Agilent 1100 LC-MSD SL instrument (Table 2.3).

**Spectra for inhibitor 6b**

$^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.50 (d, $J$ = 7.8 Hz, 1H), 8.42–8.36 (m, 1H), 8.07 (d, $J$ = 9.0 Hz, 1H), 7.74 (d, $J$ = 7.3 Hz, 1H), 7.71–7.65 (m, 3H), 7.59–7.54 (m, 4H), 7.38 (s, 1H), 7.31–7.28 (m, $J$ = 8.1 Hz, 2H), 7.15 (d, $J$ = 8.6 Hz, 1H), 6.97 (s, 1H), 6.85 (d, $J$ = 15.8 Hz, 1H), 6.81 (s, 1H), 6.68 (d, $J$ = 15.8 Hz, 1H), 4.56 (td, $J$ = 9.3, 4.3 Hz, 1H), 4.21–4.14 (m, $J$ = 6.9 Hz, 2H), 3.97 (ddd, $J$ = 10.2, 7.5, 2.9 Hz, 1H), 3.03–2.93 (m, 3H), 2.70 (ddd, $J$ = 13.4, 10.2 Hz, 1H), 2.19 (td, $J$ = 15.0, 7.6 Hz, 1H), 2.13 (td, $J$ = 15.4, 7.9 Hz, 1H), 1.92–1.86 (m, 2H), 1.82–1.68 (m, 2H), 1.63–1.55 (m, 3H), 1.55–1.48 (m, $J$ = 9.0 Hz, 3H), 1.42–1.26 (m, 4H), 1.26–1.22 (m, 1H), 1.15–1.00 (m, 5H), 0.83 (dd, $J$ = 21.5, 9.6 Hz, 1H), 0.71 (dd, $J$ = 24.8, 13.9 Hz, 1H).

$^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 195.44, 173.33 (2C), 171.35 (3C), 165.19, 164.16, 142.35, 137.28, 135.03, 132.96, 132.50 (2C), 132.03, 129.65 (2C), 129.53 (2C),

High resolution mass, calculated [M+H]^+ = 758.3872, found 758.3878, Δ = 0.791 ppm.

**Spectra for inhibitor 6bK (trifluoroacetate salt)**

^1^H NMR (600 MHz, DMSO-<i>d</i>6) δ 8.49–8.44 (m, 2H), 8.04 (d, J = 8.8 Hz, 1H), 7.72 (d, J = 7.1 Hz, 1H), 7.71–7.67 (m, J = 3.1 Hz, 2H), 7.67–7.59 (m, 3H), 7.59–7.52 (m, 4H), 7.38 (s, 1H), 7.29 (d, J = 8.2 Hz, 2H), 7.15 (d, J = 8.6 Hz, 1H), 6.96 (s, J = 2.1 Hz, 1H), 6.75 (dd, J = 81.5, 15.8 Hz, 2H), 4.58 (td, J = 9.2, 4.2 Hz, 1H), 4.23 (dd, J = 15.6, 7.8 Hz, 1H), 4.19 (ddd, J = 10.4, 8.4, 3.8 Hz, 1H), 3.98 (ddd, J = 11.1, 7.0, 3.0 Hz, 1H), 3.25 (ddd, J = 21.3, 13.6, 7.2 Hz, 1H), 2.99 (dt, J = 12.6, 7.9 Hz, 1H), 2.95 (ddd, J = 13.3, 10.5, 5.2 Hz, 1H), 2.76 (tt, J = 12.7, 6.5 Hz, 2H), 2.70 (dd, J = 13.3, 10.0 Hz, 1H), 1.81–1.74 (m, 1H), 1.73–1.65 (m, 3H), 1.63–1.48 (m, 8H), 1.45–1.38 (m, 3H), 1.37–1.20 (m, 4H), 1.18–1.11 (m, 1H), 1.11–0.96 (m, 4H), 0.89–0.79 (m, 1H), 0.73 (qd, J = 12.6, 3.6 Hz, 1H).

^1^C NMR (100 MHz, DMSO-<i>d</i>6) δ 195.57, 173.64, 171.60, 171.53, 171.45, 165.36, 164.42, 158.89 (TFA, q, J = 34.0 Hz), 142.40, 137.40, 135.16, 133.02, 132.58 (2C), 132.14, 129.75 (2C), 129.65 (2C), 129.58, 128.60 (2C), 116.50 (TFA, q, J = 294.8 Hz), 55.37, 53.68, 53.56, 50.12, 38.79, 36.46, 33.84, 33.32, 31.33, 30.94, 29.23, 29.10, 26.60, 26.07, 26.00, 25.75, 22.61, 22.03.
High resolution mass, calculated $[M+H]^+ = 758.4236$, found 758.4232, $\Delta = -0.396$ ppm.

**Spectra for bisepi-6bK (trifluoroacetate salt)**

$^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.45 (d, $J = 6.5$ Hz, 1H), 8.38 (d, $J = 8.9$ Hz, 1H), 7.73–7.65 (m, 5H), 7.65–7.59 (m, $J = 8.2$ Hz, 5H), 7.57 (dd, $J = 14.5$, 6.7 Hz, 3H), 7.39 (s, 1H), 7.30 (d, $J = 8.2$ Hz, 2H), 7.01 (s, 1H), 6.90 (s, 2H), 4.32 (ddd, $J = 14.6$, $10.0$, 4.0 Hz, 2H), 4.21 (dd, $J = 13.3$, 6.3 Hz, 1H), 3.99 (dt, $J = 8.3$, 6.4 Hz, 1H), 3.11 (dd, $J = 13.8$, 5.9 Hz, 1H), 3.08–3.00 (m, 2H), 2.82 (dd, $J = 12.7$, 5.7 Hz, 1H), 2.79–2.72 (m, 2H), 1.83–1.76 (m, $J = 7.2$ Hz, 1H), 1.73 (d, $J = 11.3$ Hz, 1H), 1.68–1.60 (m, $J = 9.4$ Hz, 4H), 1.55 (qd, $J = 14.1$, 7.5 Hz, 6H), 1.46–1.33 (m, 3H), 1.33–1.20 (m, 4H), 1.10 (d, $J = 9.2$ Hz, 3H), 1.02 (dd, $J = 23.9$, 11.8 Hz, 1H), 0.91 (dd, $J = 21.9$, 10.1 Hz, 1H), 0.79 (dd, $J = 22.0$, 10.7 Hz, 1H).

$^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 195.62, 173.82, 171.54, 171.04, 169.78, 165.09, 163.98, 158.66 (TFA, q, $J = 32.4$ Hz), 143.28, 137.35, 135.06, 133.37, 132.59 (2C), 132.07, 129.59 (4C), 129.46, 128.57 (2C), 116.80 (TFA, q, $J = 296.4$ Hz), 55.27, 54.51, 52.52, 50.23, 38.73, 38.61, 36.48, 33.79, 33.45, 31.46, 30.84, 30.45, 28.19, 26.62, 26.09, 25.77, 23.07, 22.63.

High resolution mass, calculated $[M+H]^− = 758.4236$, found 758.4232, $\Delta = -0.527$ ppm.
Expression and purification of recombinant cysteine-free hIDE (IDE-CF). We expressed cysteine-free catalytically-inactive, human IDE (IDE-CF) in pPROEX vector\textsuperscript{17}. IDE-CF contains the following substitutions: C110L, E111Q, C171S, C178A, C257V, C414L, C573N, C590S, C789S, C812A, C819A, C904S, C966N, and C974A. IDE was expressed and purified as previously described\textsuperscript{17}. Briefly, IDE-CF was transformed into \textit{E. coli} BL21-CodonPlus (DE3)-RIL, grown at 37 °C to a cell density of 0.6 O.D. and induced with IPTG at 16 °C for 19 hours. Cells were lysed and the lysate was subjected to Ni-affinity (GE LifeScience) and anion exchange chromatography (GE LifeScience). The protein was further purified by size exclusion chromatography (Superdex S200 column) three successive times, first without inhibitor then two times after addition of 2-fold molar excess of 6b.

**IDE-CF•6b co-crystallization.** Eluent from size exclusion chromatography was concentrated to 20 mg/ml in 20 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM PMSF and 2-fold molar excess of 6b was added to form the protein-inhibitor complex. The complex was mixed with equal volumes of reservoir solution containing 0.1 M HEPES (pH 7.5), 20 % (w/v) PEGMME-5000, 12 % tacsimate and 10 % dioxane. Crystals appeared after 3-5 days at 25 °C and were then equilibrated in cryoprotective buffer containing well solution and 30 % glycerol. IDE-CF•6b complex crystals belong to the space group \textit{P}6\textsubscript{5}, with unit-cell dimensions \(a = b = 262 \text{ Å}\) and \(c = 90 \text{ Å}\), and contain two molecules of IDE per asymmetric unit (see Appendix for refinement statistics).
**X-Ray Diffraction.** X-ray diffraction data were collected at the National Synchrotron Light Source at Brookhaven National Laboratories beamline X29 at 100K and 1.075 Å.

**IDE-CF•6b structure determination.** Data were processed in space group $P6_5$ using autoProc$^{22}$. The structure was phased by molecular replacement using the structure for human IDE E111Q (residues 45-1011, with residues 965-977 missing) in complex with inhibitor compound 41367 (PDB: 2YPU) as a search model in Phaser$^{23}$. The model of the structure was built in Coot$^{24}$ and refined in PHENIX$^{25}$, using NCS (torsion-angle) and TLS (9 groups per chain). In the Ramachandran plot, 100 % of the residues appear in the allowed regions, 97.2 % of the residues appear in the favored regions, and 0 % of the residues appear in the outlier regions (see Appendix for refinement statistics). The structural model of IDE and inhibitor was refined using non-crystallographic symmetry restraints due to the high degree of structural similarity between the two chains of the protein the asymmetric unit. The root-mean-square deviation between the two chains is 0.5 Å for the protein chains, and 0.9 Å for the ligand atoms, indicating high structural similarity between the two chains. Structure coordinates are deposited in the Protein Data Bank (accession number 4TLE).
**Table 2.2 | X-ray data collection and refinement statistics.**

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<td>(I / \sigma I)</td>
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Site-directed mutagenesis, expression, and purification of human IDE. The reported N-His$_6$-tagged human IDE$_{42-1019}$ construct was introduced in the expression plasmid pTrcHis-A (Invitrogen) using primers for uracil-specific excision reactions (USER) by Taq (NEB) and Pfu polymerases (PfuTurbo CX®, Agilent). The IDE gene was amplified with the primers 5’-ATCATCATATGAATAATCCAGCCA-$d$U-CAAGAGAATAGG and 5’-ATGCTAGCCATACCTCAGA $G$-$d$U-$TTTGCA$GAGCCATGAAG (underlined sequences represent overhangs, and italics highlight the PCR priming sequence). Similarly, the pTrcHis-A vector was amplified for USER cloning with the primers 5’-ATGGCTGGATTATTCATATGATGA-$d$U-$GATGATGATGAGAACCC and 5’-ACTCTGAGGTATGGCTAGCA-$d$U-$GACTGGTG$. Mutant IDE constructs were generated by amplifying the full vector construct with USER cloning primers introducing a mutant overhang (Table 2.4).

All PCR products were purified on microcentrifuge membrane columns (MinElute®, Quiagen) and quantified by UV absorbance (NanoDrop). Each fragment (0.2 pmol) was combined in a 10 µL reaction mixture containing 20 units $Dpn$I (NEB), 0.75 units of USER mix (Endonuclease VIII and Uracil-DNA Glycosylase, NEB), 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol at pH 7.9 (1x NEBuffer 4). The reactions were incubated at 37 ºC for 45 min, followed by heating to 80 ºC and slow cooling to 30 ºC (0.2 ºC/s). The hybridized constructs were directly used for heat-shock transformation of chemically competent NEB turbo E. coli cells according to
the manufacturer’s instructions. Transformants were selected on carbenicillin LB agar, and isolated colonies were cultured overnight in 2 mL LB.

The plasmid was extracted using a microcentrifuge membrane column kit (Miniprep®, Qiagen), and the sequence of genes and vector junctions were confirmed by Sanger sequencing (Table 2.5). The plasmid constructs were transformed by heat-shock into chemically-competent expression strain Rosetta 2 (DE3) pLysS E. coli cells (EMD Millipore), and selected on carbenicillin/chloramphenicol LB agar. Cells transformed with IDE pTrcHis A constructs were cultured overnight at 37 °C in 2 XYT media (31 g in 1L) containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Expression of His6-tagged IDE proteins was induced when the culture measured OD600 ~0.6 by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1 mM final concentration, incubated overnight at 37 °C, followed by centrifugation 10,000 g for 30 min at 4 °C.

Recombinant His6-tagged proteins were purified by Ni(II)-affinity chromatography (IMAC sepharose beads, GE Healthcare®) according to the manufacturer’s instructions. The cell pellets were resuspended in pH 8.0 buffer containing 50 mM phosphate, 300 mM NaCl, 10 mM imidazole, 1 % Triton X-100 and 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and were lysed by probe sonication for 4 min at <4 °C, followed by clearing of cell debris by centrifugation at 10,000 g for 25 min at 4 °C. The supernatant was incubated with Ni(II)-doped IMAC resin (2 mL) for 3 h at 4 °C. The resin was washed twice with the cell resuspension/lysis buffer, and three times with pH 8.0 buffer containing 50
mM phosphate, 300 mM NaCl, 50 mM imidazole and 1 mM TCEP. Elution was performed in 2 mL aliquots by raising the imidazole concentration to 250 mM and subsequently to 500 mM in the previous buffer. The fractions were combined and the buffer was exchanged to the recommended IDE buffer (R&D) using spin columns with 100 KDa molecular weight cut off membranes (Millipore). Protein yields were typically ~10 µg/L, and >90 % purity based on gel electrophoresis analysis (Coomassie stained). IDE-specific protease activity was >95 % as assessed by inhibition of degradation of peptide substrate Mca-RPPGFSAFK(Dnp)-OH (R&D) by 20 µM 6bK, compared with pre-quantitated commercially available human IDE (R&D). The complete list of IDE mutations assayed is shown in Table 2.6.
Table 2.3 | HPLC and high-resolution mass spectrometry analysis of IDE inhibitor analogs.

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<th>[M+H]+ found</th>
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Table 2.4 | Site-directed mutagenesis primers.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers (forward, reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A198Q</td>
<td>ACATGAGAAGAATGTGATGAATGA-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACATCATTCACTACATCTCTCTCACTdU-TCTG</td>
</tr>
<tr>
<td>A198T</td>
<td>AGACTCTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td></td>
<td>AGCTTTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td>A198C</td>
<td>AGACTCTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td></td>
<td>AGCTTTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td>A198Y</td>
<td>ACATGAGAAGAATGTGATGAATGA-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACATCATTCACTACATCTCTCTCACTdU-TCTG</td>
</tr>
<tr>
<td>W199L</td>
<td>ACATGAGAAGAATGTGATGAATGA-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACATCATTCACTACATCTCTCTCACTdU-TCTG</td>
</tr>
<tr>
<td>W199F</td>
<td>AGACTCTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td></td>
<td>AGCTTTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td>W199Y</td>
<td>AGACTCTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td></td>
<td>AGCTTTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td>F202L</td>
<td>ACATGAGAAGAATGTGATGAATGA-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACATCATTCACTACATCTCTCTCACTdU-TCTG</td>
</tr>
<tr>
<td>F202R</td>
<td>AGACTCTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td></td>
<td>AGCTTTTTCAATGGAAAAGC-dU-ACAGGG</td>
</tr>
<tr>
<td>I310R</td>
<td>ACCATTTAAGCTCATGGAATC-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACCATTTAAGCTCATGGAATC-dU-TC</td>
</tr>
<tr>
<td>Y314F</td>
<td>ACCATTTAAGCTCATGGAATC-dU-TC</td>
</tr>
<tr>
<td></td>
<td>ACCATTTAAGCTCATGGAATC-dU-TC</td>
</tr>
<tr>
<td>V360Q</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td>V360R</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td>G361Q</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td>G362Q</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
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<td>K364A</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td>I374Q</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td>A479L</td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
<tr>
<td></td>
<td>AAGGCTGGTTATATACCTCT-dU-AGGAGG</td>
</tr>
</tbody>
</table>

Table 2.5 | Sequencing primers.

<table>
<thead>
<tr>
<th>Sequencing primers</th>
<th>Primers (forward, reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq_Fw1</td>
<td>GATTAACCTTTATTATATTAAATAAAGG</td>
</tr>
<tr>
<td></td>
<td>AATCATTCACTACATCTCTCTCGTC</td>
</tr>
<tr>
<td>Seq_Re2</td>
<td>GTATGAGGTTATGATTCGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>CATGAGGTTATGATTCGAGGAGG</td>
</tr>
<tr>
<td>Seq_Fw3</td>
<td>GCAGAGTACCTCACCCCTGCT</td>
</tr>
<tr>
<td></td>
<td>GCAGAGTACCTCACCCCTGCT</td>
</tr>
<tr>
<td>Seq_Re4</td>
<td>GCTTATGTTCGCCAGAGGACTG</td>
</tr>
<tr>
<td></td>
<td>GCTTATGTTCGCCAGAGGACTG</td>
</tr>
</tbody>
</table>
**Table 2.6** | Site-directed IDE mutants used in the small molecule-enzyme mutant complementation studies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Batch</th>
<th>Relative activity</th>
<th>Ii1 IC$_{50}$ shift *</th>
<th>6b IC$_{50}$ shift</th>
<th>13 IC$_{50}$ shift</th>
<th>9 IC$_{50}$ shift</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIDE WT</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>hIDE WT</td>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>hIDE WT</td>
<td>3</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>A198Q</td>
<td>1</td>
<td>1.5</td>
<td>1.0</td>
<td>16</td>
<td>13.6</td>
<td>-</td>
<td>scaffold interaction</td>
</tr>
<tr>
<td>A198T</td>
<td>2</td>
<td>2.3</td>
<td>1.1</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>W199F</td>
<td>2</td>
<td>1.3</td>
<td>1.8</td>
<td>3.0</td>
<td>3.2</td>
<td>-</td>
<td>modest interaction</td>
</tr>
<tr>
<td>W199Y</td>
<td>2</td>
<td>0.3</td>
<td>1.4</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F202L</td>
<td>1</td>
<td>0.7</td>
<td>1.7</td>
<td>1.7</td>
<td>2.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F202R</td>
<td>2</td>
<td>0.9</td>
<td>1.1</td>
<td>3.7</td>
<td>4.1</td>
<td>-</td>
<td>modest interaction</td>
</tr>
<tr>
<td>I310R</td>
<td>2</td>
<td>3.0</td>
<td>1.9</td>
<td>0.8</td>
<td>0.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Y314F</td>
<td>2</td>
<td>3.5</td>
<td>1.0</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>V360Q</td>
<td>1</td>
<td>2.6</td>
<td>0.9</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>V360R</td>
<td>3</td>
<td>0.4</td>
<td>0.9</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>G361Q</td>
<td>3</td>
<td>0.7</td>
<td>0.9</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>G362Q</td>
<td>3</td>
<td>1.2</td>
<td>0.6</td>
<td>77</td>
<td>1.8</td>
<td>-</td>
<td>cyclohexyl ring interaction</td>
</tr>
<tr>
<td>K364A</td>
<td>2</td>
<td>4.3</td>
<td>1.0</td>
<td>26</td>
<td>10.0</td>
<td>-</td>
<td>scaffold interaction</td>
</tr>
<tr>
<td>I374Q</td>
<td>1</td>
<td>1.2</td>
<td>1.0</td>
<td>4.2</td>
<td>-</td>
<td>-</td>
<td>cyclohexyl ring interaction</td>
</tr>
<tr>
<td>A479L</td>
<td>1</td>
<td>0.5</td>
<td>0.9</td>
<td>&gt;600</td>
<td>-</td>
<td>1.0</td>
<td>p-benzoyl interaction</td>
</tr>
</tbody>
</table>

* NB: Ii1 is not known to interact with any of these residues. Significant changes in IC$_{50}$ for Ii1 were presumed to indicate misfolding or other non-inhibitor-specific protein structure changes. For example, W199L IDE displayed an IC$_{50}$ shift of 21-fold for Ii1, 13-fold for 6b, and 17-fold for analog 13. The data for mutation I374Q has been corrected after the publication, the original data was relevant to I375Q-IDE due to incorrect primer design.
Figure 2.14 | Spectra for $^1$H- and $^{13}$C-NMR of 6b, 6bK, and bisepi-6bK.
Figure 2.14 (continued)
Figure 2.15 | Alignment for human and mouse IDE sequences (95% identity), only 10/45 variations are predicted to have any impact on local structure. Aligned using CLUSTAL 2.1.
Supplementary plasmid sequence for pTrcHisA-His6-hIDE \((42-1019)\), the protein coding sequence is highlighted in blue.
2.8 – References


Chapter 3

Development of the first physiologically active IDE inhibitor


Contributions: I designed and performed the experiments and pharmacokinetic measurements described in this chapter. Edwin A. Homan and Amanda K. McFedries assisted with in vivo experiments. Help from other colleagues is acknowledged in the text.
3.1 – Introduction (and spoiler alert)

This chapter aims synthesize all the experimental evidence to evaluate the viability of 6bK as a highly specific and physiologically active IDE inhibitor probe. In practice, the process of selecting and validating an *in vivo* probe candidate, among multiple analogs available, typically relies on the results of dose-response trials and phenotypic observations that are used to “build a case” for subsequent follow up studies with the molecule. For this reason, this chapter does not recount a linear timeline of experiments of increasing complexity, and specific observations such as glucose tolerance phenotypes are not concurrently rationalized in the text from a mechanistic point of view until the next chapter. The physiological functions of IDE are discussed in detail in Chapter 4.

In this study I followed the guidelines reviewed by Workman and Collins\(^1\) to validate the suitability of an *in vivo* probe. *In vivo* probes must satisfy a number of biochemical and biophysical features, which significantly overlap but that are distinct from the properties of pharmaceutical drugs and drug-lead molecules (Table 3.1).\(^1\)
Table 3.1 | Distinct properties of pharmaceutical drugs, drug leads, and in vivo probes, compared to 6bK. Adapted from reference 1.

<table>
<thead>
<tr>
<th>Property</th>
<th>Pharmaceutical drugs</th>
<th>Drug leads</th>
<th>In vivo probes</th>
<th>6bK features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous solubility</strong></td>
<td>&gt;10–100 µg/ml</td>
<td>&gt;10–100 µg/ml</td>
<td>&gt;0.05 µg/ml in low % DMSO aqueous solutions</td>
<td>~0.5 mM / ~400 µg/mL in 1% DMSO</td>
</tr>
<tr>
<td><strong>Membrane permeability</strong></td>
<td>10× permeability of mannitol in CaCo-2 assay desirable; minimal PGP-mediated efflux</td>
<td>P_e &gt; 10^{-6} ms^{-1} (in vitro assay); minimal PGP-mediated efflux</td>
<td>Permeability essential; minimal PGP-mediated efflux in cell lines of interest</td>
<td>Not applicable, IDE is an extracellular target</td>
</tr>
<tr>
<td><strong>Chemically reactive groups</strong></td>
<td>None present unless a well characterized and selective mechanistic requirement</td>
<td>None present unless a well characterized and selective mechanistic requirement</td>
<td>None present unless a well characterized and selective mechanistic requirement</td>
<td>See Figure 2.3</td>
</tr>
<tr>
<td><strong>Molecular weight (Da)</strong></td>
<td>&lt;500–550</td>
<td>&lt;350–450</td>
<td>Likely to be &lt;450</td>
<td>758 Da</td>
</tr>
<tr>
<td><strong>Lipophilicity (LogP)</strong></td>
<td>&lt;5</td>
<td>&lt;4</td>
<td>Likely to be &lt;5</td>
<td>&lt;1 predicted</td>
</tr>
<tr>
<td><strong>H-bond donors</strong></td>
<td>= &lt;5</td>
<td>&lt;4–5</td>
<td>Likely to be &lt;3</td>
<td>8</td>
</tr>
<tr>
<td><strong>H-bond acceptors</strong></td>
<td>= &lt;10</td>
<td>&lt;8–9</td>
<td>Likely to be &lt;11</td>
<td>7</td>
</tr>
<tr>
<td><strong>Rotatable bonds</strong></td>
<td>= &lt;10</td>
<td>&lt;8</td>
<td>Likely to be &lt;10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Potency (IC_{50} or Ki)</strong></td>
<td>10^{-8}–10^{-9} M</td>
<td>10^{-6}–10^{-8} M</td>
<td>10^{-7}–10^{-9} M</td>
<td>5 x 10^{-8} M</td>
</tr>
<tr>
<td><strong>Ligand efficiency</strong></td>
<td>NA</td>
<td>&gt;0.3 kcal mol^{-1} heavy atom^{-1}</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Target selectivity</strong></td>
<td>Well-defined selectivity; polypharmacology acceptable</td>
<td>Well-defined selectivity; &gt;10-fold over related targets; minimal activity on common off-targets</td>
<td>Well-defined selectivity; &gt;10–100-fold against closely related targets; polypharmacology undesirable</td>
<td>&gt;1000-fold selective for IDE, distal binding site not present in other metalloprotease enzymes</td>
</tr>
<tr>
<td><strong>Pharmacokinetics</strong></td>
<td>Well-defined therapeutic window and in vivo pharmacokinetics</td>
<td>Stable in microsomes; no CYP450 inhibition</td>
<td>Good pharmacokinetics not essential for in vitro and cellular use, but required for in vivo animal work</td>
<td>Determined in Chapter 3</td>
</tr>
</tbody>
</table>
3.2 – Biodistribution and pharmacokinetic properties of 6bK

Theoretical studies predict that macrocyclic ligands are privileged structures for interacting with protein pockets and surfaces in complex biological mixtures. The degrees of freedom of rotatable bonds are decreased by covalently tying up a molecule in a medium-size macrocycle. In contrast to a linear molecule or peptidic scaffold of comparable structure, fewer low-energy conformations are accessible in solution or by interacting with proteins. The consequences of added rigidity include:

i) lower entropic cost for on-target binding interaction through an accessible low-energy conformation, which has a generally favorable impact on Ki values;

ii) higher selectivity, due to increased entropic cost of binding off-target sites when the accessible conformations cannot accommodate steric clashes with the protein;

iii) improved stability in the presence of proteases due to occlusion of labile bonds from nucleophile attack and/or carbonyl activation (as well as the effect of point ii).

To study the stability, physicochemical, and pharmacokinetic properties of 6bK it was first necessary to produce milligram to gram quantities. I used the synthetic route shown in Figure 3.1 to produce over 2 grams of 6bK trifluoroacetate salt in 10–15% yield based on resin loading, relying on a single HPLC purification step.

In collaboration with Dr. Stephen Johnston at the Broad Institute we determined that 6bK displayed significant stability during incubations with plasma and liver microsome preparations (74 % and 78 % remaining after 1 h, respectively), suggesting that this compound may be sufficiently stable in vivo to inhibit IDE activity (Table 3.2). Plasma protein binding assays indicated that ~6 % of 6bK
remains unbound (Table 3.2) and potentially available to engage its target at sites of insulin degradation, extracellularly or in early endosome compartments of target tissues\(^5,6\).

**Figure 3.1** | Fmoc-based solid-supported synthesis of 6bK followed by on-resin macrocyclization. This synthetic route is amenable to gram-scale production of 6bK using a single purification step.

**Table 3.2** | Plasma binding, plasma stability, and microsomal stability parameters for 6bK. Data are averages of replicate results, Dr. Stephen Johnston (Broad Institute).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plasma binding</th>
<th>Plasma Stability (1 h)</th>
<th>Microsomal stability (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>94 %</td>
<td>74 %</td>
<td>78 %</td>
</tr>
<tr>
<td>human</td>
<td>86 %</td>
<td>83 %</td>
<td>80 %</td>
</tr>
</tbody>
</table>
To measure plasma half-life *in vivo*, we treated mice by intraperitoneal (i.p.) injection of 80 mg/kg 6bK using a formulation of sterile saline and Captisol® (CyDex), a β-cyclodextrin-based agent used to improve solubilization and delivery. The aqueous solubility of 6bK is augmented more than 20-fold by formulation with Captisol®, simplifying the preparation of injection volumes such as 10 mL/kg (i.e. 250 µL for a 25 g mouse) and providing reliable bioavailability through different preparations of 6bK.

I observed that plasma and tissue concentrations of 6bK could not be determined directly owing to non-specific losses during sample preparation (see Methods). Therefore, I used isotope-dilution mass spectrometry (IDMS) to account for these losses by spiking a 13C- and 15N-labeled 6bK analog (Figure 3.2). In mice treated with 6bK, the plasma levels of inhibitor were detectable within 5 min post-injection, reached peak concentration (> 100 µM) at 60 min, and were maintained at a detectable level for at least 4 h (Figure 3.2A).

I observed prompt biodistribution of 6bK into liver, kidney, and pancreatic tissues (Figure 3.2B). In contrast, 6bK was undetectable in the brain of mice (within the limits of the LCMS assay and injection of 10-fold concentrated sample). This suggested the favorable feature of this macrocycle for proof-of-concept studies since it may not inhibit IDE within brain tissues, where IDE activity is needed for the clearance of β-amyloid peptides. Indeed, brain tissue levels of Aβ(40) and Aβ(42) peptides in mice injected with 6bK were unchanged 2 h-post injection compared to vehicle-treated controls (Figure 3.2D). While longitudinal studies may be needed,
this data is consistent with the inability of 6bK to inhibit IDE in the brain during short-term administration.

Collectively, these measurements indicate that 80 mg/kg 6bK treatment (2 mg/mouse) transiently achieves >100 to 1,000-fold IC50 levels of inhibitor in circulation and in liver and kidney, the main insulin-degrading organs, which suggest the viability of using 6bK to inhibit IDE in vivo.

![Figure 3.2](#)

**Figure 3.2 | Pharmacokinetic parameters of 6bK.** (A) Concentration of 6bK in mice tissues and plasma collected over 4 hours (n = 1-2). (B) Average biodistribution of 6bK in five lean mice at 150 min post-injection of 6bK 80 mg/kg i.p. at the endpoint of a IPGTT experiment. 6bK was not detectable in the brain even using 10-fold concentrated samples.
(Figure 3.2 cont.) for LC-MS injection compared to other tissues. (C) Heavy 6bK was synthesized with $^{15}$N,$^{13}$C-lysine for stable-isotope dilution LC-MS quantitation. (D) Treatment of C57BL/6J lean mice with 6bK (■, 80 mg/kg, n = 6) does not change brain levels of Aβ(40) or Aβ(42) peptides in the brain 2 h post injection compared to treatment with vehicle alone (■, n = 5) or inactive diastereomer bisepi-6bK (■, 80 mg/kg, n = 6). All data points and error bars represent mean ± SEM.

3.3 – Augmentation of insulin hypoglycemic effects and abundance by IDE inhibition in vivo

Our experiments determined the circulation time of 6bK is within the timescale for standard physiological experiments with live animals.$^9,10$ In particular we identified reliable exposure to 6bK in $>100$ IC$_{50}$ levels during the period 30–150 min post-injection (Figure 3.2A). To evaluate the ability of 6bK to inhibit IDE activity in vivo we subjected non-fasted mice to insulin tolerance tests (ITT)$^10$ following a single injection with 6bK (80 mg/kg) formulated in Captisol®$^7$. The insulin injections were carried out 30 min post-injection, corresponding to the time before the highest 6bK concentration in plasma (approximately 100 µM, ~1000-fold the IC$_{50}$ for mouse IDE). Following a subcutaneous insulin injection, mice treated with 6bK experienced lower hypoglycemia and higher insulin levels compared to vehicle controls ($p < 0.01$, Figure 3.3A). At a later time, we repeated this ITT experiment using a quarter dose of insulin injection to elicit a less pronounced hypoglycemic effect and we observed identical results (Figure 3.3B). In 1955, Arthur Mirsky used a similar ITT assay to suggest the feasibility of insulin stabilization by injecting rats
and mice with preparations of an undefined endogenous IDE inhibitor crudely fractionated from bovine livers (presumably a competitive substrate; Table 1.2\textsuperscript{11,12}). With our current understanding of the biochemistry and physiological roles of IDE it would be worthwhile to identify this putative “endogenous” inhibitor in the future.

Our experiments with 6bK provide the first evidence that a well-defined, selective, and physiologically stable pharmacological IDE inhibitor can augment the abundance and activity of insulin \textit{in vivo} (Figure 3.3).

**Figure 3.3 | Acute IDE inhibition affects the abundance and hypoglycemic action of insulin.** (A and B) Blood glucose responses and abundance of injected insulin (Humulin-R) in lean mice 30 min after treatment with 6bK (■, 80 mg/kg) or vehicle alone (■). (A) Insulin i.p. (1 U/kg) after 5-hour fast. (B) Insulin s.c. (0.25 U/kg) after 5-hour fast. (C) Trunk blood was collected at the last time point of this experiment for plasma hormone measurements. All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are \( p < 0.05 \) (*) or \( p < 0.01 \) (**) versus the vehicle-only control group.
3.4 – IDE inhibitor activity- and dose-response in glucose tolerance tests

Experiments that rely on the administration of substrate exogenously are subject some of the caveats of *in vitro* cleavage assays (Chapter 1, Section 1.2). The supra-physiological concentrations (>10-100-fold basal levels) can overwhelm endogenous degradation pathways, producing measurements that are not relevant to the physiological function of the hormone. Moreover, the phenotype of a hormonal treatment can depend on overall dose, rate and/or route of administration\textsuperscript{13-18}.

To explore the effect of acute IDE inhibition on endogenously elicited hormone signaling under physiologically relevant conditions we first turned to glucose challenges, which are standard for animal experimentation\textsuperscript{9,19-22}, and for diagnosis of insulin resistance in human patients\textsuperscript{23-26}. It is worth noting that mice treated with 6bK (80 mg/kg) but not given a glucose challenge do not display significant changes in baseline glucose or basal hormone levels compared to vehicle controls (Figure 3.4), suggesting this approach cannot be used to study IDE physiology on basal hormone levels. Compensatory pancreatic hormone feedback and/or redundant degradation pathways potentially overshadow any impact of IDE inhibition on basal levels of insulin, glucagon, or amylin\textsuperscript{27-29}. 
Figure 3.4 | Administration of 6bK (■, 80 mg/kg) to lean mice not followed by injection of a nutrient such as glucose (or pyruvate; see Figure 4.8) did not significantly alter basal blood glucose or basal hormone levels compared to bisepi-6bK (■) or vehicle controls (■) 30 min post-injection. C-peptide is an inactive marker of β-cell secretion of insulin and amylin used as a control. All data points and error bars represent mean ± SEM.

Figure 3.5 | Low-potency diastereomers of 6bK. Inhibition of mouse IDE activity by low potency diastereomers of 6bK. The stereocenters altered in each compound relative to those of 6bK are labeled with a star.

In order to corroborate that our phenotypic observations during glucose tolerance tests were due to on-target IDE inhibition I synthesized epimers of 6bK of diminished inhibitory potency to use as in vivo controls (Figure 3.5). For most in
*vivo* studies we favored the analog with two stereochemical changes on building block B and C, called **bisepi-6bK**, which is thus identical to **6bK** in chemical composition and bond connectivity, but is essentially inactive as an IDE inhibitor (**IC**$_{50}$ > 200 µM, **Figure 3.5**). This control is also superior in lacking any potential contamination with **6bK** arising from enantiomeric impurities in commercial Fmoc-amino acids, or from α-carbon racemization during amide coupling (**Figure 3.1**).

Testing **6bK** at several doses, in parallel with the low-activity controls (**Figure 3.6**), led us to establish that the effective dose of **6bK** is approximately 2 mg/mouse i.p., representing 80 mg/kg for lean C57BL/6J mice (25 g), and 60 mg/kg for diet-induced obese (DIO) mice (35-45 g). In particular, we performed numerous dose-response relationship experiments during i.p. glucose tolerance tests (IPGTT) studies using lean and DIO mice. We observed the phenotypic changes in glucose profiles were dependent on **6bK** dose, and no effects were observed in cohorts treated with vehicle alone or inactive **bisepi-6bK**, indicating that the both glucose tolerance effects correlate with IDE activity (**Figure 3.6**, and see **Figure 4.2** for optimal dose). We observed that doses below 1.5 mg/mouse **6bK** led to variable glucose profiles towards the end of the experiments, whereas doses above 4 mg/mouse were not well tolerated, producing lethargy.
Figure 3.6 | Low-potency diastereomers of 6bK used to determine the effective dose range and confirm on-target IDE inhibition effects during IPGTTs in lean and DIO mice. (A to E) In dose optimization experiments the effects of 6bK (■, 90 to 40 mg/kg) were compared with equal doses of either weakly active stereoisomer epi-C-6bK (■) or inactive stereoisomer bisepi-6bK (■) and vehicle controls (■) in IPGTTs using lean and obese mice. DIO mice treated with low doses of 6bK (■, 40 mg/kg) responded to IPGTT in either of two ways: improved glucose tolerance throughout the experiment (n = 3) or a hyperglycemic rebound as described in the main text (n = 4), suggesting this dose is too low to achieve a consistent effect (note the large error bars). DIO mice treated with high doses of 6bK (■, 3.5 mg/animal, 90 mg/kg) respond similarly to Figure 4.4 (2 mg/animal, 60 mg/kg), but the weak activity observed for bisepi-6bK (IC$_{50} > 100$ μM) using a matching dose (■, 90 mg/kg) compared to vehicle alone (■) suggests that 60 mg/kg (2 mg/animal) is an
(Figure 3.6 cont.) Appropriate dose for DIO mice experiments. All data points and error bars represent mean ± SEM. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are * $p < 0.05$ versus vehicle control group; ** $p < 0.01$ versus vehicle control group.

3.5 – Features and limitations of 6bK as an in vivo probe

In conclusion, we determined the dose of 6bK inhibitor that produces reliable effects on insulin hypoglycemic action as well as glucose tolerance, which is also well tolerated, did not produce adverse reactions, and did not induce body weight loss or detectable behavioral abnormalities (Figure 3.7A–B). We showed that 6bK treatment transiently achieves >100 to 1,000-fold IC$_{50}$ levels of inhibitor in the peripheral organs responsible for insulin degradation, which suggests the viability of 6bK as a potential in vivo IDE inhibitor probe to study the physiological roles of this enzyme. The features of 6bK are summarized in Table 3.3.

The 6bK validation presented in this work is limited to short terminal experiments using a single dose of the experimental agent, which is in accordance with the standing committee on the Use of Animals in Research and Teaching at Harvard University (see Methods section). The optimal dose of 6bK was determined for mice of the C57BL/6J genetic background (Jackson Laboratories). Deviations from the procedures described herein may require revalidation of the IDE on-target effects using the appropriate injection controls, dose-response analysis, as well as IDE$^{-/-}$ knockout mice (described in Chapter 4). Similarly, we have not determined if
non-specific off-target effects may appear in repeated once-daily dosing or using chronic administration paradigms due to accumulation of 6bK or other metabolites.

**Figure 3.7** | **Acute treatment with 6bK is well tolerated.** (A) Body weight measurements for C57BL/6J mice treated with 6bK (■, 80 mg/kg i.p.) or vehicle alone (■). (B) Behavioral scoring observations during a typical experiment. Mice treated with 6bK (■, 80 mg/kg) are active, display normal posture, normal grooming, and response to stimulation, compared to vehicle controls (■). All data points and error bars represent mean ± SEM.
Table 3.3 | The features of 6bK satisfy the requirements for an *in vivo* probe\(^1\).

Adapted from reference 1.

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>6bK</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td>Discrete chemical species, characterized spectroscopically; Defined structure with reproducible preparative method</td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td>Defined purity and stability in test media; Free from non-specific chemical reactivity</td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
<td>Sufficiently soluble in aqueous media; e.g. &gt;100 µM; No aggregation effects in biochemical assays</td>
</tr>
<tr>
<td><strong>Permeability</strong></td>
<td>Proven passive membrane permeability, or define active transport mechanisms</td>
</tr>
<tr>
<td><strong>Potency</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Biochemical</strong></td>
<td>Typically &lt;100 nM in an in vitro biochemical assay; Sufficient to confidently associate with cellular activity</td>
</tr>
<tr>
<td><strong>Cellular</strong></td>
<td>Typically &lt;1-10 µM in a mechanistic cell-based assay; Sufficient to confidently address hypotheses in cells; Concentration-dependent effect on the biological target</td>
</tr>
<tr>
<td><strong>Analogs</strong></td>
<td>Closely related structures identified with similar activity; Correlation of biochemical target activity and activity in cells; Correlation of biomarkers of target modulation with biochemical and cellular potency</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td>Pharmacokinetic properties sufficient to achieve levels in target tissue relevant to cellular potency</td>
</tr>
<tr>
<td><strong>Selectivity</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Profile</strong></td>
<td>Defined selectivity for related targets or known targets of chemotype; Typically &gt;10-100 fold in biochemical assay; typically &gt;50 kinases profiled for kinase inhibitors; broader pharmacology profiling desirable</td>
</tr>
<tr>
<td><strong>Inactive analog</strong></td>
<td>Analog with no biochemical target activity shows no activity in cells</td>
</tr>
<tr>
<td><strong>Other chemotypes</strong></td>
<td>Probes from a different chemical class with similar activity</td>
</tr>
<tr>
<td><strong>Chemo-informatics</strong></td>
<td>Awareness of other activities associated with the chemical class</td>
</tr>
<tr>
<td><strong>Context</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Genetic methods</strong></td>
<td>RNAi and/or mutants of target available for complimentary experiments</td>
</tr>
<tr>
<td><strong>Target</strong></td>
<td>Cellular context of the target and potential linked activities considered</td>
</tr>
<tr>
<td><strong>Application</strong></td>
<td>Fitness of the probe to test the specific biological hypothesis considered</td>
</tr>
<tr>
<td><strong>Availability</strong></td>
<td>Origin, identity and properties fully disclosed; Available for use without restrictions; Accessible in quantities (15-20 mg) for follow-up studies</td>
</tr>
</tbody>
</table>
3.6 – Methods

Dosing and formulation of macrocycle inhibitors for in vivo studies.

The doses of 6bK used in this study were chosen based on literature precedent for small molecules and drugs of similar potencies\textsuperscript{2,30-35}. Procedures using experimental compounds were in accordance with the standing committee on the Use of Animals in Research and Teaching at Harvard University, the guidelines and rules established by the Faculty of Arts and Sciences' Institutional Animal Care and Use Committee (IACUC), and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals.

Purified macrocycle inhibitors were dissolved in DMSO\textsubscript{d\textsubscript{6}} (~200 to 250 mg/mL stock solutions). A sample aliquot (5 µL) of the macrocycle solution was diluted with 445 µL DMSO\textsubscript{d\textsubscript{6}}, then combined with 50 µL of freshly prepared solution of 20 mM CH\textsubscript{2}Cl\textsubscript{2} in DMSO\textsubscript{d\textsubscript{6}} in for \textsuperscript{1}H-NMR acquisition (600 MHz, relaxation time = 2 sec). The inhibitor concentration was calculated using the integral of the CH\textsubscript{2}Cl\textsubscript{2} singlet (δ 5.76 ppm, 2H)\textsuperscript{36}, which appears in an uncluttered region of these spectra\textsuperscript{37,38}. For injectable formulations (10 mL/kg i.p. injection volume), the macrocycle inhibitor solution in DMSO\textsubscript{d\textsubscript{6}} (200 – 250 mg/mL, based on free-base molecular weight) was combined with 1:20 w/w Captisol® powder (CyDex)\textsuperscript{7}. The resulting slurry was supplemented with DMSO\textsubscript{d\textsubscript{6}} to make up to 5% of the final formulation volume, mixed thoroughly and dissolved with sterile saline solution (0.9 % NaCl). Vehicle controls were identically formulated with 5 % DMSO\textsubscript{d\textsubscript{6}} and equal amount of Captisol®. The formulated solutions of inhibitor
were clear with no visible particles, and were stored overnight at 4 °C prior to injection.

**Stable isotope dilution LC-MS, pharmacokinetics, and tissue distribution measurements.** Heavy-labeled macrocycle inhibitor (heavy-6bK) was synthesized as described above, substituting “building block C” with Nα-Fmoc-Nε-Boc-lysine $^{13}$C$_6$N$_2$ (98 atom %, Sigma-Aldrich). The product was 8 mass-units heavier than 6bK, otherwise with identical properties and IC$_{50}$. Plasma samples (15 µL) from 6bK-treated mice and vehicle controls were combined with 5 µL of heavy-6bK in PBS (final concentration of 10 µM), and incubated for 30 min on ice. Plasma proteins were precipitated with 180 µL cold 1 % TFA in MeCN, sonicated 2 min, and centrifuged 13,000 g for 1 min. The supernatant was diluted 100- and 1000-fold for liquid chromatography-mass spectrometry (LC-MS) analysis. Tissue samples (~100 mg) from 6bK-treated mice and vehicle controls were weighed and disrupted in Dounce homogenizers with PBS buffer (0.5 mL/100 mg sample), supplemented with 5 µM heavy-6bK and protease inhibitor cocktail (1 tablet/50 mL PBS, Roche diagnostics). The lysate was incubated on ice for 30 min, sonicated 5 min and centrifuged at 13,000 g for 5 min. The bulk of supernatant proteins were precipitated by denaturation at 95 °C for 5 min, removed by centrifugation. A 50 µL aliquot of the supernatant was treated with 450 µL cold 1 % TFA in MeCN, cleared by centrifugation and diluted 100-fold for LC-MS analysis as described above for plasma samples. A standard curve of 6bK and heavy-6bK (1 µM each and 3-fold
serial dilutions) were used for LC-MS quantitation using a Waters Q-TOF premier instrument.

**In vivo studies, general information.** Wild-type C57BL/6J and diet-induced obese (DIO) C57BL/6J age-matched male adult mice were purchased from Jackson Laboratories. The age range was 13 to 15 weeks for lean mice, and 24 to 26 weeks for DIO mice. All animals were individually housed on a 14-h light, 10-h dark schedule at the Biology Research Infrastructure (BRI), Harvard University. Cage enrichment included cotton bedding and a red plastic hut. Water and food were available *ad libitum*, consisting respectively of normal chow (Prolab® RHM 3000) or high-fat diet (60 kcal % fat, D12492, Research Diets Inc.). Adult IDE knock-out mice (IDE−/−) fully back-crossed to the C67BL/6J line were obtained from Mayo Clinic (Florida), and housed in the Biology Research Infrastructure (BRI), Harvard University, for 8 weeks prior to experiments as described above, and experiments were conducted on age-matched mice cohorts ranging 17 to 21 weeks old. All animal care and experimental procedures were in accordance with the standing committee on the Use of Animals in Research and Teaching at Harvard University, the guidelines and rules established by the Faculty of Arts and Sciences' Institutional Animal Care and Use Committee (IACUC), and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals. Power analysis to determine animal cohort numbers was based on preliminary results and literature precedent, usually requiring between 5 and 8 animals per group. Animals were only
excluded from the cohorts in cases when we identified occasional DIO mice with an outlier diabetic phenotype (e.g. >200 mg/dL fasting blood glucose). Age- and weight-matched mice were randomized to each treatment group.

**Glucose tolerance tests GTT and blood glucose measurements.** Prior to a glucose challenge, the animals were fasted for 14 h (8 pm to 10 am, during the dark cycle) while individually housed in a clean cage with inedible wood-chip as a floor substrate, cotton bedding and a red plastic hut. Inhibitor, vehicle or control compounds were administered by a single intraperitoneal (i.p.) injection 30 min prior to the glucose challenge. Dextrose was formulated in sterile saline (3 g in 10 mL total), and the dose was adjusted by fasted body weight. For IPGTTs, 1.5 g/kg dextrose was administered by i.p. injection at a dose of 5 mL/kg. Blood glucose was measured using AccuCheck® meters (Aviva) from blood droplets obtained from a small nick at the tip of the tail, at timepoints -45, 0, 15, 30, 45, 60, 90 and 120 min with reference to the time of glucose injection. The area of the blood glucose response profile curve corresponding to each animal was calculated by the trapezoid method⁹, using as reference each individual baseline blood glucose measurement prior to glucose administration (t = 0) or the lowest point of the curve. The sum of the trapezoidal areas between the 0, 15, 30, 45, 60, 90 and 120 minute time points corresponding to each animal were summed to obtain the area under the curve (AUC). The relative area values are expressed as a percentage relative to the average AUC of the vehicle cohort, which is defined as 100 %. Values are reported as mean ±
Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are * $p < 0.05$ versus vehicle control group or ** $p < 0.01$ versus vehicle control group unless otherwise stated.

**Insulin Tolerance Test (ITT).** For hormone challenges animals were fasted individually housed as described above. For ITT the fasting period was 6 h (7 am to 1 pm). Inhibitor or vehicle alone was injected i.p. as previously described, 30 min prior to the hormone challenge. Insulin (Humulin-R®, Eli Lilly) was injected subcutaneously (s.c.) 0.25 U/kg or intraperitoneally i.p. 1 U/kg formulated in sterile saline (5 mL/kg). Blood glucose was measured at timepoints -45, 0, 15, 30, 45, 60 and 75 min with reference to the time of hormone injection, by microsampling from a tail nick as described above. Values are reported as mean ± S.E.M. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are * $p < 0.05$ versus vehicle control group or ** $p < 0.01$ versus vehicle control group.

**Blood collection and plasma hormone measurements.** Blood was collected in EDTA-coated tubes (BD Microtainer®) from trunk bleeding (~500 µL) after CO₂-euthanasia for all hormone assays. The plasma was immediately separated from red blood cells by centrifugation 10 min at 1800 g, aliquoted, frozen over dry ice and stored at -80 °C. Insulin, glucagon, amylin and pro-insulin C-peptide fragment were quantified from 10 µL plasma samples using magnetic-bead Multiplexed Mouse
Metabolic Hormone panel (Milliplex, EMD Millipore) according to the manufacturer’s instructions, using a Luminex FlexMap 3D instrument. Plasma containing high levels of human insulin (Humulin-R) were quantified using 25 µL samples with Insulin Ultrasensitive ELISA (ALPCO). Values are reported as mean ± S.E.M. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are * $p < 0.05$ versus vehicle control group or ** $p < 0.01$ versus vehicle control group.

**Amyloid peptide measurements.** Lean C57BL/6J mice were treated with 6bK (80 mg/kg), vehicle alone, or bisepi-6bK (80 mg/kg). Two hours post-injection, hemibrains were promptly dissected after CO$_2$-euthanasia and stored at -80 °C until extraction. A hemibrain (~150 mg wet weight) was homogenized in a Dounce homogenizer in 0.2% diethylamine and 50 mM NaCl (900 µL). The homogenate was centrifuged at 20,000 × g for 1 h at 4°C to remove insoluble material. A fraction of supernatant (100 µL) was neutralized with 1:10 volume of Tris HCl, pH 6.8. The sample was diluted 1:4 in assay buffer, and analyzed for Aβ(40) and Aβ(42) levels using the respective Aβ ELISA assays (Invitrogen) following the manufacturer’s protocols.
3.7 – References


Chapter 4

Discovery of the Anti-diabetic Activity of Insulin-Degrading Enzyme Inhibitors Mediated by Multiple Hormone Substrates


Contributions: I designed and performed the experiments and hormone measurements described in this chapter. Amanda K. McFedries assisted with the in vivo experiments. Xiu Quan Du assisted with the KO mice experiments at Maureen Charron’s group (Albert Einstein College of Medicine, NY).
4.1 – Introduction: glucose tolerance tests and mice models of diabetes

To determine the physiological consequences of acute IDE inhibition in vivo, we evaluated the glucose tolerance of mice treated with 6bK. This model was chosen due to: i) the short treatment duration\(^1,2\); ii) the extensive mechanistic validation and understanding provided by decades of research\(^3-6\); and iii) its direct relevance to human disease management and diagnosis\(^7-9\). We used two standard methods of glucose delivery, either oral gavage or i.p. injection,\(^1\) and two different mouse models, lean or diet-induced obese (DIO) mice\(^3,4\). These four conditions were chosen to survey the role of IDE activity under a broad range of endogenous insulin levels and insulin sensitivity\(^1,4\). Oral glucose administration, for example, results in greater insulin secretion compared to injected glucose delivery (Figure 4.1). Passage of glucose through the gut causes the release of GLP-1, which strongly augments glucose-dependent insulin secretion\(^4,10\). This phenomenon is referred to as the ‘incretin effect’ and is magnified in DIO mice\(^4\). In addition, DIO mice display hyperinsulinemia and insulin resistance compared to lean mice, enabling us to test the consequences of IDE inhibition in a model that resembles early type-2 diabetes in humans\(^3\).
Dependence of insulin and glucagon secretion on the route of glucose administration (oral or i.p.) and the hyperinsulinemic phenotype of DIO versus lean mice. (A) The early insulin response to glucose in lean and DIO mice is higher during OGTT than IPGTT. (B) Suppression of glucagon secretion post-glucose administration is less effective after IPGTT and in DIO mice. All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.

4.2 – IDE inhibition improves blood glucose clearance following oral glucose administration

In all glucose tolerance experiments we included two control groups: vehicle alone, and the inactive isomer$^{11}$ bisepi-6bK, which is identical to 6bK in chemical composition and bond connectivity, but has virtually no IDE inhibition activity ($\text{IC}_{50} > 200 \, \mu\text{M, Figures 3.6–3.7}$). In all glucose tolerance studies I used a standardized formulation of compound dissolved in sterile saline plus Captisol®$^{12}$, and I injected an effective dose of 6bK (or inactive control) of 2 mg/mouse i.p., representing 80
mg/kg for lean C57BL/6J mice (25 g), and 60 mg/kg for diet-induced obese (DIO) mice (35-45 g), unless otherwise stated (Chapter 3, Section 3.4).

In one set of experiments we examined the effect of 6bK on blood glucose levels during an oral glucose tolerance test (OGTT). Lean or DIO mice were fasted overnight for these experiments, and then treated with a single dose of 6bK, vehicle alone, or a matching dose of inactive bisepi-6bK. After 30 minutes, glucose was administered by oral gavage, and glycemia was measured from a droplet of blood obtained from a tail nick throughout a period of 2 hours. This timeframe corresponds approximately to peak concentration of 6bK in peripheral circulation, which is sustained reliably above 100-fold (and up to 1,000-fold) the IC$_{50}$ levels of 6bK in blood, as well as the liver and the kidneys, the main sites of insulin degradation$^{13,14}$ (Figure 3.2A).

Importantly, we observed that both lean and DIO mice treated with 6bK displayed significantly improved oral glucose tolerance compared to vehicle or inactive bisepi-6bK control groups (Figure 4.2). The two control groups exhibited similar blood glucose profiles, indicating that the observed effects of 6bK on glucose tolerance are lost when the stereochemistry of 6bK is altered in a way that abolishes IDE inhibition (see Section 4.7 for IDE$^{-/-}$ knockout controls, Figure 4.10).

The literature suggests that effects of similar magnitude on oral glucose tolerance in mice have been observed using several approved human anti-diabetic therapeutics$^{8,15,16}$. We showed that the glucose tolerance improvement provided by the anti-diabetes DPP4 inhibitor drug sitagliptin (administered at a higher-than-
standard murine dosing\textsuperscript{17} was comparable to the effect of 6bK dosed to littermate DIO mice (Figure 4.3). Moreover, co-administration of sitagliptin and 6bK (low dose, 40 mg/kg) resulted in effectively lower blood glucose levels than with either inhibitor alone (Figure 4.3B). The additive effects of these inhibitors suggests that DPP4 and IDE regulate distinct nodes along the insulin signaling pathway, and furthermore that co-administration of IDE inhibitors with drugs targeting the incretin pathway could provide promising therapeutic strategies (Section 4.8).

Collectively, these observations support a model in which IDE regulates glucose-induced insulin signaling, and therefore glucose tolerance, and demonstrate that acute IDE inhibition improves post-prandial glucose control in lean and DIO mice (Figures 4.2–4.3). Together, these results represent the first time that IDE inhibition has been shown to improve blood glucose clearance through the activity of endogenous insulin in a living animal\textsuperscript{13,14,18–20}.

**Figure 4.2** | Physiological consequences of acute IDE inhibition by 6bK on oral glucose tolerance in lean and DIO mice. (A) Male C57BL/6J lean (25 g) mice were treated with a single i.p. injection of IDE inhibitor 6bK (■, 80 mg/kg), inactive control bisepi-6bK (■, 80 mg/kg, n = 7), or vehicle (■, n = 7) at time 0. Oral glucose gavage was performed at time 0 (Rx) and blood glucose levels were measured at time points (3.0 g/kg).
(Figure 4.2 cont.) mg/kg), or vehicle alone (■) 30 min prior to glucose gavage (3.0 g/kg). (B) DIO mice (35-45 g) were treated with 6bK (■, 60 mg/kg), and inactive control bisepi-6bK (■, 60 mg/kg) or vehicle alone (■) 30 min prior to glucose gavage (3.0 g/kg). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.

Figure 4.3 | Co-administration of IDE inhibitor 6bK and DPP4 inhibitor sitagliptin followed by an oral glucose challenge. (A) DIO mice were first treated with either oral gavage of sitagliptin (■■ 4 mg/kg, 5 mL/kg in sterile saline) or saline alone. After 30 min, the mice were treated either with a low dose of 6bK (■■ 40 mg/kg) or vehicle alone (■), and after an additional 30 min all mice were given a bolus of glucose by gavage (3.0 g/kg, 10 mL/kg). Mice treated with the combination of sitagliptin and 6bK displayed glucose levels lower than baseline ($t = 0$) after 60 min. (B) Blood glucose profile areas of sitagliptin and 6bK were similarly reduced by 60-64% compared to vehicle alone, and further 15% lower when sitagliptin and 6bK were co-administered together. All data points and error bars represent mean ± SEM. Statistics were performed using a one-tail Student’s t-test. Significance levels shown in the figures are: * $p < 0.017$ (Bonferroni correction) versus vehicle control group, # $p < 0.017$ versus the 6bK cohort. See the Methods section for a description of the AUC calculation.
4.3 – IDE inhibition during an injected glucose challenge leads to impaired glucose tolerance

Prior work using IDE\(^{-/-}\) mice characterized the effect of i.p. glucose injections, therefore we repeated the above experiments with 6bK followed by i.p. injected glucose tolerance tests (IPGTTs) to provide a more direct comparison with the knockout animal experiments\(^6,20\). In contrast to the observed improvement in oral glucose tolerance upon 6bK treatment (Figure 4.2A), IDE inhibition with 6bK followed by a glucose injection (1.5 g/kg i.p.) resulted in impaired glucose tolerance after 2 h in both lean and obese mice compared to vehicle alone or bisepi-6bK-treated controls (Figure 4.4A). These changes in the glucose response profiles of lean mice treated with 6bK compared to vehicle controls resemble the reported differences between IDE\(^{-/-}\) and IDE\(^{+/+}\) mice during similar IPGTTs\(^6,20\) (Chapter 1). Moreover, DIO mice treated with 6bK followed by glucose injection displayed a biphasic response: glucose levels are lower over the initial 30 minutes of the IPGTT, followed by a hyperglycemic “rebound” starting 1 h after glucose injection (Figure 4.4B). Both the suppression of peak glucose levels and the magnitude of the hyperglycemic rebound were dependent on 6bK dose, and neither effect was observed in cohorts treated with vehicle alone or inactive bisepi-6bK, indicating that the impaired glucose tolerance during an IPGTT correlates with IDE activity (Figure 4.4B and Figure 3.6).

Collectively, the results of the OGTTs and IPGTTs indicate that the route of glucose administration impacts the physiological response of 6bK-treated animals in
ways that cannot be explained by a simple model in which IDE’s physiological role is only to degrade insulin. Instead, these results strongly suggest a role for IDE in regulating other glucose-regulating peptide hormones in vivo. These results prompted us to investigate other putative substrates of IDE identified using in vitro cleavage assays (Section 4.4), and to treat IDE−/− knockout mice lacking the target of 6bK to show that glucose tolerance changes upon 6bK treatment are mediated by IDE under both OGTT and IPGTT conditions (see Section 4.7, Figure 4.10).

Figure 4.4 | Impact of the route of glucose administration on the phenotype of glucose tolerance in lean and DIO mice following treatment with IDE inhibitor 6bK. Glucose tolerance phenotypes after i.p. injection of glucose (1.5 g/kg) in, respectively, lean (A) and DIO (B) male mice treated with 6bK (■), inactive bisepi-6bK (■), or vehicle alone (■). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.
4.4 – IDE regulates multiple hormones *in vivo*

The biochemical properties of IDE and its substrate recognition mechanism\textsuperscript{21-23} enable this enzyme to cleave a wide range of peptide substrates *in vitro* (Chapter 1, \textbf{Figure 1.7}) for which experimental validation *in vivo* has not previously been possible. Two glucose-regulating hormones, beyond insulin, that are potential candidates for physiological regulation by IDE during a glucose challenge are glucagon and amylin. Despite decades of research, only purified IDE has been previously shown to cleave these two peptides *in vitro*\textsuperscript{24-26}, but neither hormone is known to be regulated by IDE activity *in vivo* or *ex vivo*. Compared to insulin, glucagon is a modest *in vitro* IDE substrate ($K_M = 3.5 \mu M$ for glucagon versus $K_M < 30$ nM for insulin)\textsuperscript{24}, although IDE is capable of degrading glucagon at a comparable rate if present in sufficiently high concentrations ($k_{cat} = 38 \text{ min}^{-1}$ for glucagon)\textsuperscript{25}. Amylin is also a substrate for IDE *in vitro* ($K_M \approx 0.3 \mu M$)\textsuperscript{26}. Other proteases suggested to degrade glucagon include nardilysin, cathepsins B and D, in cells and *in vitro*\textsuperscript{27,28}, and neprilysin, which was shown to play a role in renal clearance of glucagon\textsuperscript{29}. However, none of these enzymes are known to regulate endogenous processing of these hormones, modulate blood glucose levels, or correlate with metabolic disease susceptibility. To our knowledge, no proteases have been previously shown to degrade amylin *in vivo*\textsuperscript{26}.

To begin to probe the possibility that glucagon or amylin are regulated *in vivo* by IDE, I measured the plasma levels of these hormones at 20 and 130 minutes post-glucose injection in DIO mice treated with 6bK or vehicle alone during an IPGTT
(Figure 4.5). Plasma collected 20 minutes post-glucose injection showed elevated insulin and amylin levels, but unchanged glucagon levels, for the 6bK-treated cohort relative to the control group (Figure 4.5A). During the hyperglycemic rebound 130 min post-injection, glucagon levels for the 6bK group were strongly elevated above 200 pg/mL, compared with 90 pg/mL glucagon in control mice (Figure 4.5A).

Consistent with these elevated glucagon levels, expression of a gluconeogenesis transcriptional marker, G6Pase (glucose-6-phosphatase mRNA)\textsuperscript{30,31}, was elevated in the livers of 6bK-treated mice compared to control mice (Figure 4.5B).

Because hormone abundance measurements can be difficult to interpret during fluctuations in blood glucose that in turn affect pancreatic hormone secretion, we performed additional studies to confirm the relationship between IDE activity and glucagon and amylin levels \textit{in vivo}. To more directly establish the effect of IDE inhibition on the clearance of insulin, amylin, and glucagon \textit{in vivo}, I injected each of these three hormones into lean mice 30 min after treatment with 6bK or vehicle alone (Figure 4.6). The 6bK-treated cohorts exhibited significantly stronger blood glucose responses to each of these hormones compared to vehicle controls; mice treated with 6bK experienced hypoglycemia during insulin tolerance tests (Figure 4.6A) and hyperglycemia following challenges with either amylin (Figure 4.6B) or glucagon (Figure 4.6C) relative to control animals. Similar to glucagon, acute administration of a high dose (50–100-fold above physiological levels) of amylin to rodents is known to result in a transient increase in blood glucose levels through gluconeogenesis and activation of lactic acid flux from muscle tissue to the liver (the
Moreover, I determined that in each case the plasma level of the hormone injected remained elevated at the end of the procedure in 6bK-treated mice relative to control animals, demonstrating a role for IDE in regulating the abundance of these hormones (Figure 4.6A-C insets).

**Figure 4.5 | Acute IDE inhibition affects the abundance of multiple hormone substrates during an injected glucose challenge to DIO mice.** (A) Plasma hormone measurements at 20 and 135 minutes post-IPGTT (Figure 4.4B) for DIO mice treated with 6bK (■) or vehicle alone (■). (B) RT-PCR analysis of DIO liver samples collected at 135 min post-IPGTT reveals 50 % higher glucose-6-phosphatease (G6Pase) and 30 % lower phosphoenolpyruvate carboxykinase (PEPCK) transcript levels for the 6bK-treated cohort (■) versus vehicle-only (V) controls (■). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.
Figure 4.6 | Acute IDE inhibition potentiates multiple injected hormone substrates.

Blood glucose responses and abundance of injected hormones in lean mice 30 min after treatment with 6bK (■, 80 mg/kg) or vehicle alone (■). (A) Insulin s.c. (0.25 U/kg) after 5-hour fast. (B), Amylin s.c. (250 µg/kg) after overnight fast. (C) Glucagon s.c. (100 µg/kg) after overnight fast. Trunk blood was collected at the last time points for plasma hormone measurements (insets). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are \( p < 0.05 \) (*) or \( p < 0.01 \) (**) versus the vehicle-only control group. (D) Model showing the connection between the injected hormones in panels A–C and the corresponding changes in blood glucose levels, which are augmented by inhibition of IDE-mediated hormone degradation in vivo.
4.5 – IDE inhibition promotes glucagon signaling and gluconeogenesis

Taken together, our results strongly suggest that IDE activity regulates the stability and physiological activities of glucagon and amylin, in addition to insulin. Higher glucagon levels upon 6bK treatment provide a possible explanation for impaired glucose tolerance observed during an IPGTT. Substrates are processed by IDE at rates dependent on their relative concentrations, and we observed two- to four-fold higher insulin levels during OGTT than IPGTT, consistent with the incretin effect\(^1,4,10\). During an OGTT, IDE inhibition therefore results primarily in an increase in insulin signaling and lower blood glucose levels. In contrast, during an IPGTT the incretin pathway is circumvented, insulin secretion levels are lower\(^1,4\) and glucagon secretion is not suppressed by GLP-1\(^10\). Hence, in the context of IPGTT, the loss of IDE-mediated glucagon degradation by 6bK inhibition results in elevation of glucagon and gluconeogenesis (Figures 4.4 and 4.5). The biphasic IPGTT profile that results using DIO mice is also consistent with this model (Figure 4.4B). DIO mice have higher insulin levels\(^3,4\), and the early improvement in glucose tolerance is explained by IDE inhibition primarily potentiating insulin during this period, but this effect is short-lived as glucagon levels rise. This transient improvement is absent in lean mice, in which insulin is secreted to lower concentrations during IPGTTs, compared to either OGTT or a DIO mouse (Figure 4.1). This model predicts that abrogating glucagon signaling should reverse the elevation of blood glucose by 6bK treatment during an IPGTT, while not substantially affecting the signaling pathways through which 6bK treatment lowers blood glucose during an OGTT.
To test these hypotheses, we collaborated with Dr. Maureen Charron (Albert Einstein College of Medicine) to repeat the glucose tolerance experiments using GCGR$^{−/−}$ mice that lack the G-protein coupled glucagon receptor (Figure 4.7)$^{33-36}$. As expected, mice lacking glucagon signaling exhibited a further improvement in oral glucose tolerance upon 6bK treatment relative to vehicle controls that was similar to the oral glucose tolerance improvement observed in wild-type mice (Figure 4.7A versus Figure 4.2A). This result is consistent with a model in which insulin and amylin signaling in these mice is intact and regulated by IDE, albeit a rapid clearance of blood glucose is observed for both these cohorts due to the insulin sensitivity that characterizes GCGR$^{−/−}$ mice$^{33-36}$. In contrast, glucose tolerance in GCGR$^{−/−}$ mice following IPGTT was not impaired by 6bK treatment, consistent with a model in which glucagon signaling is responsible for driving the impaired glucose tolerance of wild-type lean and DIO mice upon 6bK treatment during an IPGTT (compare Figure 4.7B and Figure 4.4A). This observation supports a model in which IDE regulates glucagon signaling during IPGTTs. Under these conditions and using a dose of dextrose i.p. 1.5 g/kg, there was no further lowering of blood glucose in 6bK-treated GCGR$^{−/−}$ mice, possibly because IPGTT elicits modest insulin secretion, and amylin-induced gastric emptying effects cannot play a role$^{37}$. Taken together, our results suggest that IDE inhibition promotes endogenous glucagon signaling that can impair glucose tolerance during an IPGTT in wild-type mice.
The endogenous signaling activity of glucagon is required for impairment of injected glucose tolerance following acute IDE inhibition. G-protein-coupled glucagon receptor knockout mice (GCGR−/−, C57BL/6J background) treated with IDE inhibitor 6bK (■, 80 mg/kg) display altered glucose tolerance relative to vehicle-treated mice (■) if challenged with oral glucose (A) but not i.p. injected glucose (B). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.

To investigate the effect of IDE inhibition on endogenously secreted glucagon using a complementary method, I subjected mice treated with 6bK, vehicle, or inactive bisepi-6bK to a pyruvate tolerance test (PTT). This challenge evaluates the ability of the liver, under the influence of glucagon, to use pyruvate as a gluconeogenic substrate (Figure 4.8). I measured a panel of hormones and liver mRNA markers after the procedure and observed the 6bK-treated cohort displayed significantly elevated plasma glucagon and increased expression of liver gluconeogenic markers compared to both control groups (50% higher PEPCK, phosphoenolpyruvate carboxykinase, and 40% higher G6Pase, glucose-6-
However, the 6bK cohort also experienced significantly lower blood glucose during the PTT, suggesting that IDE inhibition produced a concomitant stimulation of systemic glucose clearance that outweighed the gluconeogenic output of the liver. These results collectively suggest that under conditions that favor gluconeogenesis IDE inhibition can augment endogenous glucagon signaling accounting for impaired, rather than improved, glucose tolerance during IPGTT.

Figure 4.8 | Acute IDE inhibition modulates the endogenous signaling activity of glucagon during a pyruvate/gluconeogenesis challenge. (A) Wild-type mice fasted overnight were injected i.p. with pyruvate (2.0 g/kg) 30 min after treatment with 6bK (■), inactive analog bisepi-6bK (■), or vehicle alone (■). (B) Plasma hormone measurements 60 min post-PTT reveal elevated glucagon but similar insulin levels for the 6bK-treated cohort (■) relative to bisepi-6bK (■), or vehicle (■) controls. (C) RT-PCR analysis of liver samples 60-min post-PTT revealed elevated gluconeogenesis transcriptional markers for the 6bK-treated group (■) relative to vehicle controls (■). All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.
4.6 – IDE inhibition promotes amylin signaling and gastric emptying

Amylin is co-secreted with insulin, making up 5% of the secretory granules of β-cells. Amylin is involved in glycemic control by inhibiting gastric emptying through the vagal route⁹, promoting satiety during meals³⁷,³⁹, and antagonizing glucagon signaling⁴⁰. Unlike the effect of a non-physiologically high dose, amylin’s endogenous functions supports the concept that increased amylin levels are beneficial for blood glucose control during meals. Indeed, a synthetic analog of amylin, pramlintide (Symlin) is an injectable drug used clinically to manage post-prandial hyperglycemia (Figure 4.9D)⁸,¹⁶.

However, the blood glucose effects of amylin during an oral glucose tolerance experiment would be overshadowed by the rapid absorption of glucose through the walls of the stomach/duodenum, as well as the dominant hypoglycemic action of insulin on blood sugar. Therefore, to study the effect of IDE inhibition on endogenous amylin signaling I optimized a gastric retention assay⁴¹, which is an amylin-specific process triggered by receptors in the Area Postrema (AP) of the brainstem⁹,³⁷. Using phenol red to label a glucose solution (see Methods) we measured the gastric emptying efficiency of mice pre-treated with 6bK, inactive control bisepi-6bK, or vehicle alone (Figure 4.9). Mice treated with 6bK exhibited 2-fold slower gastric emptying of the labeled glucose solution measured at 30 minutes post-gavage compared to vehicle and bisepi-6bK-treated controls (Figure 4.9). Moreover, I demonstrated that co-administration of the specific amylin receptor antagonist AC187³⁷ blocked the effects of 6bK on gastric emptying. Collectively,
these data reveal that IDE inhibition can slow post-prandial gastric emptying and demonstrate a role for IDE in modulating amylin signaling in vivo at physiologically relevant substrate levels. These results also suggest that IDE inhibition may mimic the effect of amylin supplementation with pramlintide/Symlin during meals and its beneficial effects on post-prandial glucose control. However, in a therapeutic context pramlintide/Symlin must be co-injected twice daily with insulin, and our findings raise the possibility that IDE-targeting drugs could replace these injections by raising endogenous amylin and insulin levels during meals.

Figure 4.9 | Acute IDE inhibition modulates endogenous amylin signaling revealed by gastric emptying efficiency, an amylin-specific effect. (A) Wild-type mice fasted overnight were given an oral glucose bolus, supplemented with 0.1 mg/mL phenol red,
(Figure 4.9 cont.) 30 min after treatment with 6bK alone (■), 6bK co-administered with the specific amylin receptor antagonist AC187 (■, 3 mg/kg i.p.), vehicle alone (■), or inactive bisepi-6bK (■). The stomachs were dissected at 30 min post-glucose gavage. (B) Plasma hormone measurements 15 and 30 min post-OGTT reveal modestly elevated amylin levels for the 6bK-treated cohort (■, n.s.) relative to bisepi-6bK (■), or vehicle (■) controls. (C) Model representing the mechanisms through which amylin is involved in glycemic control by antagonizing glucagon signaling\textsuperscript{40}, and by inhibiting gastric emptying through the vagal route acting on the Area Postrema of the brainstem\textsuperscript{9}, which also promotes satiety during meals\textsuperscript{42}. (D) Amino acid sequences of rodent amylin, pramlintide (Symlin), and AC187, a structurally related amylin receptor (AR) antagonist peptide. All data points and error bars represent mean ± SEM. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are $p < 0.05$ (*) or $p < 0.01$ (**) versus the vehicle-only control group.

4.7 – Knockout experiments corroborate the IDE on-target effects of 6bK

To further test if the observed effects of 6bK are specific to its interaction with IDE, we repeated the GTT experiments using IDE\textsuperscript{−/−} knockout mice\textsuperscript{6,20}. Mice lacking IDE were unaffected by 6bK treatment (Figure 4.10), and exhibited blood glucose responses and profile areas (AUC) indistinguishable to that of the vehicle-treated cohort in both OGTT and IPGTT experiments (Figure 4.12). These results show that the blood glucose profile improvement during OGTT and impairment during IPGTT of 6bK-treated wild-type mice are mediated by IDE (Figures 4.2–4.4) and support our observations from dose-response experiments and bisepi-6bK inactive control injections (Chapter 3, Section 3.4).

It has been previously proposed that IDE\textsuperscript{−/−} knockout mice undergo pronounced compensatory changes in metabolic regulation, as evidenced by changes
in insulin resistance and down regulation of insulin receptors\(^6,20\). However, our results suggest that glucagon is an IDE substrate that has not been accounted for in this model leading to impaired glucose tolerance. The prediction is that if compensation is indeed occurring in mice chronically lacking IDE, then adult IDE\(^{-/-}\) mice should display impaired tolerance both to oral and injected glucose because they have lower insulin-receptor levels. Therefore, we performed an OGTT using IDE\(^{-/-}\) mice and compared it to age-matched wild-type IDE\(^{+/+}\) mice (Figure 4.11). Unlike 6bK-treated animals, which show improved glucose tolerance during an oral GTT, IDE\(^{-/-}\) mice have impaired oral glucose tolerance when compared to similarly treated IDE\(^{+/+}\) mice (Figure 4.11A), thus corroborating that chronic deletion of IDE leads to a dysregulation of the insulin axis that is not predictive of the outcome of IDE inhibition\(^6,20\). This data supports the compensatory model described by Abdul-Hay et al. in 2011 relying on IPGTT experiments (Figure 4.11B), while also demonstrating the value of small-molecule probes to validate therapeutic targets that are masked by compensation following gene deletion. Moreover, these results further support that compensation is occurring with IDE\(^{-/-}\) mice and that, in these absence of these compensatory changes, inhibition of IDE can result in improved glucose tolerance through potentiation of insulin signaling.

Many of the concerns about long-term effects of IDE inhibition can be potentially inferred from studies using IDE knockout mice\(^6,20,43\). Mice lacking IDE activity are viable, fertile, do not display conspicuous phenotypic defects, and have normal body weight, as well as normal organ sizes and morphologies. While
additional studies are needed to progress these discoveries towards human therapeutics, these attributes of IDE\textsuperscript{\textminus/\textminus} KO mice are encouraging for the potential pharmaceutical inhibition of IDE\textsuperscript{44}. Nevertheless, it is possible that chronic inhibition of IDE is not necessarily desirable; instead, our findings suggest that transient pre-meal IDE inhibition may be a promising approach to help manage post-prandial hyperglycemia. For a competitive inhibitor that obstructs binding of all substrates to the IDE chamber (with the exception of substrate-selective IDE inhibitors developed in Chapter 5) a short lasting pre-meal therapeutic strategy would generate less concern about side effects, given that IDE’s role in degrading glucagon may modulate basal blood glucose levels between meals.

**Figure 4.10 |** Mice lacking IDE display no effects of 6bK treatment. (A) Mice lacking IDE treated with 6bK (○, 80 mg/kg) followed by oral glucose produce an identical response compared to vehicle-treated controls (●) of the same genotype (IDE\textsuperscript{\textminus/\textminus}). (B) Similarly, mice lacking IDE treated with 6bK (○, 80 mg/kg) followed by IPPGTT display an identical response compared to vehicle-treated (●) IDE\textsuperscript{\textminus/\textminus} control mice. All data points and error bars represent mean ± SEM.
Figure 4.11 | Oral glucose tolerance of mice lacking IDE compared to WT lean mice.
Mice lacking IDE (●, IDE−/−) display impaired glucose tolerance during OGTT (A) and IPGTT (B) compared to age-matched IDE+/+ controls (■), supporting the hypothesis that chronic deletion of IDE leads to pronounced compensatory changes in metabolism that are not predictive of the outcome of IDE inhibition6,20. All data points and error bars represent mean ± SEM.

4.8 – Therapeutic implications of IDE inhibition

This chapter described the application of the first potent, highly selective, and physiologically active small-molecule IDE inhibitor, revealing for the first time that acute IDE inhibition can lead to improved glucose tolerance in lean and obese mice after oral glucose administration, conditions that mimic the intake of a meal (see AUC summary in Figure 4.12). Following decades of speculation, these results establish the potential of IDE as a therapeutic target for the treatment of type-2 diabetes13,14.
Our data show that small-molecule IDE inhibitors can improve oral glucose
tolerance to an extent comparable to that of the DPP4 inhibitor sitagliptin (Figure
4.3)\textsuperscript{8,10}. The potential relevance of these animal studies to human disease is
supported by the repeated recognition of IDE as a diabetes susceptibility gene in
humans and rodents\textsuperscript{20,45-50}. Moreover, it is established that mice lacking IDE are
viable and do not display conspicuous phenotypic or health defects beyond their
glucose metabolism\textsuperscript{6,20}. While additional studies are needed to progress these
discoveries towards human therapeutics, these attributes of IDE\textsuperscript{-/-} KO mice are
encouraging for the potential pharmaceutical inhibition of IDE\textsuperscript{44}.

Equally important, our additional \textit{in vivo} and biochemical experiments using
6bK unveiled that IDE is a regulatory node for several of the key hormones in
glucose homeostasis, regulating the stability and signaling of glucagon and amylin, in
addition to its assumed role in insulin degradation (Figure 4.13)\textsuperscript{6,13,14,19,20}. The
identification of two additional pancreatic hormones as endogenous IDE substrates
advances our understanding of the role of IDE in regulating physiological glucose
homeostasis. Indeed, amylin-mediated effects on gastric emptying and satiety during
meals have been recently recognized to have therapeutic relevance in the treatment of
diabetes\textsuperscript{8,10}, and our results represent the first demonstration of a small-molecule that
can regulate both amylin and insulin signaling. Moreover, the link between IDE and
glucagon revealed in this study provides additional evidence of the importance of
glucagon regulation in human diabetes\textsuperscript{51}.
Figure 4.12 | Summary of physiological consequences of acute IDE inhibition by 6bK on glucose tolerance in lean, DIO, and IDE−/− mice.  (A) Area under the curve (AUC) calculations, during OGTT, lean and DIO mice treated with 6bK (■) display improved glucose tolerance (lower blood glucose area), compared to vehicle controls (■) and inactive bisepi-6bK (■).  (B) In contrast, during IPGTT both lean and DIO mice treated with 6bK (■) display impaired glucose tolerance (higher blood glucose area) compared to vehicle (■) or bisepi-6bK (■) controls. Significance tests were performed using two-tail Student’s t-test, and significance levels shown are \( p < 0.05 \) (*) or \( p < 0.01 \) (**) versus the vehicle-only control group.  See the Methods section for a description of the AUC calculation.

This study also reveals that the therapeutic potential of IDE inhibition using competitive inhibitors poses the pharmacological requirement to circumvent elevation of glucagon levels\(^{51}\). Based on our findings, the proposed specific strategies
to bring about a therapeutic benefit from IDE inhibition without affecting endogenous glucagon signaling and gluconeogenesis are:

(1) Development of fast- and short-acting IDE inhibitors that can be taken with/prior to food to transiently potentiate endogenous insulin and amylin secreted in response to the meal to help control post-prandial glycemia\textsuperscript{8,15}, but which is cleared or degraded before glucagon secretion resumes. Similar pre-meal therapeutic strategies with short-lived agents have already proven successful with fast-acting insulin analogs, secretagogues\textsuperscript{15}, and amylin supplementation\textsuperscript{8,9}.

(2) Combination therapy with glucagon-lowering incretin therapies and DPP4 inhibitors\textsuperscript{52}. We can speculate that these agents may also provide additive effects when co-administered with an IDE inhibitor; indeed 6bK treatment resulted in stronger improvements in oral glucose tolerance when co-administered with sitagliptin (Figure 4.3)\textsuperscript{8,10}. Alternatively, the combination of an IDE inhibitor with a glucagon receptor antagonist\textsuperscript{51}, may also prevent elevation of glucagon signaling, as suggested by our experiments with glucagon-receptor knockout mice (Figure 4.7).

(3) In addition, our structural findings presented in Chapter 2 raise the possibility of developing IDE inhibitors that selectively obstruct insulin cleavage without affecting IDE-mediated glucagon processing. The results presented in the next chapter of this thesis represent the first series of substrate-discriminating IDE inhibitors, which preferentially block insulin degradation in a way that accommodates for IDE-mediated degradation of glucagon and amylin (Chapter 5).
Figure 4.13 | Model for the expanded roles of IDE in glucose homeostasis and gastric emptying based on the results of this study. IDE inhibition increases the abundance and signaling of three key pancreatic peptidic hormones, insulin, amylin, and glucagon, with the corresponding physiological effects shown in blue and red.

More broadly, this work highlights the value of physiologically active small-molecule probes to characterize the functions of genes implicated in human disease as a key step in the drug discovery process that could prove increasingly valuable as human genomic studies become more prevalent\textsuperscript{11,53}. The identification of a highly selective IDE inhibitor using a DNA-encoded library coupled with \textit{in vitro} selection further establishes the value of this approach for the target-based discovery of bioactive small molecules. The unbiased nature of the selection led to the identification of a novel small-molecule binding site in IDE that enables
unprecedented inhibition selectivity for this enzyme. These macrocycles and structural insights therefore may also prove useful in the discovery of novel drug-like small-molecule leads to target IDE. Towards this goal, I developed a high-throughput fluorescence anisotropy assay using a fluorescent 6b analog that enables screening of large small-molecule collections against this new binding site, providing a path forward for the further discovery and development of novel therapeutic leads against IDE that share the specificity features of 6bK (Chapter 5).

4.9 – Methods

**Dosing and formulation of macrocycle inhibitors for in vivo studies.** The doses of 6bK used in this study were chosen based on literature precedent for small molecules and drugs of similar potencies\(^{54-60}\). Procedures using experimental compounds were in accordance with the standing committee on the Use of Animals in Research and Teaching at Harvard University, the guidelines and rules established by the Faculty of Arts and Sciences' Institutional Animal Care and Use Committee (IACUC), and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals.

Purified macrocycle inhibitors were dissolved in DMSO-\(d_6\) (~200 to 250 mg/mL stock solutions). A sample aliquot (5 \(\mu\)L) of the macrocycle solution was diluted with 445 \(\mu\)L DMSO-\(d_6\), then combined with 50 \(\mu\)L of freshly prepared solution of 20 mM CH\(_2\)Cl\(_2\) in DMSO-\(d_6\) in for \(^1\)H-NMR acquisition (600 MHz, relaxation time = 2 sec). The inhibitor concentration was calculated using the integral
of the CH$_2$Cl$_2$ singlet (δ 5.76 ppm, 2H)$^{61}$, which appears in an uncluttered region of these spectra$^{62,63}$. For injectable formulations (10 mL/kg i.p. injection volume), the macrocycle inhibitor solution in DMSO-$d_6$ (200 – 250 mg/mL, based on free-base molecular weight) was combined with 1:20 w/w Captisol® powder (CyDex)$^{12}$. The resulting slurry was supplemented with DMSO-$d_6$ to make up to 5 % of the final formulation volume, mixed thoroughly and dissolved with sterile saline solution (0.9 % NaCl). Vehicle controls were identically formulated with 5 % DMSO-$d_6$ and equal amount of Captisol®. The formulated solutions of inhibitor were clear with no visible particles, and were stored overnight at 4 °C prior to injection.

**In vivo studies, general procedures.** Wild-type C57BL/6J and diet-induced obese (DIO) C57BL/6J age-matched male adult mice were purchased from Jackson Laboratories. The age range was 13 to 15 weeks for lean mice, and 24 to 26 weeks for DIO mice. All animals were individually housed on a 14-h light, 10-h dark schedule at the Biology Research Infrastructure (BRI), Harvard University. Cage enrichment included cotton bedding and a red plastic hut. Water and food were available *ad libitum*, consisting respectively of normal chow (Prolab® RHM 3000) or high-fat diet (60 kcal % fat, D12492, Research Diets Inc.). Adult IDE knock-out mice (IDE$^{-/-}$) fully back-crossed to the C67BL/6J line were obtained from Mayo Clinic (Florida), and housed in the Biology Research Infrastructure (BRI), Harvard University, for 8 weeks prior to experiments as described above, and experiments were conducted on age-matched mice cohorts ranging 17 to 21 weeks old. All animal
care and experimental procedures were in accordance with the standing committee on the Use of Animals in Research and Teaching at Harvard University, the guidelines and rules established by the Faculty of Arts and Sciences' Institutional Animal Care and Use Committee (IACUC), and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals. Glucagon-receptor knock-out mice (GCGR<sup>−/−</sup>) and control WT mice (GCGR<sup>+/+</sup>) were group-housed with a 14-h light and 10-h dark schedule and treated in accordance with the guidelines and rules approved by the IACUC at Albert Einstein College of Medicine, NY. Power analysis to determine animal cohort numbers was based on preliminary results and literature precedent, usually requiring between 5 and 8 animals per group. Animals were only excluded from the cohorts in cases of chronic weakness, which occurs among GCGR<sup>−/−</sup> mice, or when we identified occasional DIO mice with an outlier diabetic phenotype (e.g. >200 mg/dL fasting blood glucose). Age- and weight-matched mice were randomized to each treatment group. Double-blinding was not feasible.

**Glucose tolerance tests GTT and blood glucose measurements.** Prior to a glucose challenge, the animals were fasted for 14 h (8 pm to 10 am, during the dark cycle) while individually housed in a clean cage with inedible wood-chip as a floor substrate, cotton bedding and a red plastic hut. Inhibitor, vehicle or control compounds were administered by a single intraperitoneal (i.p.) injection 30 min prior to the glucose challenge. Dextrose was formulated in sterile saline (3 g in 10 mL total), and the dose was adjusted by fasted body weight. For OGTT, 3.0 g/kg dextrose
was administered by gavage at a dose of 10 mL/kg, and for IPGTT, 1.5 g/kg dextrose injected at a dose of 5 mL/kg. Blood glucose was measured using AccuCheck® meters (Aviva) from blood droplets obtained from a small nick at the tip of the tail, at timepoints -45, 0, 15, 30, 45, 60, 90 and 120 min with reference to the time of glucose injection. The area of the blood glucose response profile curve corresponding to each animal was calculated by the trapezoid method\textsuperscript{1}, using as reference each individual baseline blood glucose measurement prior to glucose administration (t = 0) or the lowest point of the curve. The sum of the trapezoidal areas between the 0, 15, 30, 45, 60, 90 and 120 minute time points corresponding to each animal were summed to obtain the area under the curve (AUC). The relative area values are expressed as a percentage relative to the average AUC of the vehicle cohort, which is defined as 100 % (Fig. 2, Extended Data Figs. 8 and 9). Values are reported as mean ± S.E.M. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are *$p < 0.05$* versus vehicle control group or **$p < 0.01$** versus vehicle control group unless otherwise stated.

**Insulin Tolerance Test (ITT), Glucagon Challenge (GC) and Amylin Challenge (AC).** For hormone challenges animals were fasted individually housed as described above. For ITT the fasting period was 6 h (7 am to 1 pm), and for glucagon and amylin challenges the fasting period was 14 h (8 pm – 10 am). Inhibitor or vehicle alone was injected i.p. as previously described, 30 min prior to each hormone challenge. Insulin (Humulin-R®, Eli Lilly) was injected subcutaneously (s.c.) 0.25
U/kg formulated in sterile saline (5 mL/kg). Glucagon (Eli Lilly) was injected s.c. 100 µg/kg formulated in 0.5 % BSA sterile saline (5 mL/kg). Amylin (Bachem) was injected s.c. 250 µg/kg formulated in sterile saline (5 mL/kg). Blood glucose was measured at timepoints -45, 0, 15, 30, 45, 60 and 75 min with reference to the time of hormone injection, by microsampling from a tail nick as described above. Values are reported as mean ± S.E.M. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are *p < 0.05 versus vehicle control group or **p < 0.01 versus vehicle control group.

**Blood collection and plasma hormone measurements.** Blood was collected in EDTA-coated tubes (BD Microtainer®) from trunk bleeding (~500 µL) after CO₂-euthanasia for all hormone assays. The plasma was immediately separated from red blood cells by centrifugation 10 min at 1800 g, aliquoted, frozen over dry ice and stored at -80 °C. Insulin, glucagon, amylin and pro-insulin C-peptide fragment were quantified from 10 µL plasma samples using magnetic-bead Multiplexed Mouse Metabolic Hormone panel (Milliplex, EMD Millipore) according to the manufacturer’s instructions, using a Luminex FlexMap 3D instrument. Plasma containing high levels of human insulin (Humulin-R) were quantified using 25 µL samples with Insulin Ultrasensitive ELISA (ALPCO). Values are reported as mean ± S.E.M. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are *p < 0.05 versus vehicle control group or **p < 0.01 versus vehicle control group.
**Gastric emptying measurements.** Mice were fasted 14 h (8 pm to 10 am). Inhibitor or vehicle alone was injected i.p. as previously described, followed 30 min later by an oral glucose bolus (3.0 g/kg, 10 mL/kg) in sterile saline containing 0.1 mg/mL phenol red. At 30 min, the stomach was promptly dissected after CO₂-euthanasia and stored on ice. The stomach contents were extracted into 2.5 mL EtOH (95 %) by homogenization for 1 min using a probe sonicator. Tissue debris were decanted by centrifugation at 4000 g, followed by clearing at 15000 g for 15 min. The supernatant (1 mL) was mixed with 0.5 mL of aqueous NaOH (20 mM), and incubated at -20 °C for 1 h. The solution was centrifuged at 15,000 g for 15 min, and absorbance was determined at 565 nM. The spectrophotometer was blanked with the stomach contents of a mouse treated with colorless glucose solution. The absorbance was adjusted to the amount of glucose solution dosed to each mouse. Values are reported as mean ± S.E.M relative to the original phenol red glucose solution. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are *$p < 0.05$* versus vehicle control group or **$p < 0.01$** versus vehicle control group.

**RT-PCR analysis of liver gluconeogenesis markers phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase).** Total RNA was isolated from liver samples (~100 mg) using TRIzol® reagent (Invitrogen, 1 mL/100 mg sample), followed by spin-column purification (RNeasy® kit, Qiagen) and on-column DNAse treatment (Qiagen) according to the manufacturer’s instructions.
RNA concentrations were determined by UV spectrophotometry (NanoDrop). One microgram of total RNA was used for reverse transcription with oligo(dT) primers (SuperScript® III First-Strand Synthesis SuperMix, Life Technologies) according to the manufacturer’s instructions. Quantitative PCR reactions included 1 µL of cDNA diluted 1:100, 0.4 µM primers, 2x SYBR Green PCR Master Mix (Invitrogen) in 25 µL total volume, and were read out by a CFX96™ Real-Time PCR Detection System (BioRad). Transcript levels were determined using two known primer pairs\textsuperscript{30,31} for each gene of interest (Supplementary Table S4), which were normalized against tubulin and beta-actin transcripts ($\Delta\Delta C_T$ method), and expressed relative to the lowest sample. Duplicate control assays without reverse transcriptase treatment were run for each RNA preparation and primer set used. Statistics were performed using a two-tail Student’s t-test, and significance levels shown in the figures are *$p < 0.05$ versus vehicle control group or **$p < 0.01$ versus vehicle control group.

**Table 4.1** | RT-PCR primers for mouse liver gluconeogenesis markers.

<table>
<thead>
<tr>
<th>RT-PCR primers\textsuperscript{30,31}</th>
<th>PEPCK\textsubscript{1} Fw</th>
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<tbody>
<tr>
<td></td>
<td>PEPCK\textsubscript{1} Re</td>
<td>GTTGCAGGCCTACTTTTG</td>
</tr>
<tr>
<td>G6Pase\textsubscript{1} Fw</td>
<td>GTGCAAGCAGGAAGCAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G6Pase\textsubscript{1} Re</td>
<td>TCCGGAGCTGGCATTA</td>
</tr>
<tr>
<td>PEPCK\textsubscript{2} Fw</td>
<td>GGTGGTTACTGGGAAGCATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEPCK\textsubscript{2} Re</td>
<td>CAATAATGGGCACTGGCG</td>
</tr>
<tr>
<td>G6Pase\textsubscript{2} Fw</td>
<td>CATGGGCGCGAAGGTGATTT</td>
<td></td>
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<tr>
<td></td>
<td>G6Pase\textsubscript{2} Re</td>
<td>CAAGGTAGATCCGGGACAG</td>
</tr>
<tr>
<td>Tubulin Fw</td>
<td>CCTGCCTACAGGAAGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tubulin Re</td>
<td>TCTCATCCTGTTCCTCA</td>
</tr>
<tr>
<td>Beta-actin Fw</td>
<td>CATCGTAAAGAGCTCTATGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beta-actin Re</td>
<td>ATGGAGCCACCGATCCACA</td>
</tr>
</tbody>
</table>
Western blot for IDE in red blood cells used to corroborate the genotype of IDE$^{+/+}$ and IDE$^{-/-}$ mice. Approximately 15 µL blood was collected from a tail nick as described in previous procedures into EDTA-coated capillary tubes (Microvette 200 µL, Sarstedt). A portion of the blood sample (5 µL) was combined with 5 µL phosphate-buffered saline (PBS) and 10 µL loading buffer (LDS NuPage, Life Technologies) containing 0.125 mM dithioerythritol. The sample was heated at 95 °C for 10 min, and 2 µL of the mixture (total 0.5 µL blood) was loaded into a polyacrylamide gel (4-12% gradient NuPage, Life Technologies). Following electrophoresis and transfer, the PVDF membrane (Immobilon, Millipore) was incubated with blocking buffer (Rockland) at RT for 1 h, and washed with PBS. Primary antibody (1:200 dilution in PBS with 1% BSA, rabbit polyclonal anti-IDE #ab32216, Abcam) was incubated at RT for 1 h, and washed with 0.1% Tween-20 in PBS. Secondary antibody (1:10,000 dilution in PBS, IRDye 680LT goat polyclonal anti-rabbit IgG #827-11081, Licor) was incubated at RT for 1 h, washed with 0.1% Tween-20 in PBS, and imaged on an Odyssey fluorescence scanner (Licor). Western blot of red blood cells was used to confirm the presence of IDE in WT IDE$^{+/+}$ mice as an apparent ~100 kD band, and the absence of IDE in IDE$^{-/-}$ KO mice.

![Western blot assay](image)

**Figure 4.14 | Western blot assay to confirm IDE$^{-/-}$ mice genotypes.** Knockout IDE$^{-/-}$ mice and IDE$^{+/+}$ control littermates were obtained from the Leissring group. Western blot for IDE using whole blood lysate (0.5 µL/well) from IDE$^{-/-}$ mice and a wild-type (WT) control.
(Figure 4.14 cont.) sample. IDE is observed as a ~100 KDa band (indicated by an arrow). The major non-specific band is consistent with hemoglobin subunits (~16 KDa).

4.10 – References


29 Trebbien, R. et al. Neutral endopeptidase 24.11 is important for the degradation of both endogenous and exogenous glucagon in anesthetized pigs.


Chapter 5

Discovery of substrate-selective IDE inhibitors that obstruct insulin degradation but allow IDE-mediated glucagon proteolysis


Contributions: I designed and performed the experiments described in this chapter. Dr. Amedeo Vetere and Dr. Bridget Wager assisted with the implementation of the high-throughput screen at the Broad Institute. Zachariah H. Foda (Stony Brook University) performed the docking simulations. Help from other colleagues is acknowledged in the text.
5.1 – Introduction: the need for new substrate-selective IDE inhibitors

The first-generation IDE inhibitor 6bK acts as a competitive inhibitor that obstructs the interactions with the IDE chamber that are essential for binding and unfolding of all known in vitro and in vivo hormone substrates\(^1,2\) (>29-51 residues). Likewise 6bK completely blocks the degradation of the relatively small fluorogenic decapeptide substrate used in the standard IDE assay\(^3\). The unbiased competitive mode of inhibition of 6bK was desirable for the first in vivo-active probe tool in order to characterize all the physiological roles of IDE. However, looking towards potential therapeutic applications, a more efficacious class of IDE inhibitors would preferentially block insulin degradation, while permitting glucagon clearance at all times, to further bolster blood glucose control\(^4\). Indeed, the potentiation of the opposing physiological functions of insulin and glucagon by a traditional substrate-competitive IDE inhibitor is arguably the crucial liability of IDE as a drug target\(^5\). These observations strongly suggested that we needed to move beyond the 6b series to discover new IDE inhibitors with a focus on identifying insulin versus glucagon substrate discriminating features and drug-like properties.

Other reported inhibitors of IDE, including li1 and ML345, also block all substrates from binding the IDE chamber\(^6-8\). Because of the known homodimer cooperativity of IDE some researchers have observed modest shifting of the IC\(_{50}\) between some substrates (insulin and A\(_\beta\))\(^9,10\). Consistently with the IDE literature\(^11\), these effects appear to be biochemical assay artifacts that occur when the binding sites in the IDE homodimer are partially occupied by ligands in the presence of
excess substrate, a condition that is not relevant \textit{in vivo}\textsuperscript{12}. Modulators of IDE that discriminate among substrates throughout a range of concentrations relevant to target engagement \textit{in vivo} have not been reported. The discovery of substrate-selective IDE inhibitors that abrogate IDE’s degradation of insulin but have negligible effect on IDE-mediated degradation of glucagon would represent a major step forward towards IDE-targeted therapeutics.

\textbf{5.2 – Optimization of an anisotropy assay using a fluorescent probe based on the first-generation DNA-templated macrocyclic inhibitor}

Many groups have previously screened for inhibitors of IDE\textsuperscript{6,7,10,13,14}, yet in spite of the advances in high-throughput screening, IDE-specific inhibition among >185 zinc-dependent metalloproteases encoded in the human and mouse genomes\textsuperscript{15} represents a major challenge to mainstream drug discovery platforms that rely on activity-based screening of compound collections\textsuperscript{16,17}. Unspecific modes of inhibition, zinc chelation, and covalent adducts to the cysteine residues on the inner chamber of IDE, produce an unacceptable rate of false-positive hits that seldom discriminate specifically among homolog metalloproteases\textsuperscript{7}. Indeed, prior to this work the scope of studies towards IDE inhibitors comprised of: 1) designed zinc-chelating hydroxamic acid linear peptide inhibitors\textsuperscript{6,9} that are modestly selective for IDE and have short half-lives \textit{in vivo}\textsuperscript{7}; 2) carboxylic acid-based modestly potent inhibitors\textsuperscript{10}; and 3) a thiol-reactive covalent inhibitor identified from a high-throughput screening effort\textsuperscript{7} (Chapter 1, \textbf{Figure 1.11}). The specificity of this adduct
was tentatively determined at low concentrations <2 µM, and \textit{in vivo} activity was not reported\textsuperscript{7}. The limited results from these previous studies underscore the need for alternative methods to identify IDE inhibitors. The unbiased nature of the \textit{in vitro} selection using the DNA-templated library led to the identification of a novel small-molecule binding site in IDE that enables unprecedented inhibition specificity for this enzyme (Chapter 2). We hypothesized that these structural insights may also prove useful in the discovery of novel drug-like small-molecule leads to target IDE.

To identify new IDE inhibitors that target the distal binding site I developed a fluorescence anisotropy probe-displacement assay\textsuperscript{18} based on the structure of the first-generation IDE inhibitor 6b (\textbf{FL-6b, Figure 5.1}). Anisotropy is the property of retention of polarization in emitted light when an stationary fluorophore is excited with polarized light\textsuperscript{18}. However, solutions of fluorescent molecules of low molecular weight (<1,000 Da) display anisotropy only while physically bound to a large target (typically >10,000 Da) such that the complex tumbles slowly in solution\textsuperscript{18}. The simple tagging design of \textbf{FL-6b} is based on the general feature of the covalent linker of DNA-encoded library hits, and it has been useful to validate putative binders from \textit{in vitro} selection experiments\textsuperscript{19}. However, the possibility of retailoring a hit from a DNA-templated library selection to perform a high-throughput screen had not been demonstrated. Indeed, in order to scale up a fluorescence anisotropy probe-displacement assay that enables screening of large small-molecule collections the following conditions need to be met\textsuperscript{18}: \textit{i}) the target needs to be available in high purity and scale usually >100 µg per 384-well plate; \textit{ii}) the fluorescent probe must
Figure 5.1 | Optimization of an anisotropy-based assay using the fluorescent probe FL-6b, based on the DNA-templated IDE inhibitor 6b. Anisotropy readout from FL-6b (5 nM) by competitive binding to IDE (0.1 μM). Inhibitor 6bK (1 μM) displaces the probe lowering fluorescence anisotropy with excellent signal-to-noise ratio, on the other hand neither the catalytic-site binder II1 (1 μM) nor the low affinity bisepi-6bK (1 μM) produce a significant readout.

Figure 5.2 | Determination of the assay Z-factor. A 384-well checkerboard (raw data shown in green-white heatmap) was set up using 50 μL total volume (50 mM Tris buffer, pH 7.5, 1.0 M NaCl, 5% DMSO) in the presence of inhibitor 6bK (1 μM) or inactive bisepi-6bK (1 μM).
have $K_D < 100$ nM and be specific in the presence of high target concentrations $>0.5$ – $5$ µM of target; iii) the Z-factor must be $> 0.5$ and the readout should ideally be stable $>15$ min to $1$ hr; iv) a counter-screening method is required to identify inhibitors among the probe-displacing molecules; and v) to minimize false-positives the library collection must be non-fluorescent.

In preliminary experiments I showed that 6bK (1 µM) leads to a drop in anisotropy by displacement of FL-6b from the complex with N-His$_6$-hIDE$_{(42-1019)}$ which I expressed and purified from E. coli (Figure 5.1). The FL-6b anisotropy readout was unchanged by treatment with either DMSO alone (5%), with the low affinity ligand bisepi-6bK, or with the catalytic domain binder lii$^6$. Finally, the Z-factor $> 0.69$ was calculated for 384-wells containing either 6bK (1 µM) as the positive control, or bisepi-6bK (µM) as the negative control (Figure 5.2). To further validate the specificity of FL-6b for the IDE distal binding pocket at high protein concentration I titrated the fluorescent probe (5 nM final) using wild-type IDE and the IDE variants Ala479Leu and Gly362Gln. The anisotropy readout of FL-6b matched the inhibition potency of 6bK against each of the three protein variants (Figure 5.3). These experiments demonstrated that the fluorescein appendage does not disrupt the selectivity for the distal binding site of IDE, and that the anisotropy probe-displacement Z-factor is suitable for high-throughput screening applications.
Figure 5.3 | Validation of on-target binding for the probe FL-6b using IDE variants. The probe (5 nM) was titrated with either wild-type IDE or binding-site mutants A479L and G362Q that hinder binding of the probe to the distal inhibitory site of IDE. The pattern is comparable to 6bK inhibition potencies for the same IDE variants (see Figure 2.12).

5.3 – High-throughput screening of the Broad Institute libraries

In collaboration with Dr. Amedeo Vetere and Dr. Bridget Wagner (Broad Institute) we set up an EnVision spectrophotometer equipped with an automatic plate stacker to read the FL-6b anisotropy assay. First, we tested the assay by pin-transfer of a collection of 1000 bioactive small molecules and approved drugs in DMSO (10 mM), called the “pharmakon” plates (Figure 5.4). The anisotropy readout was converted to standard Z-scores over the average for the negative control wells (DMSO alone). This preliminary trial produced a Z-factor > 0.6 for the 6bK positive controls. As expected a number of fluorescent compounds (e.g. doxorubicin and fluorescein) appeared as false positives, and among the non-fluorescent molecules with lowest Z-score we noted the antibiotic bacitracin, which is a weak competitive inhibitor of IDE (IC$_{50}$ = 10 µM). These preliminary results were encouraging to scale
up the assay to use a collection of “informer set” plates with 10,000 compounds that are representative of multiple libraries at the Broad Institute (Figure 5.5).

**Figure 5.4 | Validation of the anisotropy assay in high-throughput mode using 1,000 drug/bioactive compounds.** First trial using pin-transfer of a collection of 1,000 drug/bioactive small molecules in DMSO (10 mM), called “pharmakon” plates. Human N-His$_6$-IDE$_{42-1019}$ (E. coli expressed) was mixed with fluorescein-labeled macrocycle FL-6b (30 nM) generating a high anisotropy signal in the presence of negative control DMSO (●), or with inactive compounds (●). The inhibitor 6bK (1 μM), used here as a positive control (●) displaces analog FL-6b lowering fluorescence anisotropy with excellent signal-to-noise ratio (Z-factor > 0.6). All the false positive hits display autofluorescence (e.g. fluorescein and doxorubicin), which is not expected for the DOS libraries of the Broad Institute.
**Figure 5.5 | High-throughput anisotropy probe-displacement assay screen using 10,000 compound informer set of the Broad Institute library collections.** Unbiased screen with pin-transfer of "DOS informer set" plates comprising 10,000 compounds originating from multiple compound collections. Human N-His$_6$-IDE$_{42-1019}$ (E. coli expressed) was mixed with fluorescein-labeled macrocycle FL-6b (30 nM) generating a high anisotropy signal in the presence of negative control DMSO (●), or with inactive compounds (●). The inhibitor 6bK (1 µM), used here as a positive control (●) displaces analog FL-6b lowering fluorescence anisotropy with excellent signal-to-noise ratio (Z-factor > 0.5).

The screen of the “DOS informer set” plates revealed 16 putative IDE binders originating from 3 compound collections (Figure 5.5). The set of analogs and stereochemical isomers available at the Broad Institute were purchased to determine the IC$_{50}$ values using the fluorogenic peptide IDE assay for counter-screening and to reveal the stereochemical structure-activity relationships (SSAR).
Figure 5.6 | Structures of hits identified as putative distal-site IDE binders and IDE inhibitors. Specific hits from the screen are highlighted with an asterisk and their average Z-scores are indicated, other structures were purchased from the Broad Institute Compound Management. The IC₅₀ values were determined using the fluorogenic peptide IDE proteolysis assay (Mca-RPPGFSAFK(Dnp)-OH). (A) Four hits from with the scaffold 4-phenoxy-N-phenylbenzamide were identified and 65 available analogs were purchased to generate the SSAR table. (B) Modestly potent hits from the tricyclic-glycal libraries were identified, this series was not explored in depth because of a lack of analogs. (C) Numerous hits identified in the “DOS informer set” plates belong to the azetidine core libraries (see Table 5.1).
First I focused on the biphenyl ether series\textsuperscript{20} that bears resemblance to the \textit{6b} benzophenone (Figure 5.6A). These hits were purchased from the Broad Institute, together with a set 75 structural analogs and stereoisomers, and I used the fluorogenic counter-screening assay to identify several hits of \(\sim1\text{-}2\ \mu\text{M}\) potency. However, the stereochemistry of the macrocyclic portion did not appear to be crucial for inhibition. This SSAR trend suggested the pharmacophore \textit{4-phenoxy-N-phenylbenzamide} bears most of the ligand efficiency. The hits \textbf{BRD-194} and \textbf{BRD-815} were further investigated in metalloprotease specificity assays, which revealed these molecules were not sufficiently selective, potentially due to aggregator properties (Section 5.4). Preliminarily, we concluded that this series may prove most useful to extract the pharmacophore \textit{4-phenoxy-N-phenylbenzamide}, given that this drug-like fragment can be easily derived into a series of analogs in the future. A second group of modestly potent hits belongs to the tricyclic-glycal libraries\textsuperscript{21}, however, a search of the Broad databases provided insufficient analogs to study the SSAR (Figure 5.6B).

Importantly, more than half of the hits among the “DOS informer set” plates belonged to the azetidine-core library collections\textsuperscript{22} (Figure 5.6C). The compounds that I initially identified were modestly potent inhibitors, however, a chemo-informatic search performed in collaboration with by Mathias Wawer (Broad Institute) using biphenyl-azetidine as the query suggested that the preliminary screen would not capture the full diversity of the azetidine libraries\textsuperscript{22} (Table 5.1). Based on these observations, we performed a second high-throughput anisotropy probe displacement screen against a collection of azetidine core libraries that was biased...
with >75% biaryl-substituted molecules (Figure 5.7). This effort led to the identification of over 100 putative IDE binding structures with Z-scores < -10 (0.1% hit rate). Next, I selected representative hit structures and determined the IC₅₀ using the fluorogenic decapetide IDE assay. The resulting collection of 115 azetidine analogs that were purchased to determine the IC₅₀ values and SSAR are shown in Figure 5.8. This rich dataset yielded over 50 low- to sub-micromolar IDE inhibitors, which demonstrates the efficiency of the FL-6b probe displacement screening method towards the identification of IDE inhibitors and IDE binders with Lipinski-compliant drug-like scaffolds. Inspection of the fluorogenic peptide assay inhibition profiles revealed a class of IDE binders that incompletely block IDE-mediated cleavage (inhibition maximum, I₅₀ < 100%). This observation provided a strong starting point to identify substrate-selective IDE inhibitors (Figure 5.9).

Table 5.1 | Chemoinformatic properties of Broad Institute azetidine libraries²².
using the fluorogenic peptide IDE proteolysis assay (Mca-RPPGFSAFK(Dnp)-OH).

Figure 5.7 | High-throughput screening of biaryl-substituted azetidine libraries. The top 100 compounds highlighted (●) display low anisotropy signal for FL-6b (Z-score < -10).

Figure 5.8 (continued in subsequent 3 pages) | Structures of azetidine hits identified as putative distal-site IDE binders and IDE inhibitors. The IC₅₀ and I₇₅₀ were determined using the fluorogenic peptide IDE proteolysis assay (Mca-RPPGFSAFK(Dnp)-OH).
Figure 5.8 (cont.)
Figure 5.8 (cont.)
Figure 5.8 (cont.)
Figure 5.9 | Surrogate proteolysis assay used to identify substrate-selective IDE inhibitors that allow IDE-mediated proteolysis of small peptide substrates. (A) Representative examples of the concentration-dependent profiles of IDE inhibition for “normal” substrate competitive inhibitors, which display complete inhibition of IDE-mediated proteolysis of the fluorogenic Mca-RPPGFSAFK(Dnp)-OH. The inhibition maximum (I_{MAX}) is approximately 100% at high inhibitor concentrations. (B) Representative examples of the concentration-dependent IDE inhibition profiles for IDE binders that allow partial IDE-mediated proteolysis of the fluorogenic nonapeptide at all concentrations (I_{MAX} <100%), and that may display proteolytic activity as a ternary IDE-inhibitor-substrate complex.
5.4 – Discovery of substrate-selective inhibitors of IDE-mediated insulin degradation that allow glucagon proteolysis

The literature suggests that substrate-selective inhibition is exceedingly rare for most enzymes classes\textsuperscript{16,17,23}. Nevertheless, we hypothesized that owing to partial occlusion of the distal inhibitory site, a small molecule-IDE ternary complex may achieve substrate discrimination based on the differences in size, structure, and proteolysis kinetics between insulin and glucagon. Towards this goal, I investigated the collection of azetidine hits (Figure 5.7), and in particular I focused on a series of compounds with low Z-scores for FL6b-probe displacement that displayed sub-maximal inhibition of IDE proteolysis of the fluorogenic decapptide substrate ($I_{\text{MAX}}$ = 70–80\%, Figure 5.8). This fluorogenic assay reports on the cleavage of Mca-RPPGFSAFK(Dnp)-OH that has weak affinity for IDE ($K_{M} > 4 \mu M$) and loosely occupies the catalytic chamber (e.g. insulin has 51 residues, and glucagon 29 residues). Therefore, this surrogate assay can be used to reveal IDE binders that partially allow IDE-mediated proteolysis of small substrates, and also provides the $IC_{50}^{\text{app}}$ parameter that is linked to the inhibitor affinity for IDE ($K_{i}$).

Next, I optimized dual antibody Homogeneous Time-Resolved FRET assays\textsuperscript{24} against insulin and glucagon to reveal the extent of IDE-mediated cleavage during incubation in the presence of a series of inhibitors. As a control I showed that 6bK displays concentration-dependent inhibition of IDE degradation of insulin and glucagon with similar $EC_{50}$ (Figure 5.10). I selected a series of 16 azetidine compounds (Z-score < -15, $IC_{50}^{\text{app}} = 0.25–2 \mu M$, $I_{\text{MAX}} = 40–80\%$), which were used
in this assay at 67 µM (>30 x IC₅₀ app) in order to ensure full occupancy of the IDE binding site, while this is also a conservative concentration that prevents assay artifacts (Figure 5.11). This focused screen revealed the desired substrate-selective inhibitory property in a small subset of azetidine IDE inhibitors, in particular BRD-297 and BRD-204 (IC₅₀ = 250 nM and 400 nM, respectively), which were superior in obstructing IDE-mediated degradation of insulin, but allowed significant glucagon degradation to occur during the IDE incubation (Figure 5.11).

Figure 5.10 | Optimization of homogeneous time-resolved FRET (HTRF) assays to report on IDE-mediated degradation of insulin and glucagon in the presence of inhibitors. The inhibitor 6bK was used as a positive control to optimize the IDE incubation parameters, in order to generate a sigmoidal profile that reports on 6bK IDE inhibition in a concentration-dependent manner.
In follow up experiments, I determined the substrate-selective inhibitory properties over a range of concentrations using the HTRF endpoint degradation assays for insulin and glucagon (Figure 5.12), using 6bK as a control. Among the structures tested, only the hits BRD-297 and BRD-204 displayed effective discrimination between the two substrates, and also completely abrogated IDE-mediated insulin degradation at high inhibitor concentration (Figure 5.11). The SAR trend of this set of molecules indicates that substrate-selectivity is perturbed by minor structural changes, and that in some cases IDE-mediated degradation of insulin is not effectively blocked by specific azetidine hits. The structure and substrate-selective inhibitory properties of the hit BRD-297 were corroborated by re-synthesis (see Methods, Figure 5.18) using an advanced intermediate azetidine core generously provided by the Broad Institute.

These findings suggest that insulin and glucagon are processed by IDE through different binding requirements that can be rationalized and exploited by distal-site small molecule binders, which clash or abrogate interactions between IDE and insulin, but that can accommodate for binding and proteolysis of glucagon within the IDE chamber (Figure 5.12).
Figure 5.11 | Focused screen for substrate-selective inhibitory properties using the insulin and glucagon degradation endpoint HTRF assays. (A) Selected hits displaying submaximal inhibition in the range of 40–80% for the surrogate fluorogenic peptide cleavage assay were chosen. All compounds (used at final concentration of 67 µM, >10x IC_{50}^{app}) and control incubations were performed in parallel using the same IDE and substrate preparations. Under these conditions, the analogs BRD-297 and BRD-204 produce a strong discrepancy in IDE-mediated degradation of glucagon (■) versus insulin (▲) compared with IDE treated with DMSO alone, or with positive control 6bK (10 µM). Other analogs display modest substrate-selective inhibitory properties.

Figure 5.12 (next page) | Concentration dependence profiles for substrate-selective IDE inhibitors. Selected hits that display substrate-selective inhibitory properties were assayed over a range of concentrations using the HTRF endpoint degradation assay for insulin (▲) and glucagon (■). Only the hits BRD-297 and BRD-204 display strong substrate selectivity, and completely abrogate IDE-mediated insulin degradation.
Figure 5.12 Concentration dependence profiles for substrate-selective IDE inhibitors (caption in previous page)
Amyloid-beta and Amylin are also in vivo substrates of IDE (see Section 4.6). Amylin is co-secreted with insulin by β-cells to exert glycemic control during meals, by slowing gastric emptying25, promoting satiety26,27, and antagonizing glucagon signaling28. Our findings with 6bK raised the possibility that IDE inhibition could potentiate the effects of amylin, which may mimic the benefits of supplementation using pramlintide/Symlin, an injectable analog based on the rodent sequence of amylin25,27. However, there is also significant concern that human amylin may produce amyloid fibrils, which have been suggested to play a role in β-cell dysfunction in the late stage of type-2 diabetes29. Substrate-selective IDE inhibitors could hypothetically be designed to either exclude or accept amylin within the IDE chamber, given that amylin is of intermediate size between insulin and glucagon. Therefore, to determine the possibility of IDE-mediated cleavage in the presence of the new inhibitors we designed a fluorogenic analog of human amylin, Amylin-fp (Figure 5.13), by judiciously replacing two residues: Gln10 with the Lys-γN-anthranilamide fluorophore and Phe23 with the 3-nitrotyrosine quencher, which are 12 residues apart and encompass the IDE cleavage site. This peptide was synthesized and HPLC purified by Dennis Dobrovolsky. Whereas IDE-mediated cleavage of Aβ40 and Amylin-fp was blocked by 6bK with comparable EC50 values to the glucagon and insulin HTRF assays, the inhibitors BRD-297 and BRD-204 displayed an intermediate level of IDE cleavage inhibition, but nonetheless permitted significant IDE-mediated processing of the Amylin-fp peptide at the highest concentration tested for each inhibitor (>40-20% the uninhibited rate). These results
suggest that **BRD-297** and **BRD-204** are insulin-selective IDE inhibitors. Therefore, the substrate-selective inhibitors can be conceptualized as re-sculpting a crucial patch of the inner surface of the IDE chamber that brings about discrimination between insulin and the three smaller substrates tested, which may enter the chamber and engage in proteolysis at rates comparable to that of the uninhibited enzyme.

**Figure 5.13 | IDE-mediated cleavage of Aβ40 and an amylin fluorogenic analog is partially allowed by insulin-selective IDE inhibitors.** The fluorogenic analog of human amylin Amylin-fp was designed by replacing two residues: Gln10 with the Lys-γN-anthranilamide fluorophore and Phe23 with the 3-nitrotyrosine quencher. The IDE cleavage site of human amylin is between Phe15-Leu16. Aβ40 was measured by HTRF (CisBio).

Amylin (fp): KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH₂ (X = Lys-γN-anthranilamide; Z = 3-NO₂-Y)
hAmylin:    KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH₂

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5.5 – Metalloprotease specificity of IDE inhibitor hits

A key advantage of the FL-6b probe-displacement screening approach is the enrichment for hits that target the distal binding pocket that distinguishes IDE from other metalloproteases (Chapter 2). Similarly to 6bK, the azetidine inhibitors BRD-204 and BRD-297 (IC$_{50}$ = 250 nM and 400 nM, respectively) displayed remarkable ≥250 to 1,000-fold preference for inhibition of IDE over all other metalloproteases tested: thimet oligopeptidase (THOP), neurolysin (NLN), neprilysin (NEP), matrix metalloprotease 1 (MMP-1), and angiotensin converting-enzyme (ACE) (Figure 5.14). The specificity profiles of the structurally related inhibitors BRD-779 and BRD-504 displayed comparable >80 and 100-fold preference for IDE inhibition,

Figure 5.14 | Analysis of metalloprotease specificity for substrate-selective IDE inhibitors (continued on the next page).
Figure 5.14 (cont.) | Analysis of metalloprotease specificity for substrate-selective IDE inhibitors. The substrate-selective inhibitors BRD-297 and BRD-204 achieve remarkable ≥250 to 1,000-fold specificity for IDE (■) over all other metalloproteases tested: thimet oligopeptidase (■ THOP), neurolysin (■ NLN), neprilysin (■ NEP), matrix metalloprotease 1 (■ MMP1), and angiotensin converting-enzyme (■ ACE). (B) The specificity profiles of the structurally related inhibitors BRD-779 and BRD-504 suggest that hits of high selectivity for IDE are enriched by displacement of the anisotropy probe FL-6b. (C) The hits from the 4-phenoxy-N-phenylbenzamide series were not as specific for IDE.
suggesting that high specificity for IDE over other enzymes is a general feature of this series. On the other hand, the biphenyl ether analogs BRD-194 and BRD-815 identified from the “DOS informer set” plates (Figure 5.6A) displayed only modest selectivity for IDE versus various enzymes, suggesting that these hits may have non-specific aggregator properties.

5.6 – Binding site mutagenesis and structural basis for substrate-selective IDE inhibition

Next, to demonstrate that the new hits interact with the IDE distal binding site I determined the loss of potency against IDE variants known to disrupt the binding of 6bK, including Ala479Leu, Gly362Gln and Ile374Gln (Figure 5.15). As expected, mutagenesis of the residue Ala479Leu had the strongest impact on hindering the inhibition potency of BRD-297 and BRD-204 by at least >150-fold. This mutation similarly blocks 6bK, 6b and FL-6b, from binding IDE, suggesting that the inhibitors share the same binding site. The nearby mutations Ile362Gln and Gly362Gln, which are residues of the exo-site involved in unfolding hormone substrates, also reduce the inhibition potency of BRD-297 and BRD-204.

Together, these biochemical studies provide strong evidence for the proposed distal binding site for BRD-297 and BRD-204. Based on this data Zack Foda and Prof. Markus Seeliger (Stony Brook Universtiy) produced a docking model of the ligand BRD-297 into the 6b distal binding pocket using the IDE co-crystal structure (PDB: 4LTE, Figure 5.15B). The highest scoring pose of the ligand structure is fully
consistent with the IC$_{50}$ potency of **BRD-297** against the IDE variants Ala479Leu, Gly362Gln, and Ile374Gln (Figure 5.15A).

Next, I investigated the impact of the azetidine IDE inhibitors on the rate of glucagon processing compared to IDE alone. I determined the initial rates of glucagon cleavage in the presence of **BRD-297, BRD-204, BRD-504, BRD-799**, and **BRD-591**, again using high inhibitor concentrations (67 µM, >50–200x IC$_{50}$ app) to promote full occupancy of the IDE binding site. The initial concentration of glucagon used in this assay was 1.15 nM (4 ng/mL) and owing to the low affinity of glucagon for IDE ($K_M = 3.5 \mu$M)$^{30,31}$ the cleavage of glucagon can only proceed through a ternary complex. The data suggests that the IDE-glucagon-inhibitor ternary complexes of **BRD-297** and **BRD-204** retain significant apparent rates of glucagon degradation compared to IDE alone (Figure 5.16). Furthermore, I observed that compounds **BRD-591** and **BRD-799** accelerate the rate of IDE-mediated glucagon cleavage, suggesting that substrate-selective IDE inhibitors that make favorable interactions within the IDE-glucagon ternary complex could also be developed.
Figure 5.15 | Confirmation of the distal binding site for substrate-selective IDE inhibitors, and a docking model within human IDE. Activity assays for wild-type or mutant human IDE variants in the presence of BRD-297 and BRD-204. (A) Mutagenesis of the residue Ala479Leu (■) hinders the inhibition potency of BRD-297 and BRD-204 by at least >150-fold. The nearby mutations Ile362Gln (■) and Gly362Gln (■) also reduce the inhibition potency. (C) These results are consistent with the highest docking score pose for BRD-297 within human IDE (PDB: 4LTE, ligand 6b removed).
Figure 5.16 | IDE-mediated degradation of glucagon is permitted by the ternary complexes of substrate-selective IDE inhibitors. Selected hits displaying substrate-selective inhibitory properties were chosen. All compounds and control incubations were performed in parallel using the same IDE and preparation of glucagon (4 ng/mL, 1.15 nM final). The enzymatic reaction was incubated in the presence of compounds (67 µM, >50x IC$_{50}^{app}$), DMSO alone, or 6bK positive control (10 µM), at 0 °C for 10 min to promote incomplete IDE-mediated degradation of glucagon, which was measured using the HTRF assay. The IDE complexes with hits BRD-297 and BRD-204 display significant rates (60 and 30% respectively) of IDE-mediated glucagon proteolysis compared to IDE alone (DMSO control). It is noteworthy that the compounds BRD-504 and BRD-591 accelerate IDE-mediated glucagon cleavage, potentially by making favorable interactions that increase the modest affinity of glucagon for IDE (K$_{M}$ ~ 3.5 µM).
Figure 5.17 | Structural basis for substrate-selective IDE inhibition. Caption in the next page.
Figure 5.17 (cont.) | (A and B) The IDE-insulin X-ray co-crystal structure (PDB: 2WBY) was superimposed with (A) the model of BRD-297 docked in the IDE structure, and with (B) the IDE-6b inhibitor co-crystal structure. Insulin is shown as a yellow surface, and the inhibitors as stick models. These models predict multiple steric clashes (red circles) that abrogate the interactions of insulin when either (A) BRD-297 or (B) 6b are bound to IDE. (C and D) Similarly, the IDE-glucagon X-ray co-crystal structure (PDB: 2G49) was superimposed with (A) the model of BRD-297 docked in the IDE structure and (B) the IDE-6b inhibitor co-crystal structure. A 16-residue segment of glucagon (orange surface) is disordered and unresolved in the crystal structure, and only the terminal segments of glucagon are bound to the catalytic site (right) and exo-site (left). The superimposed models predict steric clashes (red circles) between 6b and the IDE-bound segments of glucagon, in contrast BRD-297 does not abrogate any interactions between IDE and glucagon. The insets in A-D represent the IDE-mediated cleavage sites (major red arrow, minor black arrows), the IDE-bound segments of the substrate are underlined, and the symbols (○) highlight the steric clashes with each inhibitor.

To understand the structural basis of BRD-297 substrate-selective IDE inhibition and how the ternary complex allows glucagon proteolysis we superimposed the co-crystal structures of IDE with glucagon and insulin\(^1\) with our model of BRD-297 docked in the 6b binding pocket of IDE (Figure 5.17A and C). The superimposed models predict that the bulk of the macrocycle structure of 6b obstructs multiple points of IDE-insulin interactions within the chamber (Figure 5.17B see also Figure 2.13), and similarly 6b obstructs the interactions predicted for the electronic density model of glucagon\(^1\) (Figure 5.17D). Similar superimposed models produced by Zack predict that 6b clashes with all substrates that have been co-crystalized within IDE, including amylin\(^32\), A\(\beta\)^1, and ANP\(^33\). Remarkably, this
preliminary model of IDE-BRD-297 does not predict any obstructions with the superimposed IDE-glucagon interactions (Figure 5.17A), supporting the biochemical data indicating that BRD-297 may accommodate for glucagon binding to IDE as a ternary complex that is proteolytically competent for IDE-mediated glucagon cleavage (Figure 5.16).

In conclusion, these results offer new insights into the biochemistry of IDE, and provide a first-in-class series of inhibitors capable of substrate discrimination, which clash or abrogate interactions with insulin, but that simultaneously accommodate for binding and proteolysis of glucagon and amylin within the IDE chamber (Figure 5.13). The hits BRD-297 and BRD-204 are drug lead-compliant (Table 5.2) and may prove useful to translate these findings in vivo based on their sub-micromolar IDE inhibition potencies, excellent metalloprotease specificities, and the promising in vitro glucagon cleavage rates of the respective IDE ternary complexes.

More broadly, these findings establish an unprecedented strategy for altering the physiologically active substrates degraded by IDE, rather than merely blocking the enzyme. Altering the IDE ‘degradome’ in vivo constitutes a new therapeutic strategy for the treatment of high blood sugar by selectively potentiating insulin over glucagon signaling, and favorably shifting the insulin-glucagon ratio, which has broad implications for glucoregulation in multiple stages of Type-2 Diabetes. Furthermore, the identification of IDE binders that accelerate IDE-mediated glucagon cleavage may provide an opportunity to directly suppress glucagon signaling, while simultaneously potentiating insulin signaling, as a useful adjunct to
conventional antihyperglycemic agents in the management of diabetic hyperglycemia.

**Table 5.2** | Calculated drug-like properties of substrate-selective hits versus 6bK.

<table>
<thead>
<tr>
<th>Inhibition properties</th>
<th>BRD-297</th>
<th>BRD-204</th>
<th>6bK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDE IC(_{50}) app</td>
<td>250 nM</td>
<td>400 nM</td>
<td>50 nM</td>
</tr>
<tr>
<td>I(_{\text{MAX}}) peptide assay</td>
<td>65%</td>
<td>73%</td>
<td>100%</td>
</tr>
<tr>
<td>I(_{\text{MAX}}) insulin assay</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>I(_{\text{MAX}}) glucagon assay</td>
<td>27%</td>
<td>49%</td>
<td>100%</td>
</tr>
<tr>
<td>IDE selectivity</td>
<td>&gt;1000x</td>
<td>250x</td>
<td>&gt;1000x</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipinski properties</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw (&lt;500 Da)</td>
<td>490 Da</td>
<td>448 Da</td>
<td>758 Da</td>
</tr>
<tr>
<td>cLog(P) (&lt;5)</td>
<td>4.5</td>
<td>4.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H-donors (&lt;5)</td>
<td>1</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>H-acceptors (&lt;10)</td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Rotatable bonds (&lt;10)</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Polar area (&lt;140 Å(^2))</td>
<td>70</td>
<td>60</td>
<td>250</td>
</tr>
</tbody>
</table>

5.7 – Perspective for the future of IDE-based therapeutics

The discovery and development of the first highly-selective and physiologically active IDE inhibitor enabled the detailed study of the physiological
functions of IDE in living animals, illuminating novel IDE substrates in vivo, and leading us to delineate new therapeutic strategies for modulating IDE activity to improve post-prandial glucose tolerance. This new understanding of the physiological roles of IDE then led us to develop a more efficacious class of substrate-selective IDE inhibitors that preferentially block insulin degradation, while simultaneously permitting IDE-mediated clearance of glucagon and amylin.

These studies provide the essential foundation towards validating IDE as a drug target. The detailed biochemical characterization of the inhibitory distal site that distinguishes IDE from other enzymes, and the fluorescence anisotropy assay that enables screening of large small-molecule collections against this new binding site offers a path toward the discovery and development of additional therapeutic leads against IDE. Moreover, I demonstrated that by applying this screening strategy it is possible to identify substrate-selective IDE inhibitors that circumvent the intrinsic liability of IDE as a drug target. Indeed, traditional competitive inhibitors would potentiate the opposing physiological functions of glucagon and insulin in vivo, restricting the therapeutic benefit of IDE inhibitors to periods of low glucagon secretion, or in co-administration with anti-diabetic agents. For the purpose of this work I have specifically focused on drug target validation for the peripheral pool of IDE and peripheral circulating substrates. IDE in the brain may not prove to be a significant liability (Figure 5.13), due to the multiple redundant pathways for degradation of amyloid-beta peptides\(^{37}\), and because impermeability to the blood-
brain barrier is a feature either inherent or designed into most drugs, as exemplified by Neprilysin inhibitors that are used for the treatment of hypertension\textsuperscript{38}.

The IDE inhibitor probes developed herein may become useful tools to discover and validate other physiologically relevant substrates of IDE. For instance, preliminary data obtained during the writing of this thesis suggests that the vasodilator CGRP\textsuperscript{39,40} and the natriuretic hormone ANP\textsuperscript{41,42} are \textit{bona fide} substrates of IDE \textit{in vivo}, making IDE a \textit{vasopeptidase} like ACE and Neprilysin. Mounting evidence suggests that therapies that increase the levels of the hormones released by the heart in response to high blood pressure, called the natriuretic peptides,\textsuperscript{41} lead to synergistic improvements in blood pressure control and lower risk of cardiovascular disease when used in combination with classical anti-hypertensive drugs.\textsuperscript{38,43} The ongoing validation experiments will determine if IDE inhibition may produce an anti-hypertensive effect secondary to the anti-diabetic activity, given that the vast majority of patients with diabetes would benefit from preventative and synergistic treatments for blood glucose control and hypertension\textsuperscript{44,45}. In turn, the IDE-mediated degradation of these novel substrates may be characterized in the presence of substrate-selective IDE inhibitors.

Finally, based on the principles established herein, we will be able to develop insulin-selective IDE inhibitors that accelerate IDE-mediated glucagon degradation, simultaneously potentiating insulin signaling, and suppressing glucagon to bolster the management of diabetic hyperglycemia. This is, in my view, the ideal IDE inhibitor.
5.8 – Methods

**Fluorescence anisotropy high-throughput screening assay.** Human N-His$_6$-IDE$_{42-1019}$ (*E. coli* expressed) was mixed with fluorescein-labeled macrocycle FL-6b in 50 mM Tris buffer pH 8.0, with 1 M NaCl, at 25°C. Optimum signal was obtained using a mixture of 30 mM probe FL-6b and 0.5 µM IDE. The “DOS informer set” compound plates were pinned onto 384-well plates containing the enzyme-probe mixture (50 µL/well). The azetidine screen was pre-printed on 384-well plates and the enzyme-probe mixture was added (50 µL/well). The final compound concentration was 20 µM, and IDE inhibitor 6bK was used as a positive control at 1 µM final concentration. After 30 min equilibration, the increase in fluorescence anisotropy was recorded using an EnVision spectrophotometer (excitation 492 nm, emission 523 nm).

**HTRF assay for IDE-mediated degradation of insulin.** A solution of 0.8 µg/mL IDE (R&D) in pH 7.5 buffer containing 20 mM HEPES, 135 mM NaCl (24 µL) was transferred to a 200 µL tube strip, and combined with 1 µL of each inhibitor (10 mM in DMSO, or as a 3-fold dilution series). A solution of insulin in “Assay Dilutent” (25 µL) was added to a final concentration of 20 ng/mL, and incubated at 30 °C for 15 min. This procedure was optimized to result in ~75% degradation of insulin. The reaction was terminated by adding 25 µL of inhibitor IiI (200 nM) and chilled on ice. The remaining insulin was quantified using 10 µL of the quenched enzymatic reaction using the sensitive-range protocol Homogeneous Time-Resolved FRET.
Insulin assay (CisBio® 62INSPEB) in 20 µL total volume according to the manufacturer’s instructions (384 well-plate Greiner 784904 non-binding). Fluorescence was measured using a Tecan M1000Pro plate reader (excitation = 320 nm, emission = 665 and 620 nm, lag time = 60 µs) according the assay manufacturer’s recommendations. Blank wells and insulin standard curve were included in the assay.

**HTRF assay for IDE-mediated degradation of glucagon.** A solution of 0.05 µg/mL IDE (R&D) in 1x assay “Diluent #5” (24 µL) was transferred to a 200 µL tube strip, and combined with 1 µL of each inhibitor (10 mM in DMSO, or as a 3-fold dilution series). A solution of glucagon in the same buffer (25 µL) was added to a final concentration of 4 ng/mL, and incubated at RT for 10 min. This procedure was optimized to result in ~75% degradation of glucagon. The reaction was terminated by 1 µL of inhibitor Ii1 (5 µM) and chilled on ice. The remaining glucagon was quantified using 10 µL of the quenched enzymatic reaction using the sensitive-range protocol Homogeneous Time-Resolved FRET Glucagon assay (CisBio® 62GLCPEF) in 20 µL total volume according to the manufacturer’s instructions (384 well-plate Greiner 784904 non-binding). Fluorescence was measured using a Tecan M1000Pro plate reader (excitation = 340 nm, emission = 665 and 620 nm, lag time = 60 µs) according the assay manufacturer’s recommendations. Blank wells and glucagon standard curve were included in the assay.
HTRF assay for IDE-mediated degradation of Aβ40. A solution of 1.76 µg/mL IDE (R&D) in pH 7.5 buffer containing 20 mM HEPES, 135 mM NaCl (24 µL) was transferred to a 200 µL tube strip, and combined with 1 µL of each inhibitor (10 mM in DMSO, or as a 3-fold dilution series). A solution of insulin in “Assay Dilutent” (25 µL) was added to a final concentration of 1.6 ng/mL, and incubated at 30 °C for 15 min. This procedure was optimized to result in ~80% degradation of Aβ40. The reaction was terminated by adding 25 µL of inhibitor II1 (200 nM) and chilled on ice. The remaining Aβ was quantified using 10 µL of the quenched enzymatic reaction using the sensitive-range protocol Homogeneous Time-Resolved FRET Aβ40 assay (CisBio® 62B40PEB) in 20 µL total volume according to the manufacturer’s instructions (384 well-plate Greiner 784904 non-binding). Fluorescence was measured using a Tecan M1000Pro plate reader (excitation = 320 nm, emission = 665 and 620 nm, lag time = 60 µs) according the assay manufacturer’s recommendations. Blank wells and insulin standard curve were included in the assay.

Macrocycle docking simulations. Receptor and ligand preparation was performed in the standard method. DOCKing was performed using version 6.6 with default parameters for flexible ligand and grid-based scoring, and the van der Waals exponent was 9. Because of the mutagenesis data strongly pointing to a role of Ala479, we limited docking of the inhibitor to an area within 15 Å of Ala479.
**Synthesis of anisotropy probe FL-6b.** Approximately 250 mg of O-Bis-(aminoethyl)ethylene glycol trityl resin (NovaPEG Novabiochem®, ~0.8 mmol/g, 0.2 mmol) was swollen with ~10 volumes of anhydrous DMF for 1 h in a peptide synthesis vessel with mixing provided by dry nitrogen bubbling. In a separate flask, Nα-allyloxycarbonyl-Nε-2-Fmoc-L-lysine (5 equiv.) and 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU, 4.75 equiv.) were dissolved in anhydrous DMF (~10 vol.), then treated with N,N'-diisopropylethylamine (DIPEA, 10 equiv.) for 5 min at RT. The solution was combined with the pre-swollen resin and mixed with nitrogen bubbling overnight. The vessel was eluted and the resin was washed three times with N-methyl-2-pyrrolidone (NMP, ~10 vol.). Following each coupling step, Fmoc deprotection was effected with 20% piperidine in NMP (~20 vol.) for 20 min, repeated three times, followed by washing three times with NMP (~10 vol.) and twice with anhydrous DMF (~10 vol.). The general procedure for amide coupling of building blocks A (Nα-Fmoc-4-benzoyl-D-phenylalanine), B (Nα-Fmoc-3-cyclohexyl-L-alanine) and C (Nα-Fmoc-Nδ-trityl-L-glutamine) was treatment of the resin with solutions of HATU-activated Nα-Fmoc amino acids (5 equiv.) for 3-5 hours in anhydrous DMF, mixing with dry nitrogen bubbling. The general procedure for HATU-activation was treating a solution of Nα-Fmoc amino acid (5 equiv.) and HATU (4.75 equiv.) in anhydrous DMF (10 vol.) with DIPEA (10 equiv.) for 5 min at RT. Following the final Fmoc deprotection procedure, the α-amine of glutamine was coupled with allyl fumarate monoester (10 equiv.) using activation conditions as previously described with
HATU (9.5 equiv.) and DIPEA (20 equiv.) in anhydrous DMF (~10 vol.) plus the addition of N-hydroxysuccinimide (11 equiv). Allyl fumarate coupling was accomplished by overnight mixing with dry nitrogen bubbling, followed by washing five times with NMP (~10 vol.) and three times with CHCl₃ (~10 vol.). Simultaneous allyl ester and N-allyloxycarbonyl group cleavage in solid support was effected with three consecutive treatments with a solution of tetrakis(triphenylphosphine)palladium(0) (0.5 equiv.) dissolved in degassed CHCl₃ containing acetic acid and N-methylmorpholine (40:2:1 ratio, ~20 vol.), mixing by nitrogen bubbling for 30 min. The resin was then washed twice subsequently with ~20 vol. of 5 DIPEA in DMF, then twice with a 5% solution of sodium diethylthiocarbamate trihydrate in DMF (~20 vol.), twice with 5% solution of hydroxybenzotriazole monohydrate in DMF, and finally washed with 50% CH₂Cl₂ in DMF and re-equilibrated with anhydrous DMF (~10 vol.).

Treating the resin with pentafluorophenyl diphenylphosphinate (5 equiv.) and DIPEA (10 equiv.) in anhydrous DMF (~10 vol.) mixing by nitrogen bubbling overnight produced the macrocyclized products. The resin was washed with NMP (~20 vol.) and CH₂Cl₂ (~20 vol.) and dried by vacuum. The macrocyclized product was cleaved from the resin by two 15 min treatments of the macrocycle-bound resin with 95% TFA containing 2.5% water and 2.5% triisopropylsilane (~20 vol.), followed by two TFA washes (~5 vol.). The TFA solution was dried to a residue under rotatory evaporation, and the peptide was precipitated by the addition of dry Et₂O. The ether was decanted and the remaining solid was dried and dissolved in a
minimum volume of 3:1 DMF-water prior to purification by HPLC. Purification was
performed on a C18 21.2x250 mm column (5 µm particle, 100 Å pore size,
Kromasil®), using a gradient of 30 to 80% MeCN/water over 30 min, and solvents
containing 0.1% TFA. Purity was determined by HPLC (Zorbax SB-C18 2.1x150
mm column, 5 µm particle) with UV detection at 230 nm, using a gradient of 30 to
80% MeCN/water over 30 min, and solvents containing 0.1% TFA. The formula of
final product was confirmed by accurate mass measurements using an Agilent 1100
LC-MSD SL instrument.

Fractions containing the desired PEGylated macrocyclic peptide were
combined and freeze-dried to produce a white powder (11.2 mg, 5.6% based on resin
loading). The PEGylated macrocyclic peptide (TFA salt, 1.5 mg) was dissolved in
dry DMF and treated with DIPEA (10 µL) and 5-carboxyfluorescein N-succinimidyl
ester (1.12 mg, 1.5 equiv.) for 2 hours. The mixture was acidified with TFA (10 µL)
and purified by HPLC. Fractions containing the desired fluorescein-labeled
macrocyclic peptide **FL-6b** were combined and freeze-dried to produce a white
powder (1.26 mg, 67.5%), which was dissolved in DMSO-*d*$_6$ (3.35 mM).

$^1$H NMR (600 MHz, DMSO-*d*$_6$) δ 10.15 (s, 3H), 8.91 (s, 1H), 8.77 (s, 1H), 8.53 –
8.40 (m, 3H), 8.24 (s, 1H), 8.11 – 8.05 (m, $J = 9.9$ Hz, 1H), 8.03 – 7.95 (m, 1H), 7.78
– 7.73 (m, 1H), 7.73 – 7.62 (m, $J = 26.5$, 16.3 Hz, 5H), 7.61 – 7.50 (m, 5H), 7.37 (d, $J = 8.0$
Hz, 1H), 7.31 (s, 1H), 7.28 (d, $J = 7.4$ Hz, 2H), 7.20 (s, 2H), 7.14 (d, $J = 7.6$
Hz, 2H), 6.88 – 6.79 (m, $J = 25.2$ Hz, 2H), 6.72 – 6.62 (m, 6H), 6.62 – 6.50 (m, 6H),
5.37 – 5.28 (m, 4H), 4.57 (mz, 1H), 4.23 – 4.13 (m, 2H), 4.06 – 3.97 (m, 1H), 2.04 – 1.94 (m, J = 7.4 Hz, 9H), 1.92 – 1.85 (m, 1H), 1.65 – 1.41 (m, 12H).

High resolution mass, calculated for C_{67}H_{74}N_{8}O_{16} [M+H]^+ = 1247.5296, found 1247.542, Δ = 9.93 ppm.

**Figure 5.18 | Synthesis of BRD-297.** Intermediate 1 was provided by the Broad Institute.

*N-((2R,3S,4S)-1-allyl-3-(4-bromophenyl)-4-((trityloxy)methyl)azetidin-2-yl)methyl)-2-methylbenzenesulfonamide (2).* A solution of (2R,3S,4S)-1-allyl-3-(4-bromophenyl)-4-((trityloxy)methyl)azetidine -2-carbonitrile (2 g, 3.64 mmol) in 200 mL of CH_{2}Cl_{2} was cooled to 0 °C. To this solution DIABAL (1 M in CH_{2}Cl_{2}) was added dropwise (18.2 mL, 5 equiv., 18.2 mmol), and stirred at 0 °C to RT for 3 h. The solution was again cooled to 0 °C, and added MeOH dropwise (100 µL) until no
gas evolved, and worked up using the Fieser method: first 0.73 mL water was added slowly, and stirred vigorously for 5 min 0 ºC. Then 0.73 mL 15% NaOH was added and stirred 5 min; followed by addition of 1.8 mL water and stirred vigorously 30 min 0 ºC to RT. Approximately 1.5 g MgSO₄ was added and stirred vigorously for 15 min. The solids were filtered, and washed with 50 mL EtOAc five times, and the filtrates were combined, dried over 3 g MgSO₄ and refiltered. The solution was evaporated to a residue under vacuum, and dissolved in 100 mL CH₂Cl₂. This solution was treated with 2,6-lutidine (5.1 mL, 43.7 mmmol), followed by o-toluenesulfonyl chloride (5.3 mL, 36.4 mmmol), and stirred overnight at RT. The solution was treated with ethylenediamine (2.67 mL, 40 mmol) for 10 min, washed with saturated NaHCO₃, dried over NaSO₄, and evaporated to a residue under vacuum. Purification by column chromatography (5 → 40% EtOAc/hexanes) and evaporation of the collected fractions gave 2.487 g of the title compound 2 (96.5%, 3.51 mmol), a white solid. High resolution mass, calculated for C₄₃H₄₃BrN₂O₃S [M+H]+ = 707.1938, found 707.196, Δ = -1.47 ppm.

![Image](image_url)

(8R,9S,10S,Z)-9-(4-bromophenyl)-10-(phenoxyethyl)-6-(o-tolylsulfonyl)-1,6-diazabicyclo[6.2.0]dec-3-ene (3). A solution of sulfonamide intermediate 2 (1.175 g, 1.66 mmol) in 150 mL of DMF was treated with K₂CO₃ (2 g, 13.3 mmol, 8 equiv.)
followed by allyl bromide (570 µL, 6.64 mmol, 4 equiv.) and stirred overnight at RT. The solution was evaporated to a residue under vacuum, and dissolved in 100 mL EtOAc. The solids were filtered, and washed with 50 mL EtOAc five times, and the filtrates were combined and evaporated to a residue under vacuum. Purification by column chromatography (5 → 40% EtOAc/hexanes) and evaporation of the collected fractions gave 1.07 g of the \( N \)-allylated product (86%, 1.43 mmol), a white solid. High resolution mass, calculated for \( \text{C}_{43}\text{H}_{43}\text{BrN}_{2}\text{O}_{3}\text{S} \) [M+H]+ = 719.1938, found 719.196, \( \Delta = 3.06 \) ppm. The bis-allyl intermediate (420 mg, 0.564 mmol) was dissolved in benzene (250 mL, 2.25 mM) and purged vigorously with argon gas for 30 min. The solution was added Grubbs-Hoveyda catalyst (110 mg, 0.135 mmol, 0.25 equiv.) and heated to 90 °C overnight (16 h). The solution was evaporated to a residue under vacuum, followed by purification by column chromatography (5 → 40% EtOAc/hexanes) and evaporation of the collected fractions gave 322.5 mg of cyclized title compound 3 (79.5%, 0.448 mmol), a white solid.

\(^1\)H NMR (600 MHz, DMSO-\( \text{d}_6 \)) \( \delta \) 8.32 (s, \( J = 20.1 \) Hz, 1H), 7.72 (d, \( J = 7.8 \) Hz, 1H), 7.54 (t, \( J = 7.4 \) Hz, 1H), 7.50 (s, \( J = 8.3 \) Hz, 1H), 7.49 (s, 1H), 7.43 (d, \( J = 7.5 \) Hz, 1H), 7.40 – 7.34 (m, \( J = 7.5 \) Hz, 6H), 7.32 (s, 1H), 7.32 – 7.29 (m, \( J = 18.5 \) Hz, 3H), 7.29 (s, 1H), 7.26 (s, 1H), 7.25 (t, \( J = 7.1 \) Hz, 2H), 7.20 (s, \( J = 8.4 \) Hz, 1H), 7.19 (s, \( J = 10.8 \) Hz, 1H), 5.69 – 5.60 (m, 2H), 4.28 – 4.23 (m, \( J = 3.0 \) Hz, 1H), 3.86 (dd, \( J = 14.3, 6.8 \) Hz, 1H), 3.76 (d, \( J = 17.9 \) Hz, 1H), 3.51 (dd, \( J = 13.2, 1.8 \) Hz, 1H), 3.46 (d, \( J = 17.7 \) Hz, 1H), 3.29 – 3.25 (m, 2H), 3.23 – 3.15 (m, 2H), 3.05 (dd, \( J = 13.4, 10.3 \) Hz, 1H), 3.01 – 2.97 (m, 2H), 2.50 (s, \( J = 1.1 \) Hz, 3H).
High resolution mass, calculated for C_{43}H_{43}BrN_{2}O_{3}S [M+H]+ = 719.1938, found
719.196, Δ = 3.06 ppm.

(8R,9S,10S,Z)-9-(3'-methyl-[1,1'-biphenyl]-4-yl)-6-(o-tolylsulfonyl)-10-
((trityloxy)methyl)-1,6-diazabicyclo[6.2.0]dec-3-ene (3). A solution of bromoaryl
intermediate 3 (100 mg, 0.139 mmol) was dissolved in THF (30 mL), added 10 mL
of K_{3}PO_{4} solution (0.5 M, >20 equiv.), followed by m-tolylboronic acid (37.7 mg,
0.278 mmol, 2 equiv.), and the heterogeneous mixture was purged vigorously with
argon gas for 30 min. The solution was added X-Phos-Pd G3 catalyst (25 mg, 0.028
mmol, 0.2 equiv.), and stirred overnight (16 h) at 50 °C. The solution was added
EtOAc (50 mL), followed by 2 g MgSO_{4}, and the organic layer was decanted over a
filter with a pad of Celite®. The aqueous slurry was washed 3 times with 50 mL
EtOAc, and these fractions were used to wash the Celite®. The combined organic
fraction was dried over MgSO_{4} and evaporated to a residue under vacuum, followed
by purification by column chromatography (5 → 40% EtOAc/hexanes) and
evaporation of the collected fractions gave 60 mg of title compound 4 (59%, 0.082
mmol), a white solid.

^{1}H NMR (600 MHz, DMSO-\textit{d}_{6}) δ 7.74 (d, J = 7.8 Hz, 1H), 7.58 (d, J = 8.0 Hz, 2H),
7.54 (t, J = 7.5 Hz, 1H), 7.46 – 7.43 (m, 2H), 7.43 – 7.36 (m, 9H), 7.35 – 7.28 (m,
9H), 7.27 – 7.23 (m, J = 7.1 Hz, 3H), 7.16 (d, J = 7.2 Hz, 1H), 5.70 – 5.63 (m, 2H), 4.33 – 4.25 (m, 1H), 3.93 – 3.85 (m, 1H), 3.81 (d, J = 18.0 Hz, 1H), 3.52 (dd, J = 24.0, 14.6 Hz, 2H), 3.30 – 3.25 (m, 2H), 3.21 (dd, J = 9.5, 6.9 Hz, 1H), 3.09 (dd, J = 13.4, 10.4 Hz, 1H), 3.06 – 2.99 (m, 2H), 2.52 (s, J = 2.7 Hz, 3H), 2.36 (s, 2H).

High resolution mass, calculated for C_{48}H_{46}N_{2}O_{3}S [M+H]+ = 731.3302, found 731.330, Δ = -0.27 ppm.

\[
\text{((8R,9S,10S)-9-(3'-methyl-[1,1'-biphenyl]-4-yl)-6-(o-tolylsulfonyl)-1,6-diazabicyclo[6.2.0]decan-10-yl)methanol (BRD-297, BRD-K29788283). A solution of intermediate 3 (60 mg, 0.082 mmol) was dissolved in THF (10 mL), added 1 mL MeOH, 10 drops of AcOH, and 20 mg 20% Pd(OH)_{2}/C, and stirred vigorously overnight (16 h) under an atmosphere of H\textsubscript{2} gas from a balloon. The solution was evaporated to a residue under vacuum, and treated with 10 mL of TFA solution containing 2.5% water and 2.5% triisopropyl silane (v/v). After 10 min the solution was evaporated to a residue under vacuum, and the mixture was purified by HPLC (10 → 70\% MeCN/water containing 0.1\% TFA). Fractions containing BRD-297 were combined and freeze-dried to produce a white powder (33.8 mg, 0.0689 mmol, 84\% yield).}
\]
$^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 9.21 (s, 1H), 7.13 (m, 2H), 7.10 (d, $J = 7.8$ Hz, 1H), 7.00 (t, $J = 7.4$ Hz, 1H), 6.97 – 6.92 (m, 3H), 6.90 (d, $J = 7.1$ Hz, 2H), 6.85 (t, $J = 7.6$ Hz, 1H), 6.79 (t, $J = 7.6$ Hz, 1H), 6.63 (d, $J = 7.4$ Hz, 1H), 5.03 (s, 1H), 4.14 (s, 1H), 3.81 (s, 1H), 3.38 – 3.22 (m, $J = 17.3$ Hz, 3H), 3.20 – 3.09 (m, 3H), 3.07 – 2.95 (m, $J = 27.6$ Hz, 1H), 2.62 (d, $J = 18.7$ Hz, 1H), 1.95 (s, 3H), 1.82 (s, 3H), 1.50 – 1.27 (m, $J = 43.9$, 24.5, 8.9 Hz, 3H), 1.26 – 1.18 (m, 1H).

High resolution mass, calculated for C$_{29}$H$_{34}$N$_2$O$_3$S [M+H]$^+$ = 491.2363, found 491.237, $\Delta = 1.42$ ppm.

**Figure 5.19** | Spectra for $^1$H-NMR of BRD-297.
References


Properties calculated with the online tool Chemicalize.org (ChemAxon). &lt;<http://www.chemicalize.org/%3E.&gt;


Appendix

Perl scripts used for deconvolution of in vitro selections

Contributions: The following Perl scripts were coded by Dr. Ralph Kleiner, and they were used in Chapter 2 with minor modifications.
#!/usr/bin/perl
use warnings;

open (FILE01, "~/Users/FILE.fastq");
open (SEQ1, "~/Users/FILE.fastq.txt");
$i = 0;

while (!<$line1 = <FILE01>>) {
    if ($line1 =~ /^@/) {
        $line2 = <FILE01>;
        chomp($line2);
        if (primer($line2) == 1) {
            $i = $i + 1;
            $revcom = reverse $line2;
            $revcom =~ tr/ACGT/TGCA/;
            print SEQ1 ">$i"
            print SEQ1 "$revcom"
        }
    }
}

sub rev_com {
    my ($DNA) = @_; 
    my ($revcom);
    $revcom = reverse $DNA;
    $revcom =~ tr/ACGT/TGCA/;
    return $revcom;
}

sub primer{
    my ($line) = @_; 
    my (@chars1, @chars2);
    my ($compval1);
    @chars1 = split(/,/,$line);
    # @chars2 = split(/,, "GAGTGGGATG"); # search for index with offset 0 for selections 1 and 7
    # @chars2 = split(/,, "TGAGTGGGAT"); # search for index with offset 1 for selections 2 and 8
    # @chars2 = split(/,, "CTGAGTGGGA"); # search for index with offset 3 for selections 3 and 9
    # @chars2 = split(/,, "ACTGAGTGGG"); # search for index with offset 4 for selections 4 and 10
    # @chars2 = split(/,, "CACTGAGTG"); # search for index with offset 5 for selections 5 and 11
    @chars2 = split(/,, "TCACTGAGTG"); # search for index with offset 6 for selections 6 and 12
    if ($compval1 > 7) {
        return 1;
    } else {
        return 0;
    }
}

print "Sequence trimming completed.\n";
#!/usr/bin/perl
use warnings;
open (FILE1, "~/Users/FILE.fastq.txt");
open (SEQ, "~/Users/FILE.txt");

my $offset = 5; my $dots = "....."; # for selections 1 and 7
my $offset = 4; my $dots = "...." ; # for selections 2 and 8
my $offset = 3; my $dots = "..."; # for selections 3 and 9
my $offset = 2; my $dots = ".." ; # for selections 4 and 10
my $offset = 1; my $dots = "." ; # for selections 5 and 11
my $offset = 0; my $dots = "" ; # for selections 6 and 12

my @codon1 = ("TTCCTC", "AGCTCA", "ATCGGA", "TGTGCA", "AGACTC", "CTTCAG", "AGTCGA", "ATGACG", "ACTAGC", "CAACCT", "TCCGTA", "GCTTAC");
my @codon2 = ("GCTGAA", "AACGGT", "GTCGAT", "GATTGC", "GGACTT", "ACGGAT", "AGGACT", "TCGAGT", "GCAAGA", "CTTGTG", "GGCTAA", "CTGGAA");
my @codon3 = ("GGCTTT", "AGGCTT", "GCCAAA", "AGGAAC", "CGTATG", "CATGAG", "GCAGTA", "GCTCTT", "GAGACA", "CTGTAG", "GGAATC", "TAGCTG");

sub codon_match {
    my ($codon, $line, $pos, $mis) = @_; 
    my ($n, @chars1, @chars2);
    my ($id) = 0;
    for ($n=0; $n<14; $n++) {
        @chars1 = split(/\s/, $codon->{[$n]});
        @chars2 = split(/\s/, $line);
            $id = $n+1;
            last;
        }
    }
    return $id;
}

sub scaffold_match_perfect_for {
    my ($scaffold, $line) = @_; 
    my ($n);
    my ($id) = 0;
    for ($n=0; $n<8; $n++) { 
        if ( $line =~ /^...................................................................................$dots$scaffold->{[$n]}/ ) {
            $id = $n+1;
            last;
        }
    }
}

my @scaffold = ("AAC", "CTA", "AGA", "TAC", "CAA", "TGA", "ACA", "GAA");
sub scaffold_match_perfect_rev {
    my ($scaffold, $line) = @_; 
    my ($n1); 
    my ($id) = 0; 
    for ($n1=0; $n1<8; $n1++) {
        if ($line =~ /\$dots$ scaffold->[$n1]/) {
            $id = $n1+1; 
            last; 
        }
    }
    return $id;
}

sub rev_com {
    my ($DNA) = @_; 
    my ($revcom); 
    $revcom = reverse $DNA; 
    $revcom =~ tr/ACGT/TGCA/; 
    return $revcom;
}

sub codon {
    my ($codon, $line, $pos) = @_; 
    if (codon_match($codon, $line, $pos, 1) >=1) {
        return codon_match($codon, $line, $pos, 1); 
    } elsif ((codon_match($codon, $line, $pos, 1) == 0) && (codon_match($codon, $line, $pos, 2) >=1)) {
        return codon_match($codon, $line, $pos, 2); 
    } else { return 0; }
}

$i = 0;
while ($line1 = <FILE1>) {
    $line3 = <FILE1>; 
    chomp($line3);
    #print SEQ "$i$line3"; 
    if ($i > 0) {
        if ($s1 = barcode_match($s1, $line3, 1, 2)); 
        $a1 = codon_match($a1, $line3, 53 + $offset, 1); 
        $b1 = codon_match($b1, $line3, 64 + $offset, 1); 
        $c1 = codon_match($c1, $line3, 75 + $offset, 1); 
        $d1 = scaffold_match_perfect_for($d1, $line3);
        print SEQ "$i$line3";
        #print SEQ "$s$A$c1 B$b1 C$c1 D$d1\n"; #with internal barcode
        print SEQ "$sA$c1 B$b1 C$c1 D$d1\n"; #without internal barcode
    }
}
sub primer{
    my ($line) = @_; 
    my (@chars1, @chars2, @chars3);
my ($compval1, $compval2);
@chars1 = split(//, $line);
# this is the new primer
@chars2 = split(//, "CCATTACTCGCCCTGTACAC"); #this is the original primer
@chars3 = split(//, "CCATGACTACAGAGTGGGATG");


if ($compval1 >= 15) {
  return 1;
} elsif ($compval2 >= 15) {
  return -1;
} else {
  return 0;
}

print "Codon assignment complete.\n\n";
#!/usr/bin/perl
use warnings;
open SEQCODONS, "~/Users/FIle.txt"
open MORECODONS, "~/Users/Codonlist.txt"

@filename = qw(Final/Sample_4-2_JMJD2A.R1.fastq_freq.txt Other/Bc2.txt Other/Bc3.txt Other/Bc4.txt Other/Bc6.txt Other/Bc7.txt Other/Bc8.txt Other/Bc9.txt Other/Bc10.txt Other/Bc11.txt Other/Bc12.txt Other/Bc13.txt Other/Bc14.txt Other/Bc15.txt Other/Bc16.txt Other/Bc17.txt Other/Bc0.txt);

@arraylist = (@array1, @array2, @array3, @array4, @array5, @array6, @array7, @array8, @array9, @array10, @array11, @array12, @array13, @array14, @array15, @array16, @array17, @array18);

@arrayname = qw(Bc1 Bc2 Bc3 Bc4 Bc5 Bc6 Bc7 Bc8 Bc9 Bc10 Bc11 Bc12 Bc13 Bc14 Bc15 Bc16 Bc17 Bc0);

for (my $i = 0; $i<18; $i++) {
  open ($arrayname[$i], ">$filename[$i]" or die "$! error trying to overwrite";
}

while ($line3 = <MORECODONS>) {
  $line4 = <MORECODONS>
  chomp($line4);
  ($A2, $B2, $C2, $D2) = split(/\t/, $line4);
  $D3 = substr $D2, 0, 2;
  $code2 = join("\t", $A2,$B2,$C2,$D3);
  if ($A2 =~ "A") {
    push (@array1, $code2);}
}

while ($line1 = <SEQCODONS>) {
  $line2 = <SEQCODONS>
  chomp($line2);
  ($s, $A, $B, $C, $D) = split(/\t/, $line2);
  $code = join("\t", $A,$B,$C,$D);
  if ($s =~ "S1") {
    push(@array1, $code);}
  else {
    push(@array1, $code);}
}

@arraylist = (\@array1, \@array2, \@array3, \@array4, \@array5, \@array6, \@array7, \@array8, \@array9, \@array10, \@array11, \@array12, \@array13, \@array14, \@array15, \@array16, \@array17, \@array18);

sub count {
  my ($array_ref, $fh) = @_;
  my ($n);
  my %per;
  my %count;
  my @array = @$array_ref;
  map { $count{$_}++ } @array;
  $n = scalar(@array);
  foreach $$_ (keys(%count)){
    $per($_) = ($count{$_})/$n;
  }
map { print {$fh} "$_ = $count{$_} $per{$_}\n" } sort {$count{$_} <=> $count{$_} }
keys($count);
print {$fh} "total reads = $n\n";

for (my($j) = 0; $j<18; $j++) {
    count($arraylist[$j], $arrayname[$j]);
}

print "Codon counting completed.\a\n";
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