

UNIVERSITÉ DU QUÉBEC  
INSTITUT NATIONAL DE LA RECHERCHE SCIENTIFIQUE  
INSTITUT ARMAND-FRAPPIER

**THE *NAJA KAOUTHIA* SNAKE VENOM CONTAINS A  
POTENT INSULINOTROPIC PEPTIDE**

**Isolation, identification and characterization**

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**Thèse présentée pour obtenir le grade de  
Philosophiae Doctor**

Doctorat en Biologie  
Programme offert conjointement par l'INRS et l'UQAM

**Jury d'évaluation**

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Montreal 2014

*For my lovely daughter Bùi Anh Thư and my husband Bùi Quang Dũng*

## ACKNOWLEDGEMENTS

Foremost, I would like to express my deepest gratitude to my research director Professor Alain Fournier for his patience, caring, excellent continuous support and providing me with a perfect atmosphere for doing research. Without his supervision and constant help, this dissertation would not have been possible. I would like to thank Professor Truong Nam Hai, my research supervisor in Vietnam, for giving me the opportunity to perform my PhD in Quebec, and for supporting me during my study in Vietnam.

I would like to thank Professor David Chatenet for his advices, motivation, and enthusiasm. His guidance helped me during my research. Very special thanks go to Myriam Létourneau who, not only as a colleague but also as a good friend, was always willing to help and give her best suggestions. I could not have imagined having a better collaborator for my PhD studies.

I would like to thank Professor Dang Vu Minh and Professor Le Quoc Sinh who put the first rock to build this PhD training co-program between Vietnam Academy of Science and Technology and Institut National de la Recherche Scientifique. I wish to thank professor Chau Van Minh and professor Vu Thi Bich who made it work, therefore giving me an opportunity to participate in the program. I would like to thank Professor Le Thanh Hoa, and Professor Dinh Duy Khang for encouraging me.

I thank Dr. Benjamin Folch for his contribution to my project. His peptide modeling studies made the project more complete.

I would like to thank my committee members, Professor Pedro D'Orléans-Juste and Professor Martin Sirois for their insightful comments for my thesis.

I would like to thank my parents, my parents-in-law, my husband, Bui Quang Dung and my lovely daughter, Bui Anh Thu for their love and encouragement. Without their love, I would not have finished this thesis. I would also like to thank my family for always supporting me and encouraging me with their best wishes.

Many thanks go to all colleagues in the Institute of Biotechnology, Viet Nam Academy of Science and Technology and all friends in Canada for providing a stimulating and fun environment in which we learn and grow. Warm thanks to my colleague Nguyen Hong Thanh for supporting me during my studies in Vietnam.

Last but not least, I wish to express my gratitude to the Ministry of Education and Training of Vietnam, the Institut national de la recherche scientifique, and the Vietnam Academy of Science and Technology for the financial assistance.

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## LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E - binding protein 1
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
nAChR	Nicotinic acetylcholine receptor
mAChR	Muscarinic acetylcholine receptor
ADP	Adenosine diphosphate
$\beta$ -AR	$\beta$ -adrenoreceptors
AGEs	Advanced glycosylated end products
ALS	Amyotrophic lateral sclerosis
AMPA	$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	5' adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BAS	Bile acid sequestrants
BK channel	Large-conductance $\text{Ca}^{2+}$ -activated $\text{K}^{+}$ channel
BmKTX	<i>Buthus martensii</i> kaliotoxin
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
Ctri9577	Peptide 9577 from <i>Chaerilus tricostatus</i>
CTXs	Cardiotoxins
CVF	Cobra venom factor

DAG	Diacylglycerol
DM	Diabetes mellitus
DPP-IV	Dipeptidyl peptidase - IV
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FA	Fatty acid
FDA	Food and Drug Administration
FFA	Free fatty acid
Glucose 6-P	Glucose-6-phosphate
GABA	$\gamma$ -Aminobutyric acid
GDIS	Glucose-dependent insulin secretion
GDM	Gestational diabetes mellitus
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLP	Glucagon-like peptide
GLP-1R	Glucagon-like peptide 1 receptor
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
GPIb	Glycoprotein Ib
GPVI	Glycoprotein VI
GRP	Gastrin-releasing peptide
GRPP	Glicentin-related pancreatic peptide
GS	Glycogen synthase

GxTX	Guangxitoxin
HaTx	Hanatoxin
HbA1c	Glycated hemoglobin
HK	Hexokinase
hUll	Human urotensin II
[Ca <sup>2+</sup> ] <sub>i</sub>	Intracellular Ca <sup>2+</sup>
IP	Intermediate peptide
IP <sub>3</sub>	Inositol trisphosphate
IR	Insulin receptor
IRS	Insulin-receptor substrate
JZTX-I	Jingzhaotoxin-I
K <sub>ATP</sub>	ATP-dependent potassium channel
K <sub>v</sub>	Voltage-gated potassium channel
LCCoA	Long-chain acyl-CoA
LDL	Low-density lipoproteins
LKB1	Liver kinase B1
LTX	Latrotoxin
M3R	Muscarinic acetylcholine receptor M3
MgTX	Margatoxin
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartic acid
NPY	Neuropeptide Y

OEA	Oleoylethanolamide
PACAP	Pituitary adenylate cyclase-activating polypeptide
PDPK-1	3-phosphoinositide-dependent protein kinase
PDK-1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PI3K	Phosphatidylinositol 3 kinase
PIP <sub>2</sub>	Phosphatidylinositol 4,5 bisphosphate
PIP <sub>3</sub>	Phosphatidylinositol 3,4,5 trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLGA	Poly (lactide-co-glycolide)
PLI	PLA2 inhibitor
PP	Pancreatic polypeptide
PPAR- $\gamma$	Peroxisome proliferator-activated receptor gamma
PPRE	PPAR $\gamma$ response elements
SP	Signal peptide
PVD	Peripheral vascular disease
RER	Rough endoplasmic reticulum
RVV-X	Russellysin
RXR	Retinoid X receptor
SGLT	Sodium-coupled glucose co-transporter
SGTx	<i>Scodra griseipes</i> toxin

SK channels	Small conductance $\text{Ca}^{2+}$ -activated $\text{K}^+$ channels
SUR	Sulfonylurea receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TDZ	Thiazolidinedione
TSC	Tuberous sclerosis complex
UDP	Uracil-diphosphate
VDCC	Voltage-dependent calcium channels
VIP	Vasoactive intestinal polypeptide

## RÉSUMÉ EN FRANÇAIS

La prévalence du diabète s'accroît à un rythme alarmant. En 2015, cette maladie affectera plus de 350 millions de personnes à l'échelle mondiale et touchera approximativement 550 millions d'individus en 2030. Le diabète de type 2 (DT2) est la forme la plus répandue, représentant de 90% à 95% des cas de diabète dans le monde entier. Le DT2 est un désordre qui touche le système endocrinien et le métabolisme, et qui conduit à l'incapacité de maintenir une glycémie normale. Afin de traiter cette maladie progressive, les traitements doivent être modifiés en fonction de ces divers stades. Plusieurs médicaments sont actuellement utilisés pour maintenir une glycémie normale et leur diversité a élargi les options pour traiter le DT2. Cependant, malgré leur puissance et leur spécificité, ils présentent encore des carences et des effets secondaires tels que des épisodes d'hypoglycémie, une prise de poids, certains problèmes cardiovasculaires, des troubles gastro-intestinaux et un oedème périphérique. En outre, la plupart des améliorations initiales de la glycémie produites par la prise de médicaments ne sont pas maintenues en raison de la dégradation de la fonction des cellules  $\beta$  du pancréas. Par conséquent, de nouveaux agents doivent être créés afin de contrôler efficacement la glycémie, de prévenir le déclin de la fonction des cellules  $\beta$ , de favoriser la perte de poids, d'améliorer l'action de l'insuline et de produire un effet favorable sur les maladies cardiovasculaires qui pourraient être en développement.

Il a été observé depuis longtemps que les venins, qui sont des mélanges complexes de protéines, peptides, enzymes et autres agents aux propriétés biologiques diverses, représentent une source immense et variée de molécules. Ces dernières peuvent notamment offrir des options nouvelles de traitements grâce à leur puissance et efficacité unique. Les venins sont le fruit d'une sélection naturelle associée à des millions d'années d'évolution. Les substances qui les composent servent par exemple pour la capture des proies et la digestion et/ou la défense contre les prédateurs. En particulier, les peptides présents dans les venins sont reconnus pour leur grande variété de cibles systémiques. Ceci les identifie comme des ligands aux propriétés inestimables pour étudier les mécanismes physiologiques et/ou pharmacologiques dans différents paradigmes expérimentaux liés à des maladies telles que le cancer, l'inflammation, les maladies neurodégénératives, les atteintes neuromusculaires, les troubles hématologiques, les infections autoimmunes et les problèmes cardiovasculaires. À ce propos, un exemple frappant est celui de

l'exendine 4, un peptide extrait du venin du monstre de Gila, qui possède une activité insulinothrompe marquée. Ainsi, une étude pharmacologique exhaustive a permis de l'exenatide, une forme synthétique du peptide qui est maintenant utilisée pour traiter le diabète. Cette découverte image bien les nouvelles perspectives qu'offrent les composants extraits de venins, et tout particulièrement pour le traitement de pathologies telles que le diabète. En effet, il a été montré que les toxines suivantes, soit *Agelalia* MP-I, konkunitzine-S1 et  $\alpha$ -latrotoxine, sont capables de déclencher l'exocytose de l'insuline. De plus, de façon plus générale, l'étude des mélanges complexes de polypeptides et de protéines de venins a donné lieu à la découverte de molécules biologiques clés comme le BPP (*bradykinin-potentiating peptide*), le NGF (*nerve growth factor*), la dendrotoxine, le CVF (*cobra venom factor*) et la convulxine. Ces composés ont contribué à faciliter la compréhension de systèmes physiologiques et ont été utilisés comme gabarit pour concevoir de nouveaux dérivés affichant des fonctions pharmacologiques précises.

Le monstre de Gila, duquel a été isolé l'exendine 4, est un lézard qui ne mange qu'environ quatre fois par an. Il évite l'hypoglycémie en stoppant sa production d'insuline. Lorsque vient le moment de manger, il tue une proie avec son venin. L'ingestion de celle-ci entraîne aussi l'absorption de son propre venin et conséquemment de l'exendine 4, ce qui réactive sa production d'insuline. De façon similaire, plusieurs espèces de serpents ont la capacité de réduire considérablement leur métabolisme et de survivre sans nourriture pour des périodes pouvant aller jusqu'à deux ans. Cette observation suggère qu'un ou des facteurs équivalents à l'exendine 4 se retrouveraient aussi dans le venin de serpents, d'autant plus que ces animaux sont phylogénétiquement reliés au monstre de Gila. Par conséquent, en collaboration avec un partenaire vietnamien qui étudie les propriétés du venin du cobra à monocle *Naja kaouthia*, nous avons initié dans notre laboratoire un projet visant à identifier un peptide présentant des caractéristiques pharmacologiques similaires à celles de l'exendine 4. Ultiment, cette molécule ou un dérivé pourrait s'ajouter à l'arsenal développé pour le traitement du diabète.

Nos travaux ont conduit à l'développer de peptide insulinothrompe, dont NTTN-16, à partir du venin de *Naja kaouthia* du Vietnam. Ce composé s'est montré capable d'induire la sécrétion d'insuline par les cellules INS-1E sans affecter leur viabilité. Sa caractérisation a montré que sa séquence est identique à celle d'un autre peptide précédemment isolé à partir de l'espèce *Naja kaouthia* vivant à Taiwan, soit la cardiotoxine I (CTX-I), qui compte 60 acides aminés et forme

une structure compacte maintenue par 4 ponts disulfures. Malgré la complexité de la molécule, sa synthèse a été réalisée avec succès comme montré entre autres par l'activité insulino trope concentration-dépendante de la forme synthétique sur des cellules INS-1E et ce, et il est important de le signaler, tant en absence qu'en présence de glucose. De plus, cette toxine ne provoque pas la lyse des globules rouges humains. Notre étude a aussi montré que la sécrétion d'insuline par la CTX-I est dépendante du calcium. Également, nous avons démontré que les mécanismes insulino tropes associés aux canaux potassiques sensibles à l'ATP ( $K_{ATP}$ ) ou au récepteur du GLP-1 ne sont pas impliqués dans l'action de la CTX-I. En effet, l'inhibition du flux potassique suite à la fermeture du canal  $K_{AIP}$  par une absence de glucose extracellulaire n'a pas modifié la sécrétion d'insuline. De même, des tests de liaison de la CTX-I au récepteur du GLP-1 se sont avérés négatifs.

Dans les cellules  $\beta$ , l'inhibition des courants  $K^+$  accroît la durée du potentiel d'action. Ceci maintient la membrane plasmique dans un état dépolarisé et provoque l'afflux du  $Ca^{2+}$  par l'ouverture des canaux calciques, un phénomène qui améliore la sécrétion d'insuline dépendante du glucose. Ainsi, indirectement, au cours de nos travaux, le rôle de canaux  $K^+$  autres que  $K_{AIP}$  a aussi été étudié. En effet, dans les cellules  $\beta$ , nous retrouvons également des canaux appelés BK (canal  $K^+$  à conductance élevée, activé par le  $Ca^{2+}$ ) qui, même s'ils ne jouent pas un rôle déterminant dans l'exocytose de l'insuline, participent au contrôle de l'amplitude du potentiel d'action. Il a été montré que ces canaux modulent la mobilisation du calcium dans diverses actions biologiques dont notamment lors de la contraction d'un tissu vasculaire tel que l'aorte. Nous avons montré au moyen d'un bioessai que la CTX-I est incapable d'induire la contraction d'anneaux d'aorte de rat, ce qui suggère que cette toxine ne serait pas en mesure d'interagir avec ce type de canal. Néanmoins, la littérature indique que les effets exercés sur le cœur par les CTXs sont causés par une modulation de canaux ioniques et tout spécialement par une amplification de l'interaction entre la calséniline (protéine KChIP1) et le canal potassique sensible au voltage ( $K_v$ ). Par conséquent, l'effet insulino trope de la CTX-I pourrait vraisemblablement se produire par l'entremise de ces canaux. Dans les faits, nous observons que la CTX-I possède des homologies structurelles avec des toxines à canaux potassiques. Par exemple, les boucles II et III de la CTX-I sont similaires à celles de nombreuses toxines à canaux potassiques. De plus, la superposition de certains résidus jouant un rôle fonctionnel majeur tels



que la Lys<sup>23</sup>, la Lys<sup>50</sup> et l'Asn<sup>55</sup> de la CTX-I avec la Lys<sup>27</sup>, l'Arg<sup>25</sup> et l'Asn<sup>30</sup> de toxines à canaux potassiques suggère que la CTX-I pourrait agir comme un bloqueur de canal K<sub>v</sub> *via* un mécanisme similaire.

La CTX-I déclenche la libération du calcium dans différentes lignées cellulaires, y compris les cellules INS-1E, HEK293, HeLa et CHO. L'effet maximal est observé avec les cellules INS-1E, tandis que la libération la plus faible est mesurée avec les cellules CHO. En outre, même à 10<sup>-6</sup> M, la CTX-I n'a pas causé l'hémolyse d'érythrocytes humains, lesquels se sont révélés être les plus sensibles parmi les globules rouges de mammifères. La CTX-I a également montré le même effet sur la lignée cellulaire myogène murine C2C12. En fait, avec les cellules C2C12, aucune augmentation significative du nombre de cellules apoptotiques en phase précoce n'a été observée. Cependant, en phase retardée, le pourcentage de cellules apoptotiques s'est avéré légèrement augmenté.

Comme décrit précédemment, la CTX-I présente au niveau de ses boucles II et III, des homologies de structure avec celle des inhibiteurs des canaux K<sub>v</sub>. Ces boucles comptent généralement de 25 à 40 acides aminés. Cependant, les activités biologiques de la CTX-I sont réparties non pas sur deux mais essentiellement sur trois boucles. Ainsi, les boucles I et II sont connues pour leur capacité à endommager les cellules tandis que la boucle III est identifiée comme celle responsable de la dépolarisation des cellules musculaires et de la mobilisation du calcium qui y est associée. Par ailleurs, notre étude a montré que l'activité calcique de la CTX-I est l'amorce nécessaire pour le déclenchement de la libération d'insuline par les cellules INS-1E. Nous avons donc exploré plus à fond les propriétés biologiques de ces boucles au moyen de dérivés synthétiques tronqués de la CTX-I produits dans notre laboratoire en appliquant une stratégie de protection orthogonale des cystéines, afin d'obtenir les ponts disulfures dans la configuration présente dans la toxine native. Ainsi, à 10<sup>-6</sup> M, le fragment CTX-I<sub>1-39</sub> a causé la mort d'environ 50% des cellules INS-1E, tandis que le fragment CTX-I<sub>41-60</sub>, à cette même concentration, n'a pas affecté leur viabilité. Le fragment toxique 1-39 n'a donc pas été étudié davantage. Toutefois, nous avons évalué les propriétés du fragment CTX-I<sub>41-60</sub> dans quelques paradigmes et avons observé que ce peptide parvient à stimuler la mobilisation calcique d'une manière concentration-dépendante et entraîne la libération de l'insuline sans l'intervention d'un

canal  $K_{ATP}$ . Ces effets sont en nature et intensité très similaires à ceux de la molécule complète, bien que ce fragment n'améliore pas la sécrétion d'insuline, en absence de glucose.

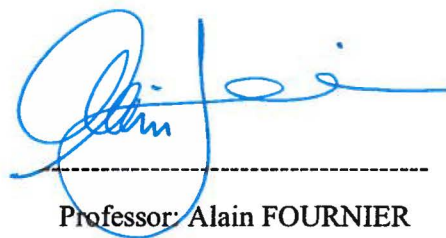
Des analyses structurales de la CTX-I ont dévoilé que la valine à la position 52 est considérablement exposée et accessible. De même, l'évaluation des homologies de séquences présentes au niveau d'antagonistes des canaux  $K_v$ , a montré qu'une lysine est fréquemment localisée dans la partie responsable de l'action inhibitrice sur les canaux. Par conséquent, nous avons émis l'hypothèse que la substitution de Val<sup>52</sup> par une lysine pouvait reproduire un élément structural clé des antagonistes des canaux  $K_v$ . Nous avons alors synthétisé l'analogue tronqué [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> et mesuré ses effets. Tout comme le fragment non substitué, ce dérivé n'a pas d'effet cytotoxique sur les cellules INS-1E. Par contre, il stimule la libération de l'insuline aussi bien en absence qu'en présence de glucose. Cette activité insulinothèque est liée au  $[Ca^{2+}]_i$  et n'est pas médiée par l'activation du canal  $K_{ATP}$  ou du récepteur du GLP-1. À 10  $\mu$ M, la [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> a diminué de 25% l'influence des courants de canaux de type  $K^+$ . Ces canaux sont membres d'une famille contenant de nombreux sous-types et l'analogue est peut-être un bloqueur sélectif de l'un de ces sous-types dans les cellules INS-1E. Par ailleurs, nous avons observé que le traitement avec [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> de cellules CHO transfectées avec le récepteur UT (CHO-UT) réduit/empêche l'hypertrophie induite par l'urotensine II, un effet qui pourrait être associé à des sous-types de canaux  $K_v$ . On doit aussi souligner la similitude des résidus importants, tels que la Lys<sup>52</sup>, l'Asn<sup>45</sup> et l'Arg<sup>58</sup> présents dans le dérivé [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, ce qui lui confère des caractéristiques structurales similaires à celles des antagonistes des canaux de sous-type  $K_{v1.3}$ , *i.e.* 1) une lysine hautement conservée se trouvant au centre de la toxine 2) une asparagine ou un résidu polaire se trouvant à une extrémité et 3) une arginine ou un résidu chargé positivement à l'autre extrémité. Donc, globalement, il semble plausible que le dérivé tronqué de la CTX-I, développé dans notre laboratoire, soit un ligand du canal potassique sensible au voltage, et plus précisément du canal du type  $K_{v1.3}$ .

En conclusion, le criblage de substances biologiques est extrêmement prometteur pour trouver de nouveaux composés thérapeutiques. En combinant cette approche à des études de relation structure-activité de molécules-mères, il est possible de favoriser le développement de composés puissants et sélectifs. Dans ce contexte, à partir de la CTX-I, un peptide de 60 acides aminés isolé du venin d'une espèce de cobra vivant au Vietnam, nous avons conçu un analogue

tronqué, la [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, qui possède la capacité de libérer l'insuline et ce, en présence ou non de glucose. De plus, certaines caractéristiques structurales et pharmacologiques suggèrent que ce dérivé serait un antagoniste des canaux K<sub>v</sub> et plus spécifiquement des canaux du type K<sub>v1.3</sub>. Si cette hypothèse s'avère exacte, en bloquant les K<sub>v1.3</sub>, cet antagoniste stimulerait aussi la translocation de GLUT4 qui transporte le glucose à l'intérieur des cellules, ce qui réduirait davantage la résistance à l'insuline. Finalement, la [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> a également empêché l'hypertrophie cellulaire. Cette caractéristique remarquable s'ajoute aux propriétés déjà décrites et contribuerait à limiter les complications cardiovasculaires qui accompagnent souvent le DT2. Nos résultats ont été obtenus à partir de modèles cellulaires. Afin de déterminer pleinement le potentiel de cette molécule, il sera indispensable d'évaluer son activité *in vivo*.



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## INTRODUCTION

During the last decade, drug development, which involves various scientific areas of expertise such as chemistry, pharmacology, microbiology, and biochemistry, has significantly decreased in the pharmaceutical industry. As a matter of fact, the traditional generation of new chemically based small molecules is slowing down, and at the same time advancements in biotechnology and genetic interventions are calling for a renewal of discovery approaches. Hence, with the crucial and increasing need for new therapies in several pathologies, pharmaceutical companies are changing their research strategy to actually rely more and more on discoveries made in academic labs and industrial spin-offs. This situation has also led researchers to look at what “Dame Nature” has to offer, and during the last century, several compounds with a therapeutic potential have been discovered in nature (Lewis, Dutertre et al. 2012; Vyas, Brahmabhatt et al. 2013). For instance, among the key medical breakthroughs, the discovery of insulin in 1921 changed diabetes from a death sentence to a survivable disease.

More recently, venoms that are a rich source of bioactive molecules, such as peptides, proteins and enzymes, were investigated. Indeed, venomous animals, represented by zoological groups including some mollusks, arthropods, reptiles, and a number of fishes, are often characterized by a well-developed organ derived from the salivary or other exocrine glands, and injection devices. Compounds produced in these glands are usually secreted with an aim to reach specific targets within the inflicted organism. For this reason, these toxins represent potential therapeutic and biotechnological drugs since their primary molecular targets include receptors, membranes and enzymes (Rocha, Rostelato-Ferreira et al. 2013; Safavi-Hemami, Moller et al. 2013). Therefore, the continuous need for new and improved pharmacological tools has led the scientific community to investigate venoms and toxins as a template for drug design. With this approach, a number of venom- or toxin-based compounds have produced some useful drugs that are currently used or under investigation for several human diseases. For example, from the initial discovery of captopril, the first oral angiotensin-I converting enzyme (ACE) inhibitor (Odaka and Mizuochi 2000; Ben Henda, Labidi et al. 2013), to the recent application of disintegrins for the potential treatment of cancer (Thangam, Gunasekaran et al. 2012), the various components of snake venoms have never failed to reveal amazing new properties. Most

notably, the disintegrins (eptifibatide and tirofiban) were shown both *in vitro* and *in vivo* to be potent antiplatelet aggregates (Conrotto, Scacciati et al. 2012; Sisk, Palma et al. 2012). Also, special ion channel toxins, like conantokin G (Kunda, Cheriyan et al. 2013), chlorotoxin (Costa, Cardoso et al. 2013),  $\omega$ -conotoxin (Adams and Berecki 2013) and so on, have helped to understand critical mechanisms in several human pathologies, thus representing a major driving force for continuing the prospective search for specific drugs capable of modulating the channel function.

While original native toxins are usually unsuitable as therapeutics, interventions by medicinal chemists and clinicians in pharmaceutical R&D made their use possible as therapeutics or pharmacological tools for multiple disorders. However, many aspects must be observed to fulfill all requirements needed for a drug candidate to finish as a drugstore shelf product, including *in vivo* stability, pharmacodynamics and pharmacokinetics aspects. Venoms, with their cocktail of components, have been tested by millions of years of evolution in bloodstream and other circulatory systems of animals suggesting that their *in vivo* chemical stability could be controlled by modifying for their therapeutic application. Moreover, with the growing data, it became evident that these substances were exerting remarkable biological properties associated with their ability to act on specific targets. In fact, many venom components target the cardiovascular (Chaisakul, Isbister et al. 2013), endocrine and nervous systems (Wolz-Richter, Esser et al. 2013), and modulate the action potentials by acting at various molecular sites such as central or peripheral receptors, enzymes, neurons, axons, synapses and neuromuscular junctions (Kang, Roh et al. 2013; Zhao, Zhao et al. 2013). Therefore, the discovery of molecules in venoms with selective activities represents a new methodology to the search for new drugs. As mentioned above, peptide toxins have been biochemically and pharmacologically refined by the process of evolution to exhibit optimal and finely tuned activities. In this sense, nature has already pre-screened huge combinatorial libraries of potential therapeutic drugs. These toxins can be probed for novel pharmacological tools or as a treatment for pain, as well as for the development of anti-arrhythmics, anti-convulsants, anti-microbials, anti-diabetic, anti-neoplastic and even insecticidal agents. Hence, these examples indicate that the exploration of “venoms”, with the goal of discovering new therapeutics, represents a significant endeavor.

Diabetes is a syndrome caused by sugar metabolic disordering, resulting in several cases from insufficient levels of the hormone insulin. According to the World Health Organization, every year in the world, there are about 3.8 million deaths (or 6% of world mortality) by complications of diabetes. Following the prediction of the World Health Organization, by 2030, unless preventive measures are taken, 552 million people worldwide will have diabetes, with the largest increase occurring in developing countries (Whiting, Guariguata et al. 2011). In healthy nondiabetic subjects, oral administration of glucose produces a substantially enhanced insulin response compared with intravenous administration. This discrepancy was called the *incretin effect*, and it is diminished in people with type 2 diabetes. Subsequent studies identified two primary gut-derived hormones that are responsible for most of the incretin effect: glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) (Mari, Bagger et al. 2013). GLP-1 is a better therapeutic target and has accordingly been studied to a greater extent. GLP-1 inhibits postprandial glucagon release and stimulates glucose dependent insulin secretion. This hormone also slows gastric emptying and enhances satiety. At least in animals, at the cellular level, GLP-1 appears to stimulate  $\beta$  cell growth and survival, and reduces apoptosis, leading to an increase of  $\beta$  cell mass (Meier 2012). Unfortunately, GLP-1 has an *in vivo* half-life of less than 2 minutes, which prevents it from being an attractive therapeutic candidate (Meier 2012). The currently available incretin mimetics, which involve GLP-1 agonists or analogs that are resistant to DPP-IV-mediated degradation, is exenatide (Byetta<sup>®</sup>), a GLP-1 agonist that was approved by the FDA in 2005 as adjunctive therapy for patients with type 2 diabetes. Exenatide is a synthetic analog of exendin-4, a peptide found in the Gila monster venom, which binds to and activates the human GLP-1 receptor. Exenatide provokes a sustained reduction in hemoglobin *A1c* (a good measure of diabetes care), probably related to its effects on pancreatic  $\beta$  cells, accompanied by a dose-dependent weight loss. Consistent with the known activities of GLP-1, exenatide promotes  $\beta$  cell proliferation and differentiation in animal models and also enhances  $\beta$  cell function in patients with type 2 diabetes. Exenatide enhances endogenous insulin secretion in a glucose-dependent manner. Another incretin mimetic, liraglutide, is an acylated GLP-1 analog that is non-covalently bound to albumin and has a half-life of about 12 hours. Liraglutide was also effective in improving glycemic control in treated patients. As with exenatide, liraglutide has been reported to improve  $\beta$  cell function and is associated with

decreases in body weight (Barnett 2009; Fineman, Cirincione et al. 2012). The choice of diabetic therapies will likely continue to expand in the coming years with the discovery of new oral antihyperglycemic agents.

The Gila monster is able to reduce its metabolic rate in combination with a large capacity to store fat. It is this observation that led researchers to investigate the venom of this animal and finally to discover a new therapeutic drug for type II diabetes (Christel and DeNardo 2006). Likewise, snakes were known to survive up to two years without food, but it was not clear how. Recently, it was demonstrated that snakes could lower their metabolic rate by up to 70%, allowing them to survive prolonged periods without food (Mccue 2007). In this respect and since snake venom is an abundant resource of bioactive compounds, we screened venom peptide fractions from the cobra *Naja kaouthia* in an insulin secretion assay to identify new compounds that could have a therapeutic value in the design of new drugs for diabetic patient.

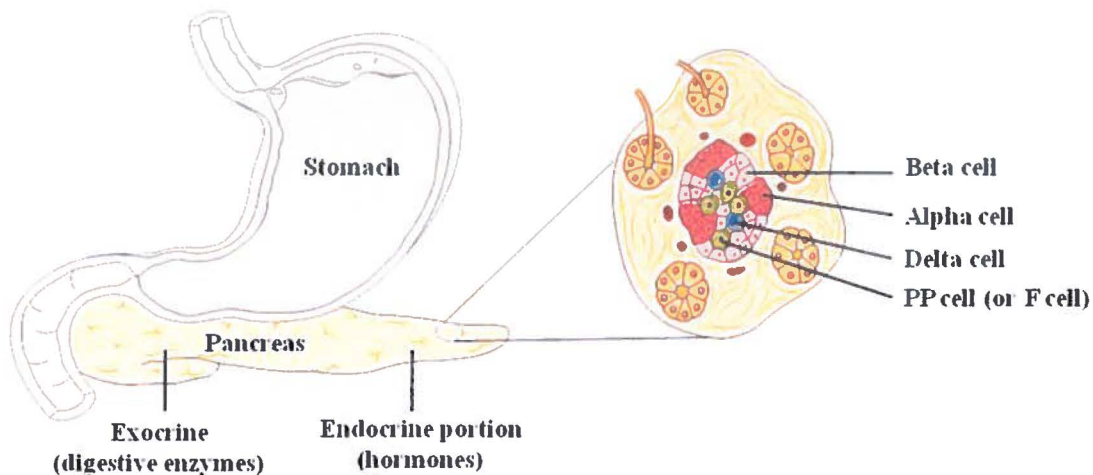
## **CHAPTER I. LITERATURE**



# 1. General characterization of pancreas and type 1 and 2 diabetes

## 1.1. The pancreas and its endocrine function

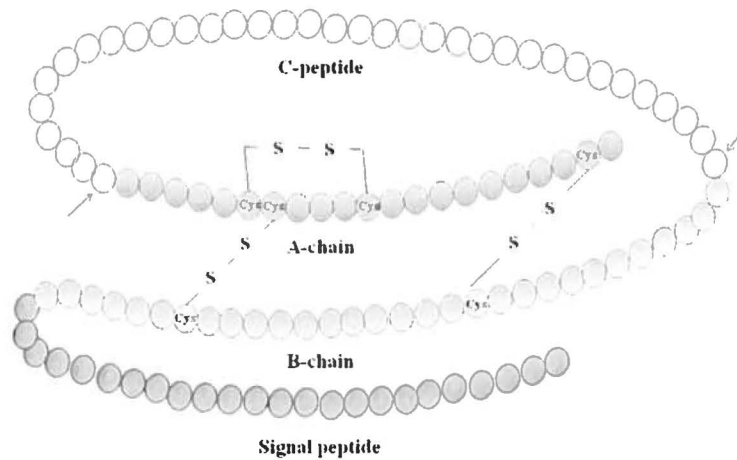
The pancreas is a unique structure that performs both exocrine and endocrine functions. On one hand, the exocrine cells, present within the pancreas, secrete digestive enzymes including for instance trypsin, carboxypeptidases and lipases that will break down carbohydrates, proteins, and lipids in the chyme. On the other hand, the endocrine portion of the pancreas consists of four distinct cell types, found in substructures called the islets of Langerhans. They are mostly composed of  $\beta$  cells (60-80%), which produce insulin and amylin, and  $\alpha$  cells (20%), which secrete glucagon. The remaining cells are the somatostatin-secreting  $\delta$  cells and the PP cells (or F cells) producing pancreatic polypeptide (Guo and Hebrok 2009) (Fig.1-1).



**Figure 1-1. Cellular structure of the pancreas**  
(modified from Nabeel Bardeesy & Ronald A. DePinho, 2002)

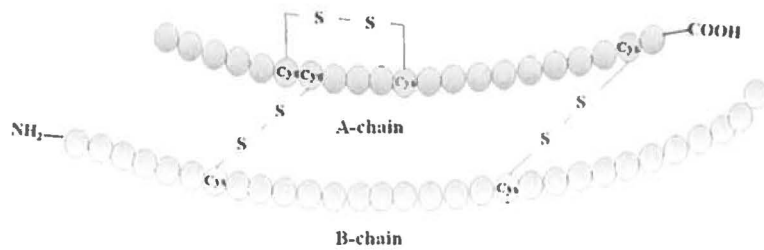
### 1.1.1. Insulin hormone

Insulin is a peptide hormone composed of 51 amino acids. In the body, insulin is synthesized as a proinsulin precursor. Proinsulin is synthesized in the rough endoplasmic reticulum (RER) from mRNA as preproinsulin. Preproinsulin is a 110-amino acid structure containing a signal peptide, a B chain, a connecting C peptide and an A chain (Fig. 1-2). Removal of the signal peptide forms proinsulin, which acquires its characteristic 3 dimensional structure in the endoplasmic reticulum. Then, secretory vesicles transfer proinsulin from the RER to the Golgi apparatus, whose aqueous zinc and calcium rich environment favors formation of soluble zinc-containing proinsulin hexamers.



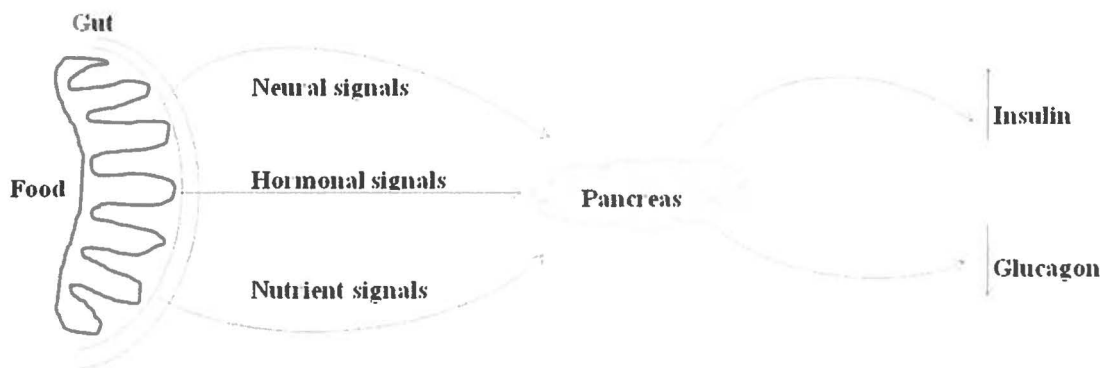
**Figure 1-2. Diagrammatic representation of the amino acid sequence of human preproinsulin**

As immature storage vesicles form from the Golgi, enzymes acting outside the Golgi convert proinsulin into insulin. When mature granules are secreted into the circulation by exocytosis, insulin, and an equimolar ratio of C-peptide are released (Fig. 1-3).



**Figure 1-3. Mature human insulin**

Synthesis and secretion of insulin is regulated by both nutrient and non-nutrient secretagogues, in the context of environmental stimuli and the interplay of other hormones (Fig. 1-4). The exocytosis of insulin is mostly triggered in response to the rising of plasma glucose (nutrient secretagogue). Glucose enters the  $\beta$  cells where it is metabolized to generate ATP, increasing ratio of ATP to ADP, therefore activating  $K_{ATP}$ . Closure of ATP-dependent  $K^+$  channels ( $K_{ATP}$ ) results in membrane depolarization and activation of voltage-gated dependent calcium channels (VDCC) leading to an increase in intracellular calcium  $[Ca^{2+}]_i$  concentration. This increase triggers insulin exocytosis. Non-nutrient secretagogues may act via neural stimuli such as cholinergic and adrenergic pathways, or through peptide hormones and amino acids.



**Figure 1-4. Signals controlling insulin and glucagon secretion**

It has been well recognized that vagus nerve stimulation results in pancreatic insulin secretion. Insulin secretion by these mechanisms only occurs in the feeding state when food is seen, smelled or acutely ingested. Hence, acetylcholine, generated by parasympathetic activity, triggers insulin secretion through the muscarinic acetylcholine receptor M3 (M3R) stimulation, which produces a diacylglycerol (DAG) and protein kinase C (PKC) activation (Nakajima, Jain et al. 2013). The  $\beta$ -adrenoreceptors ( $\beta$ -AR) are also able to transfer signals from neural stimuli to initiate insulin secretion via cyclic adenosine monophosphate (cAMP), but this effect appears to be minor. Also, the incretin hormone glucagon-like peptide (GLP-1), secreted after detection of nutrients in the gut, enhances insulin secretion. Its mechanism of action appears to be mediated by cAMP and activation of a cAMP-responsive protein kinase A (PKA). Infusion of several amino acids such as arginine and leucine leads to significant increases in plasma insulin. The insulinotropic effect of these amino acids is associated to depolarization of the cell membrane and gating of VDCC (Kim and Egan 2008). Some fatty acids (FAs) stimulate the G protein-coupled receptor (GPCR) 40 (GPR40) or GPR119 to augment the  $[Ca^{2+}]_i$  and consequently cause insulin exocytosis (Mancini and Poitout 2013) (Fig. 1-5).

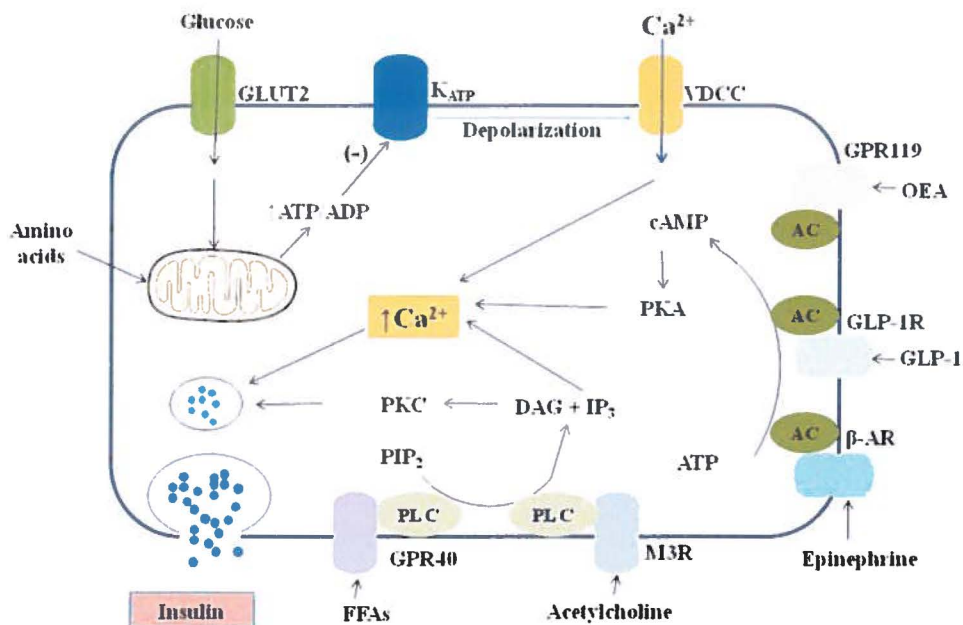
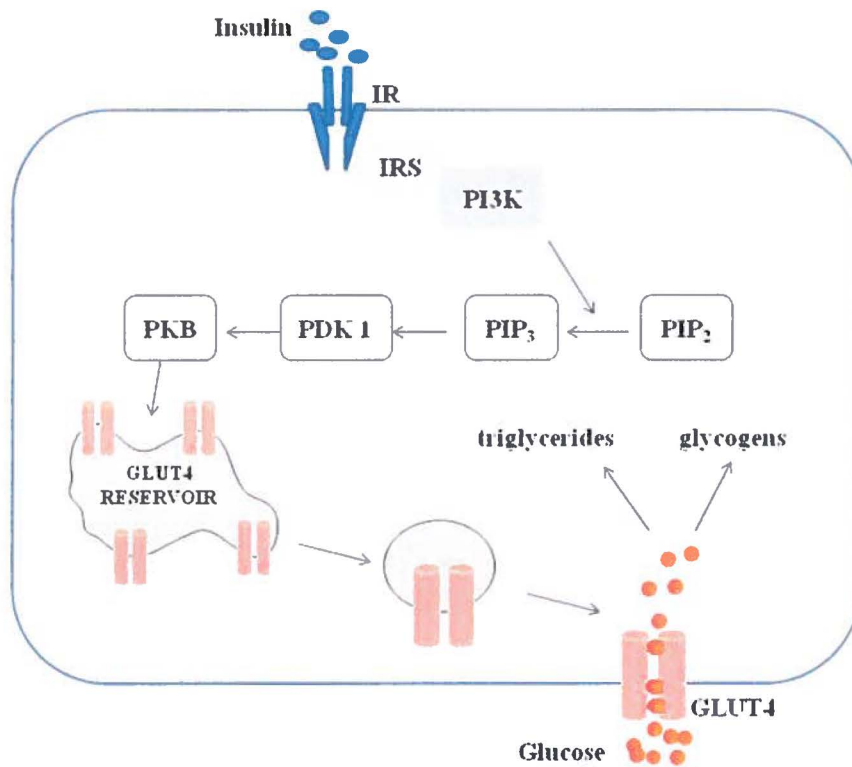


Figure 1-5. Mechanism of insulin secretion in  $\beta$  cell (Adapted from Kahn, Hull et al. 2006)

Like other peptide hormones, insulin interacts with a membrane receptor on its target cells. The insulin receptor (IR) exhibits tyrosine kinase activity. The activated IR phosphorylates proteins called the insulin-receptor substrates (IRS). These proteins act through downstream signals to influence transport and cellular metabolism (Grote, Ryals et al. 2013). In the body, insulin is central to regulating carbohydrate and fat metabolism.

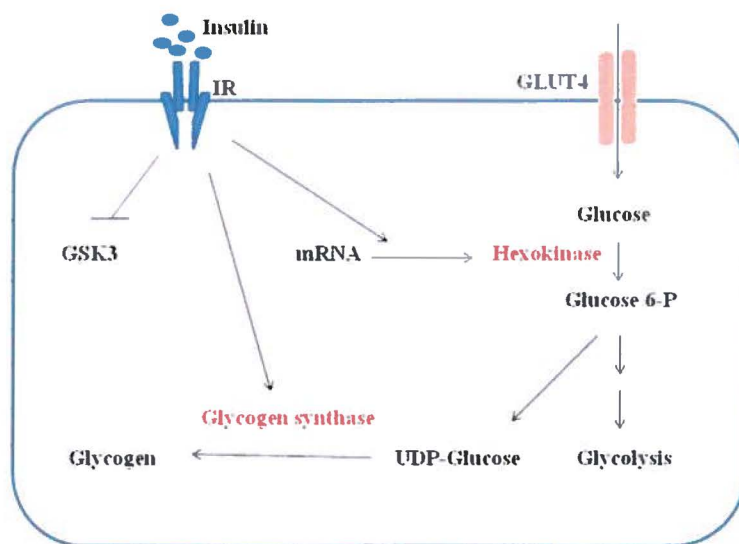


**Figure 1-6. Role of insulin in GLUT4 translocation**

Insulin causes cells in the liver, skeletal muscles, and fat tissues to absorb glucose from the blood. In the liver and skeletal muscles, glucose is stored as glycogen, and in fat cells (adipocytes), it is stored as triglycerides. Insulin stimulates glycogen accumulation through a coordinated increase in glucose transport and glycogen synthesis. Glucose transport into fat and muscle cells is largely mediated by a specific protein known as glucose transporter 4 (GLUT4). Insulin increases glucose transport in these cells by stimulating the translocation of the

transporter GLUT4 from intracellular sites to the plasma membrane. Insulin activates the IR, which phosphorylates IRS. Phosphorylated IRS activate phosphatidylinositol-3-kinase (PI3K) in the cytoplasm, which afterwards converts phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) into phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> activates PDK-1 (3-phosphoinositide-dependent kinase 1), and PDK-1 in turn phosphorylates protein kinase B (PKB) in the cytosol. PKB then stimulates the relocation of the storage vesicles to the plasma membrane (Kahn, Hull et al. 2006) (Fig. 1-6).

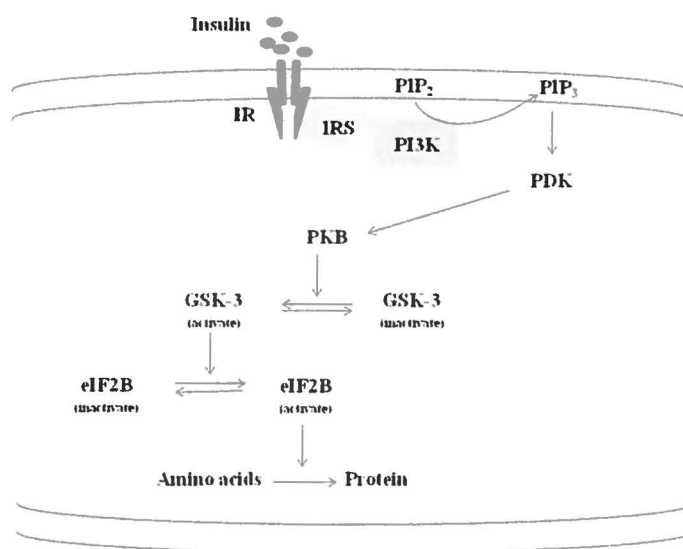
The synthesis of glycogen firstly requires glucose to be transported into muscle cells, and then glucose is phosphorylated by hexokinase to form glucose 6-phosphate (G-6-P). G-6-P is converted to uridine diphosphate glucose (UDP-glucose), and glycogen synthase (GS) subsequently catalyzes the synthesis of glycogen by transferring a glucosyl moiety from UDP-glucose to a preexisting glycogen molecule. In skeletal muscle, two isoforms of hexokinase (type I and type II) are expressed. Between these two isoforms, hexokinase II (HK-II) predominates, accounting for 90% of the total. Gene transcription of this type of enzyme is regulated acutely by insulin. This hormone also promotes activation of GS by inactivating glycogen synthase kinase-3 (GSK-3) through phosphorylation (Hoffman and Elmendorf 2011) (Fig. 1-7).



**Figure 1-7. Role of insulin in glycogen synthesis**

Insulin also inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis. This occurs through a direct effect of insulin on the liver, as well as by indirect effects of insulin on substrate availability. Insulin can also influence glucose metabolism indirectly by changes in free fatty acids (FFAs) generated from visceral fat.

Insulin rapidly activates protein synthesis by activating components of the translational machinery including eIFs (eukaryotic initiation factors) and eEFs (eukaryotic elongation factors). In the long term, insulin also increases the cellular content of ribosomes to augment the capacity for protein synthesis. The rapid activation of protein synthesis by insulin is mediated primarily through PI3K. This involves the activation of PKB. In one case, PKB phosphorylates and inactivates GSK-3, which in turn phosphorylates and inhibits eIF2B. Insulin elicits the dephosphorylation and activation of eIF2B. Because eIF2B is required for recycling of eIF2, a factor required for all cytoplasmic translation initiation events, this contributes to overall activation of protein synthesis (Fig. 1-8). PKB also phosphorylates the TSC1 (tuberous sclerosis complex 1)-TSC2 complex to relieve its inhibitory action on the mTOR (mammalian target of rapamycin). The protein mTOR controls translation initiation and elongation. The cap-binding factor eIF4E can be sequestered in inactive complexes by 4E-BP1 (eIF4E-binding protein 1).



**Figure 1-8. Role of insulin in protein synthesis**

Insulin elicits phosphorylation of 4E-BP1 and its release from eIF4E, allowing eIF4E to form initiation factor complexes. Insulin induces dephosphorylation and activation of eEF2 to accelerate elongation. Insulin inactivates eEF2 kinase by increasing its phosphorylation at several mTOR-regulated sites. Insulin also stimulates synthesis of ribosomal proteins by promoting recruitment of their mRNAs into polyribosomes.

As in the case of carbohydrate metabolism, insulin also promotes the synthesis of lipids, and inhibits their degradation. In adipocytes, glucose is stored primarily as lipids, owing to increased uptake of glucose and activation of lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase. Insulin also profoundly inhibits lipolysis in adipocytes, primarily through inhibition of the hormone sensitive enzyme lipase. This enzyme is acutely regulated by control of its phosphorylation state, which is activated by PKA-dependent phosphorylation, and inhibited as a result of a combination of kinase inhibition and phosphatase activation. Insulin inhibits the activity of the lipase primarily through reductions in cAMP levels, owing to the activation of a cAMP-specific phosphodiesterase in fat cells. In general, insulin exerts multiple effects upon target cells, especially skeletal muscle, liver, and adipose tissue (Wong and Sul 2010).

The hormone amylin, in addition to insulin, is secreted from the pancreatic  $\beta$  cells. This hormone is co-secreted with insulin from the pancreatic  $\beta$  cells in the ratio of approximately 1:100. It is a 37-residue peptide hormone. Amylin decreases food intake, slows the emptying of the stomach and activates the satiety center in the brain. Amylin reduces the level of glucose in the blood by inhibiting the secretion of glucagon.

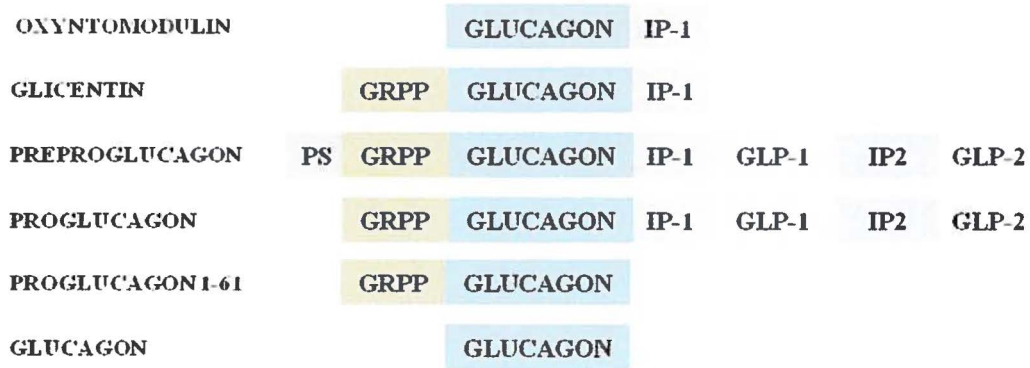
### *1.1.2. Glucagon hormone*

Glucagon is a 29-amino acid peptide that is generated from the cleavage of proglucagon secreted by pancreatic islet  $\alpha$  cells.

In intestinal L cells, proglucagon is cleaved to the alternate products glicentin, GLP-1, intervening peptide 2 (IP-2), and glucagon-like peptide 2 (GLP-2) (Fig. 1-9), which promotes intestinal growth. In the body,  $\alpha$  cells are equipped with a specific set of channels that generate action potentials of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  at low levels of glucose. Although most of the  $\text{Ca}^{2+}$  current



goes through L-type channels in  $\alpha$  cells, N-type channels mediate the  $\text{Ca}^{2+}$  required for exocytosis at low glucose levels. At low glucose levels, the activity of  $\text{K}_{\text{ATP}}$  channels produces a membrane potential of about -60 mV.

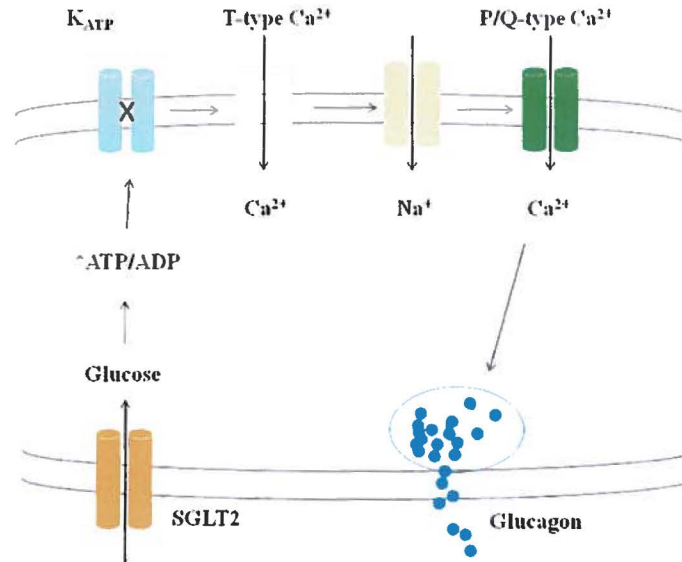


**Figure 1-9. Processing of glucagon. PS: Signal peptide; GRPP: Glicentin-related pancreatic peptide; IP: Intermediate peptide; GLP: Glucagon-like peptide**

At activated state, T-type channels open, which depolarizes the membrane potential to levels where  $\text{Na}^+$  and P/Q-type  $\text{Ca}^{2+}$  channels are activated, leading to regenerative action potentials.  $\text{Ca}^{2+}$  entry through P/Q-type channels induces glucagon secretion (Fig. 1-10). However, the increase in extracellular glucose levels rises the cytosolic ATP/ADP ratio, which blocks  $\text{K}_{\text{ATP}}$  channels, thus depolarizing  $\alpha$  cells to a membrane potential range where the channels involved in action potentials become inactivated. As a consequence, electrical activity,  $\text{Ca}^{2+}$  signals and glucagon secretion are inhibited. Moreover, secretion studies prove that glucose inhibits glucagon release at concentrations below the threshold for  $\beta$  cell activation and insulin release.

Glucagon secretion is also stimulated by fatty acids. Palmitate and oleate enhance glucagon secretion and triglyceride accumulation is time- and dose-dependent. Short-term exposure to these fatty acids stimulates the release of this hormone via enhancing  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels and also by relief of the inhibitory paracrine action of the somatostatin secreted from  $\delta$  cells. The principal level of control on glycaemia by the islets of Langerhans depends largely on the coordinated secretion of glucagon and insulin from  $\alpha$  and  $\beta$  cells, respectively. Both cell types respond oppositely to changes in blood glucose concentration:

while hyperglycemic conditions induce insulin secretion from  $\beta$  cells,  $\alpha$  cells release glucagon when glucose levels decrease.



**Figure 1-10. Glucagon synthesis**

Insulin and glucagon have opposite effects on glycaemia as well as on the metabolism of nutrients. Glucagon induces a catabolic effect, mainly by activating liver glycogenolysis and gluconeogenesis, which results in the release of glucose into the bloodstream (Gaisano, Macdonald et al. 2012).

### 1.1.3. Somatostatin hormone

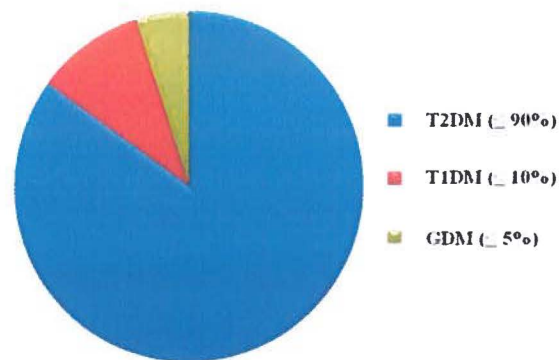
Somatostatin (SS) is a peptide hormone produced by many tissues in the body, principally in the nervous system. There are two different forms of somatostatin molecules: one contains 14 amino acids and the other 28. In the pancreas,  $\delta$  cells produce SS. This hormone inhibits the secretion of insulin and glucagon. SS is also produced in the gastrointestinal tract where it acts locally to reduce the secretion of gastrointestinal hormones, including gastrin and secretin. Moreover, somatostatin from the hypothalamus inhibits the secretion of growth hormone and thyroid stimulating hormone from the pituitary gland (Weckbecker, Lewis et al. 2003).

#### 1.1.4. Pancreatic polypeptide hormone

Pancreatic polypeptide (PP) is a 36-amino acid peptide hormone secreted by both PP and acinar cells of the pancreas. Release of PP by protein-rich meals, fasting, exercise, and acute hypoglycemia occurs in a biphasic manner. The first rapid release occurs as a result of vagal stimulation; the second, more prolonged rise, occurs predominantly in response to hormonal stimulation. PP inhibits gastric emptying of solid food and delays the postprandial rise in plasma glucose and insulin. It also reduces the appetite (Guo and Hebrok 2009).

### 1.2. Types of diabetes mellitus

The most important disease of the pancreatic endocrine system is diabetes mellitus (DM). DM is characterized by abnormally elevated plasma glucose concentrations (hyperglycemia) resulting from either inadequate insulin secretion or abnormal target cell responsiveness. Chronic hyperglycemia and its associated metabolic abnormalities cause the various complications of DM, including damage to blood vessels, eyes, kidneys and the nervous system (Le Roith and Zick 2001; DeFronzo and Tripathy 2009).



**Figure 1-11. Types of diabetes**

There are three main types of DM (Fig. 1-11). Type 1 diabetes mellitus (T1DM) is a condition of insulin deficiency resulting from  $\beta$  cell destruction. T1DM is most commonly an autoimmune disease in which the body fails to recognize the  $\beta$  cells as "self" and destroys them

with antibodies and white blood cells (Buschard 2011). T2DM is known as insulin-resistant diabetes because in most patients, insulin levels in the blood are normal or even elevated initially. Later in the disease process, many T2DM become insulin-deficient and require insulin injection. T2DM is actually a whole family of diseases with a variety of causes (Schofield and Sutherland 2012). The third type of DM is gestational diabetes, a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy.

### *1.2.1. Type 1 diabetes mellitus*

T1DM is a complex disorder whose onset in genetically susceptible individuals is sometimes preceded by a viral infection. Many T1DM diabetics develop their disease in childhood. About 10% of all diabetics have T1DM. Because individuals with T1DM are insulin deficient, the only treatment is insulin injections (Leslie, Williams et al. 2006). Until the arrival of genetic engineering, most pharmaceutical insulin came from swine, cow and sheep pancreas. However, once the gene for human insulin was cloned, biotechnology companies began to manufacture artificial human insulin for therapeutic use (Werner and Chantelau 2011). In addition, scientists have developed techniques for implanting encapsulated  $\beta$  cells in the body, in the hope that individuals with T1DM will no longer need to rely on regular insulin injections (Beck, Angus et al. 2007; Tuch, Keogh et al. 2009; Dufrane and Gianello 2012).

### *1.2.2. Type 2 diabetes mellitus*

T2DM accounts for 90% of all diabetics. The disease is more common in people over the age of 40, but there is growing concern about the increase diagnosis of T2DM in children and adolescents. About 80% of T2DM diabetics are obese (Dixon and O'Brien 2002). A common hallmark of T2DM is insulin resistance, demonstrated by a delayed response to an ingested glucose load. Some T2DM diabetics have both resistance to insulin action and decreased insulin secretion (Schofield and Sutherland 2012). In addition, although T2DM diabetics are hyperglycemic, they often have elevated glucagon levels as well (Shah, Vella et al. 2000). The glucagon then contributes to hyperglycemia by promoting hepatic glycogenolysis and gluconeogenesis (Quesada, Tuduri et al. 2008).

### *1.2.3. Gestational diabetes mellitus*

Gestational diabetes mellitus (GDM) is high blood glucose occurring exclusively in pregnant women. About 7% of pregnant women develop GDM. It usually occurs when the placenta produces large amounts of hormones to help the baby grow. These hormones cause insulin resistance and, unless the woman can produce more insulin to overcome the resistance, the blood glucose will rise. High blood glucose levels may cause the baby to grow large. GDM usually disappears once the baby is born. However, after the birth, 5 to 10 percent of women are found to have DM, usually T2DM (Buchanan, Xiang et al. 2012).

## **1. 3. Type 2 diabetes mellitus**

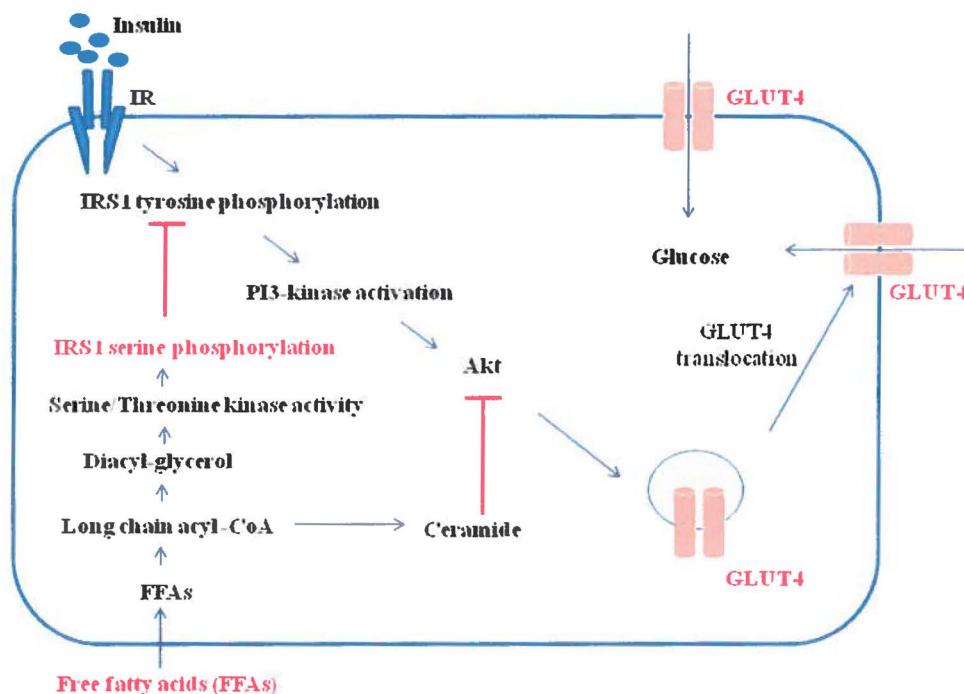
### *1.3.1. Prevalence of type 2 diabetes mellitus*

T2DM affects 90% of people with DM around the world, and is largely the result of excess body weight and physical inactivity. The prevalence of T2DM is very high and increasing. More than 80% of diabetes deaths occur in low- and middle-income countries. Until 2011, with an estimated 366 million people struggling with the disease, 4.6 million deaths were due to it each year, and annual health-care spending was pegged at \$465 billion. Following the prediction of the World Health Organization, by 2030, unless preventive measures are taken, 552 million people worldwide will have diabetes, with the largest increase occurring in developing countries (Grote, Ryals et al. 2013). Over time, diabetes can cause complications such as blindness, heart disease, kidney problems, nerve damage and erectile dysfunction.

### *1.3.2. Causes of type 2 diabetes mellitus*

T2DM is characterized by a slow, progressive loss of  $\beta$  cell function and/or the inability of the body to use insulin (which is called insulin resistance). T2DM typically occurs in individuals over the age of 40 and in individuals who are considered overweight. Normally, insulin activates the glycogen synthesis from glucose by stimulation of the translocation of GLUT4 that transports glucose into the cells and expression of hexokinase, which is essential in the first step of the synthesis. Among the target tissues of insulin, skeletal muscle accounts for

the majority of insulin-stimulated glucose uptake and over 80% of this glucose is stored as glycogen. Glucose transportation into the cells is largely mediated by glucose transporter 4 (GLUT4). In obese patients with T2DM, fatty acids (FA) accumulation causes insulin resistance. Fatty acids compete with glucose for substrate oxidation in muscle. Increased fatty acid oxidation would cause an increase in the long-chain acyl-CoA (LCCoA) and diacylglycerol (DAG), which triggers a serine/threonine kinase cascade. This ultimately induces serine/threonine phosphorylation of critical IRS-1 sites; thereby inhibiting IRS-1 binding and activation of PI 3-kinase, resulting in reduced insulin-stimulated glucose transport (Fig.1-12).



**Figure 1-12. Mechanism of fatty acid-induced insulin resistance**

Fatty acid infusion could alter insulin-regulated GLUT4 traffic between intracellular compartments and the cell membrane. In insulin-resistant skeletal muscle exposed to high fatty acid levels, glucose oxidation and glycogen synthesis were 50% to 60% lower than control. The increase of the fatty acid metabolite also induces a rise in intracellular citrate levels, leading to inhibition of phosphofructokinase and glucose-6-phosphate accumulation. Because glucose-6-

phosphate inhibits hexokinase activity, this would result in intracellular glucose accumulation and decreased glucose uptake (Savage, Petersen et al. 2005).

T2DM is a progressive disease also characterized by the  $\beta$  cell dysfunction. It is generally accepted that  $\beta$  cell failure is caused by the increasing demands associated with insulin resistance; however, recent findings have suggested that impaired  $\beta$  cell function may be the primary genetic defect in patients with T2DM, with affected individuals being less capable of overcoming insulin resistance (Cnop, Welsh et al. 2005; Bonadonna, 2013). The  $\beta$  cell function in many patients is already markedly compromised by up to 50% at the time of diagnosis. Furthermore, recent data suggest that  $\beta$  cell function might have decreased by up to 80% (Abdul-Ghani, 2011). Consequently, the pancreatic islet function declines, and this is thought to be due primarily to a significant increase in  $\beta$  cell apoptosis, ultimately resulting in a deficit in  $\beta$  cell mass. In patients with T2DM, chronically elevated blood glucose levels have a detrimental effect on  $\beta$  cells (glucose toxicity). This results in a perpetual cycle of reduced insulin production, hyperglycemia and  $\beta$  cell damage.

### *1.3.3. Complications of type 2 diabetes mellitus*

Generally, the harmful effects of hyperglycemia are separated into microvascular complications (diabetic retinopathy, nephropathy and neuropathy) and macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke). Diabetic retinopathy is the most common microvascular complication of diabetes. The high concentration of glucose leads to the increase of sorbitol. Osmotic stress from sorbitol accumulation induces the development of this disease. Advanced glycosylated end products (AGEs), promoted by high glucose concentrations, were also associated with the formation of microaneurysms and pericyte loss. Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nephrotic syndrome and diffuse glomerulosclerosis. Although diabetic nephropathy remains the most common cause of renal failure, retinopathy is the leading cause of visual loss in adults and diabetic neuropathy is the leading cause of lower limb amputations. Improvement in the management of these complications has been substantial. The pathological changes to the kidney include increased glomerular basement membrane thickness, microaneurysm formation, mesangial nodule formation, and other changes. The

underlying mechanism of injury may also involve some or all of the same mechanisms as diabetic retinopathy. Diabetic neuropathy is thought to result from diabetic microvascular injury involving small blood vessels that supply nerves. The precise cause of injury to the peripheral nerves from hyperglycemia is related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress.

The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. Moreover, the combination of increased coagulability and impaired fibrinolysis in T2DM further increases the risk of vascular occlusion and cardiovascular events. Peripheral vascular disease (PVD), commonly referred to as peripheral artery disease, refers to the obstruction of large arteries not within the coronary, aortic arch vasculature, or brain. PVD can result from atherosclerosis, inflammatory processes leading to stenosis, an embolism, or thrombus formation. It causes either acute or chronic ischemia. T2DM causes endothelial and smooth muscle cell dysfunction in peripheral arteries. The risk of developing lower extremity peripheral arterial disease is proportional to the severity and duration of diabetes. Up to 70% of non-traumatic amputations are performed on diabetics.



#### 1.4. Therapeutic targets used in treatment of type 2 diabetes

Drugs used to treat type 2 diabetes may (1) stimulate  $\beta$  cell secretion of insulin, (2) make target tissues more responsive to insulin, (3) inhibit hepatic glucose output, or (4) slow the digestion or absorption of carbohydrates in the intestine (Bennett, Wilson et al. 2011).

**Table 1-1. Available medications for the treatment of T2DM**

Agents	Approval	Target	Mode of action
Insulin	1921	Insulin receptor	Stimulates glucose uptake
K <sub>ATP</sub> blocker (Sulphonylurea)	1955	Sulphonylurea receptor 1 (SUR1)	Stimulates insulin secretion
AMP-activated protein kinase activator (Metformin)	1958	AMP-activated protein kinase (AMPK)	Inhibits hepatic gluconeogenesis
$\alpha$ -glucosidase inhibitor	1995	$\alpha$ -glucosidase	Delays carbohydrate digestion
Thiazolidinedione	1997	Peroxisome proliferator-activated receptor $\gamma$ (PPAR $\gamma$ )	Increases fat cell proliferation and fatty acid uptake
Meglitinide	1998	Sulphonylurea receptor	Stimulates insulin secretion
Amylin receptor agonist (Pramlintide)	2005	Brain amylin receptors	Inhibits glucagon release and slows gastric emptying
GLP-1 analogs (Exenatide)	2005	GLP-1 receptor	Stimulates insulin secretion and inhibits glucagon release
DPP-IV inhibitors	2006	Dipeptidyl peptidase IV	Prolongs half-life of GLP-1
Bile-acid sequestrant	2008	Bile acid	Increases cholesterol uptake
Sodium/glucose co- transporter 2 inhibitor	2012	Sodium/glucose co-transporter 2	Inhibits reabsorption of glucose in kidney

#### 1.4.1. $K_{ATP}$ blockers

The  $K_{ATP}$  channels play an integral role in glucose-dependent insulin secretion (GDIS). Closure of these channels, as a result of increased glucose metabolism, leads to the release of insulin. In pancreatic  $\beta$  cells, the  $K_{ATP}$  channel is composed of two subunits: a sulfonylurea receptor (SUR1) and an inward rectifying potassium channel ( $K_{ir6.2}$ ) (Fig. 1-13).

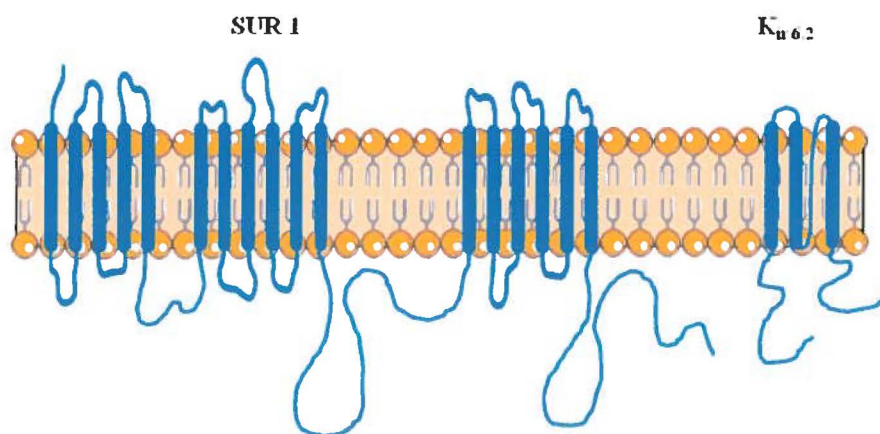
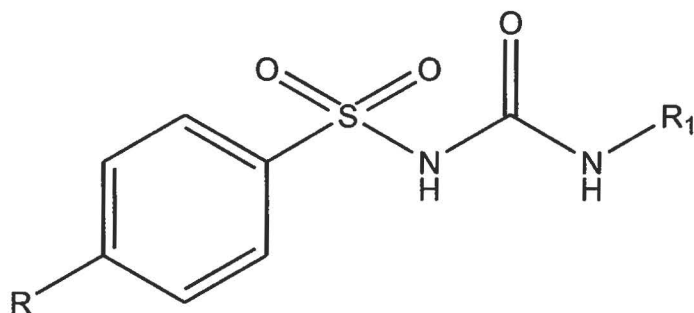


Figure 1-13. Structure of  $K_{ATP}$  in  $\beta$  cell

Sulfonylureas derive from sulfonic acid and urea. Sulfonylureas contain a central S-phenylsulfonylurea structure with a *p*-substituent (R) on the phenyl ring and various groups terminating the urea N end group (R1). Their differences reside in the types of substitutions at both ends of the molecule.

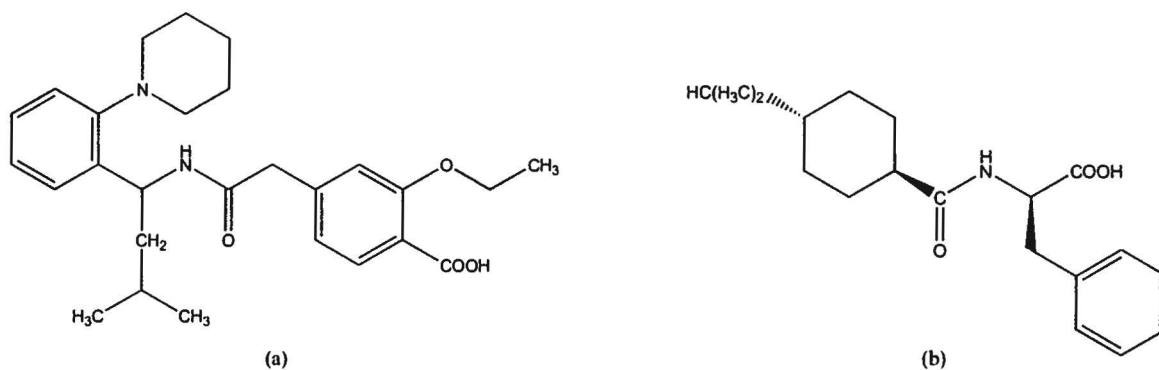
Introduced in 1955, the sulfonylureas belong to the first group of drugs used for the treatment of type 2 diabetes. The first generation of sulfonylureas includes acetohexamide, chlorpropamide, tolazamide and tolbutamide. These medicines bind to the SUR1 subunit of  $K_{ATP}$  and keep the channel closed. This causes an influx of  $Ca^{2+}$  into the cell that results in an increased release of insulin. Because these drugs of first generation are not potent, a high dose is always suggested. In fact, this first generation of sulfonylureas has been largely replaced in

routine use by agents of second generation, which include glyburide (also known as glibenclamide), gliclazide, glipizide, and glimepiride. These drugs of second generation bind more tightly to the sulfonylurea receptor, which makes them more potent, thus requiring a lower dose to produce the secretion of an adequate amount of insulin. Although this new generation of drugs outperforms the first group, side effects such as headache, dizziness, paresthesias, abdominal discomfort, and nausea are observed.



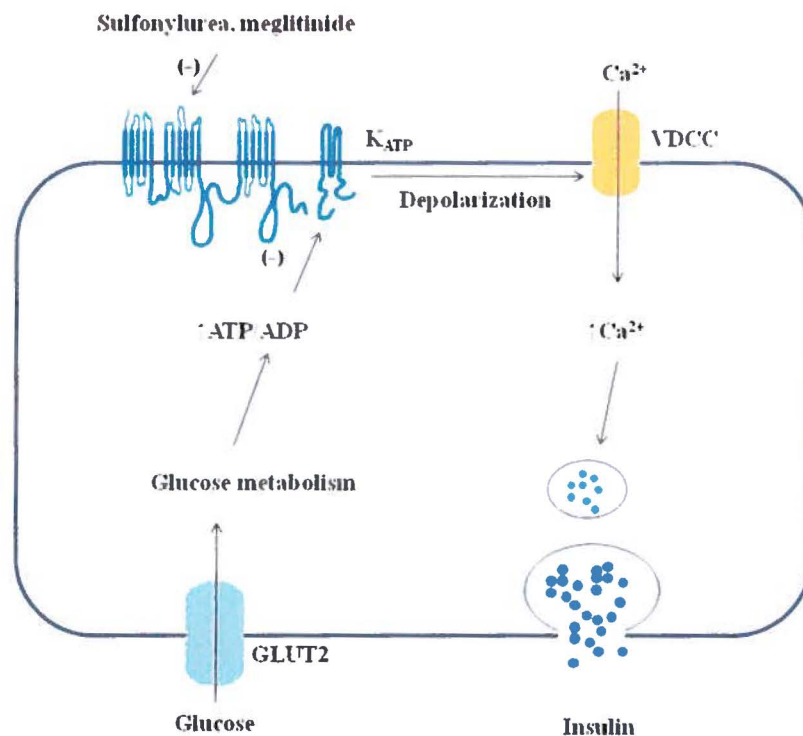
**Figure 1-14. Structure of sulfonylureas (adapted from Margolskee, 2012)**

Furthermore, all sulfonylureas stimulate insulin secretion in a glucose-independent manner, and this explains why hypoglycemia is reported frequently. To solve this hypoglycemic activity observed with all sulfonylureas, meglitinides were developed.



**Figure 1-15. Structure of repaglinide (a) and nateglinide (b)**

This is a novel class of non-sulfonylurea insulin secretagogues, including repaglinide and nateglinide (Fig. 1-15). Repaglinide (Prandin<sup>®</sup> in USA and GlucoNorm<sup>®</sup> in Canada) is a benzoic acid derivative introduced in 1998, and was the first member of the meglitinide class, whereas nateglinide (Starlix<sup>®</sup> in USA and Canada), a derivative of the amino acid D-phenylalanine, was introduced to the market in 2001. Meglitinides also bind to the sulfonylurea receptor (Fig. 1-16) in  $\beta$  cells but to a different part of the receptor than sulfonylureas. The interaction of meglitinides with the receptor is not as “tight” as that of the sulfonylureas. Because they are shorter-acting, they do not cause  $\beta$  cells to push out as much insulin between meals. They are therefore less likely to cause hypoglycemia.

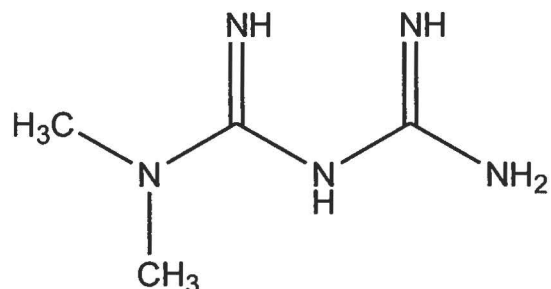


**Figure 1-16. Mechanism of action of sulfonylureas and meglitinides in  $\beta$  cell**

#### 1.4.2. AMP-activated protein kinase (AMPK) activators

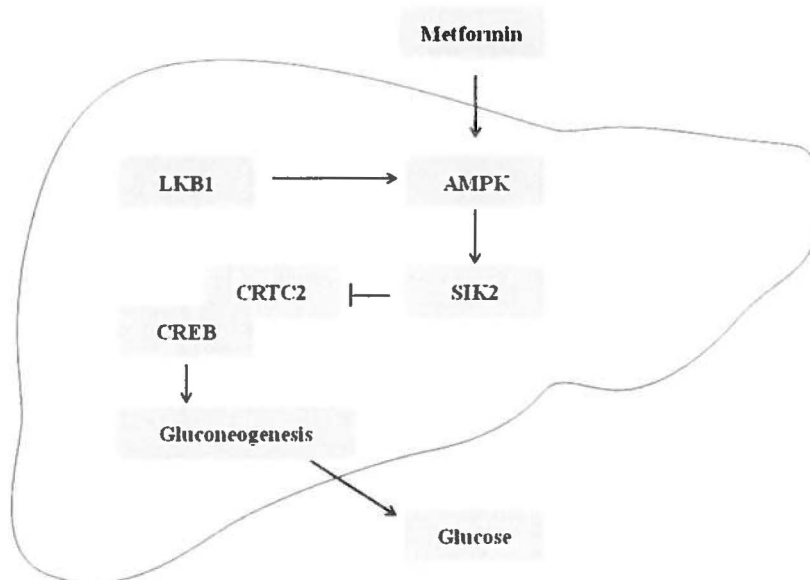
Insulin resistance is a characteristic feature in T2DM and plays a major role in the pathogenesis of the disease. The application of insulin-sensitizers will help the body to reduce

the resistance. Metformin (Glucophage<sup>®</sup>), a synthetic analog of guanidine (Fig. 1-17), was first introduced to the United Kingdom in 1958 (Setter, Iltz et al. 2003). This medicine acts as an insulin-sensitizer.



**Figure 1-17. Structure of metformin**

The antihyperglycemic properties of metformin are mainly attributed to suppressed hepatic glucose production and lowered serum lipid release by activation of the AMPK, through the liver kinase B1 (LKB1), thereby reducing fasting plasma glucose and increasing peripheral tissue insulin sensitivity (Hallows, Mount et al. 2010) (Fig. 1-18).

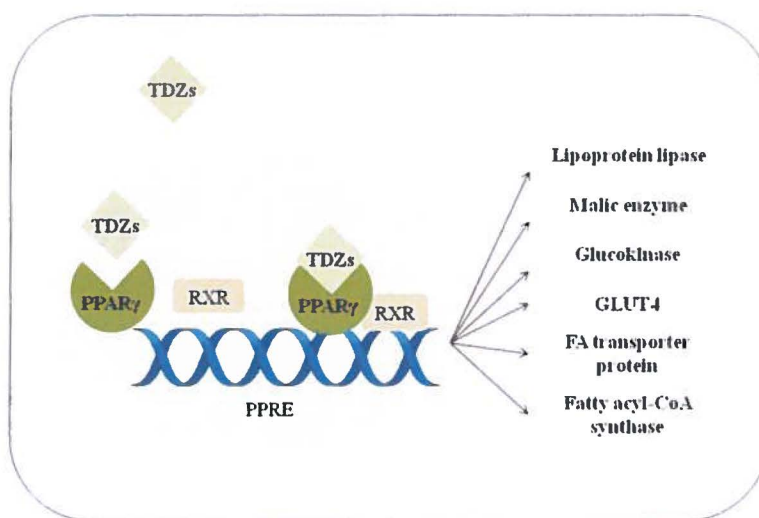


**Figure 1-18. Effect of metformin in liver**

Metformin can be used alone or in combination with certain other types of blood glucose lowering medication (including TDZ and DPP-IV inhibitor drugs; described below) (Setter, Iltz et al. 2003). Unlike sulfonylurea treatment, metformin causes neither significant weight gain nor hypoglycemia and was reported to reduce the risk of developing macrovascular complications. However, because it favors the accumulation of lactic acid in tissues, metformin can cause a rare but serious condition called lactic acidosis, as well as other side effects such as nausea, abdominal cramping, and diarrhea (Blonde 2009).

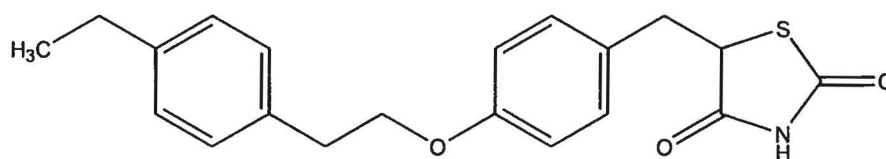
#### 1.4.3. Peroxisome proliferator-activated receptor (PPAR) gamma agonist

The fatty acids are absorbed from the blood into fat cells, muscle cells and liver cells. In these cells, under stimulation by insulin, fatty acids are made into fat molecules and stored as fat droplets. Fat cells also take up glucose and amino acids, and convert those into fat molecules. The conversion of carbohydrates or proteins into fat is 10 times less efficient than simply storing fat in a fat cell. Given a choice, a fat cell will capture the fat and store it rather than the carbohydrates because fat is much easier to store. In obese T2DM diabetics, the presence of too high levels of fatty acids inhibits the glucose absorption, leading to hyperglycemia.

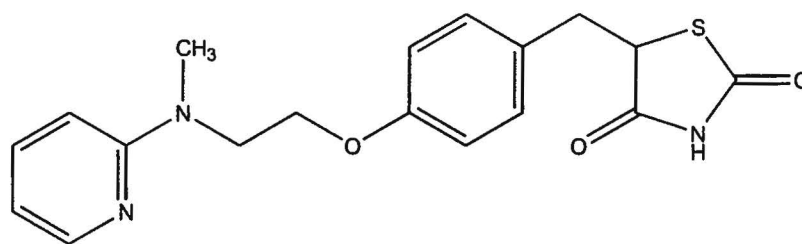


**Figure 1-19. Effect of TDZs in fat and muscle cells**

The increase of fat cell mass will help to uptake more fatty acids. As a result, thiazolidinediones (TDZs), the most potent triggers of adipose differentiation, were developed. The TDZs target PPAR- $\gamma$ , a member of the nuclear receptor superfamily of transcription factors. Two isoforms of the protein exist. While PPAR $\gamma$ 1 is found in many tissues, including skeletal muscle and liver, PPAR $\gamma$ 2 is almost exclusively expressed in adipose tissue and this receptor plays a key role in the induction of adipose differentiation. In the cell, PPAR $\gamma$  forms a heterodimer with the retinoid X receptor (RXR). TDZs activate the binding of PPAR $\gamma$ -RXR complex to PPAR $\gamma$  response elements (PPRE) in target genes (Fig.1-19). This action alters the transcription of a number of genes involved in lipid metabolism and energy balance, including those encoding for lipoprotein lipase, fatty acid transporter protein, adipocyte fatty acid binding protein, fatty acyl-CoA synthase, malic enzyme, glucokinase and the GLUT4 glucose transporter (Hauner 2002).



(a)



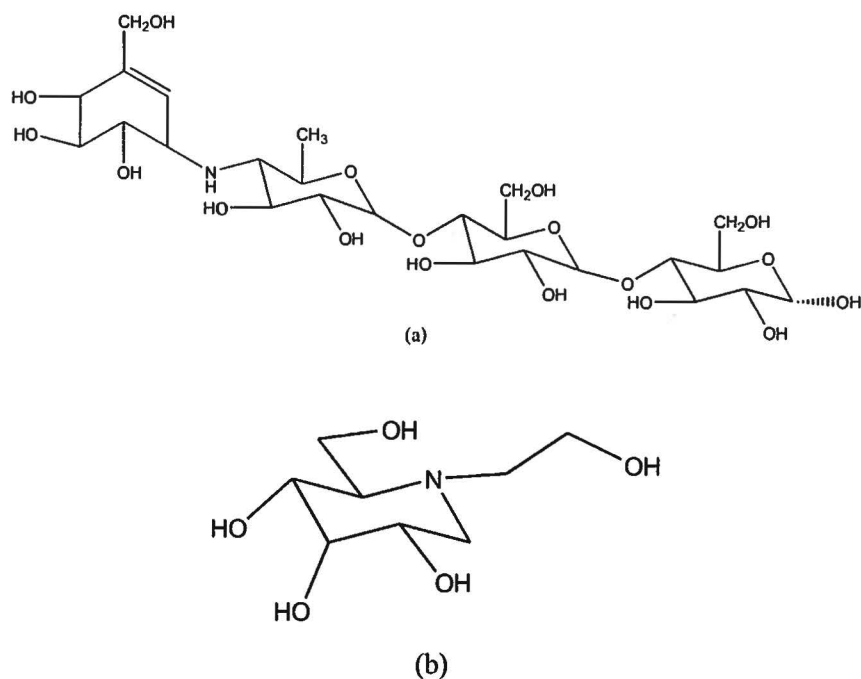
(b)

**Figure 1-20. Structure of pioglitazone (a) and rosiglitazone (b)**

TDZs include pioglitazone and rosiglitazone (Fig. 1-20). The affinity of individual TDZs for PPAR $\gamma$  is diverse. Rosiglitazone binds to the receptor with the greatest affinity, ten-fold greater than pioglitazone. This may explain apparent differences in the safety and efficacy profiles of these agents. Among the side effects of TDZs are edema, decreased hematocrit and hemoglobin, and elevated alanine aminotransferase activity (Sood, Collieran et al. 2000). In addition, treatment with TDZs results in a moderate weight gain because of increased fat cell mass (Hauner 2002).

#### 1.4.4. Alpha-glucosidase inhibitors

Alpha-glucosidase is an enzyme that acts upon 1,4-alpha bonds to break down starch and disaccharides to glucose. Inhibition of the activity of this enzyme results in delayed digestion of carbohydrates in the stomach.



**Figure 1-21. Structure of the the alpha-glucosidase inhibitors acarbose (a) and miglitol (b)**



This process slows down the absorption of glucose from foods, giving the pancreas more time after meals to secrete enough insulin to lower blood glucose levels. Because of that,  $\alpha$ -glucosidase inhibitors (Fig. 1-21) including acarbose (a nitrogen-containing pseudo-tetrasaccharide) and miglitol (pseudo-carbohydrate) were approved in 1991 as oral anti-hyperglycemic compounds. Although these compounds do not increase the risk of weight gain or hypoglycemia, their anti-hyperglycemic effectiveness is 50% lower than either sulfonylureas or metformin. Hence, these medications are generally suggested to prevent the development of diabetes in prediabetics. Significant side effects of the  $\alpha$ -glucosidase inhibitors are related to gastrointestinal disturbances occurring in approximately 25-30% of diabetic patients.

#### 1.4.5. GLP-1 receptor agonists

GLP-1 is a 37-amino acid peptide hormone produced in the intestinal epithelial endocrine L cells after eating. Two circulating biologically active species exist *i.e.* GLP-1(7-37) and GLP-1(7-36)-NH<sub>2</sub>. Both forms are able to stimulate GDIS and to inhibit glucagon secretion.

They also slow gastric emptying and reduce food intake, thereby contributing to limit postprandial glucose. Because of these multiple effects, the GLP-1 receptor system has become an attractive target for T2DM therapies. However, the GLP-1 peptides are rapidly metabolized and inactivated by the enzyme DPP-IV, which limits their clinical relevance for the treatment of T2DM.

Two main classes of GLP-1-mediated therapies are now in use: the GLP-1R agonists and the DPP-IV inhibitors, which reduce the degradation of GLP-1. The first GLP-1R agonist to be approved was exenatide. Exenatide is a synthetic form of exendin-4, a 39-amino acid peptide isolated from the salivary gland of the Gila monster (*Heloderma suspectum*). It shares 53% sequence identity with GLP-1(7-36). For exenatide, substitution of alanine at position 2 with glycine prevents the degradation by DPP-IV. This results in an extended half-life of 6-7h. Like GLP-1, exenatide reduces food intake, slows gastric emptying, suppresses glucagon and enhances GDIS. Exenatide even improves  $\beta$  cell proliferation (Fig. 1-22).

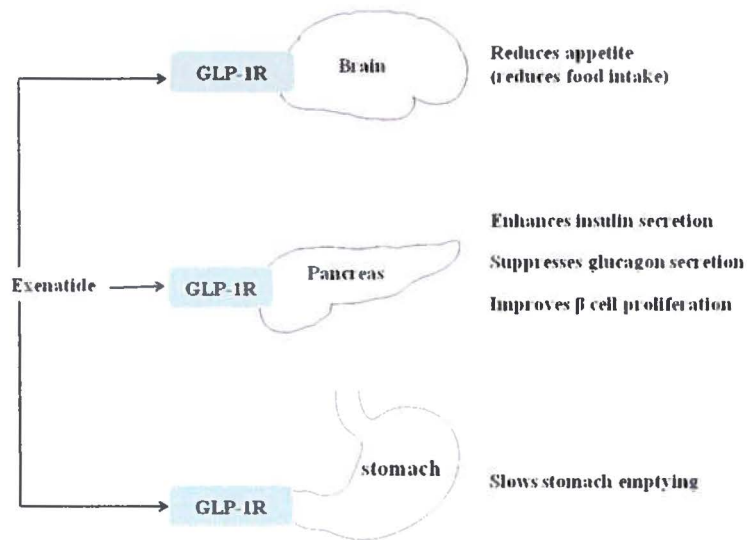


Figure 1-22. Effects of exenatide in the body

Although the plasma half-life of exenatide is a significant improvement over native GLP-1, the duration of exposure is still limited. Thus, multiple strategies were employed to extend the half-life of GLP-1R agonists (Fig. 1-23).

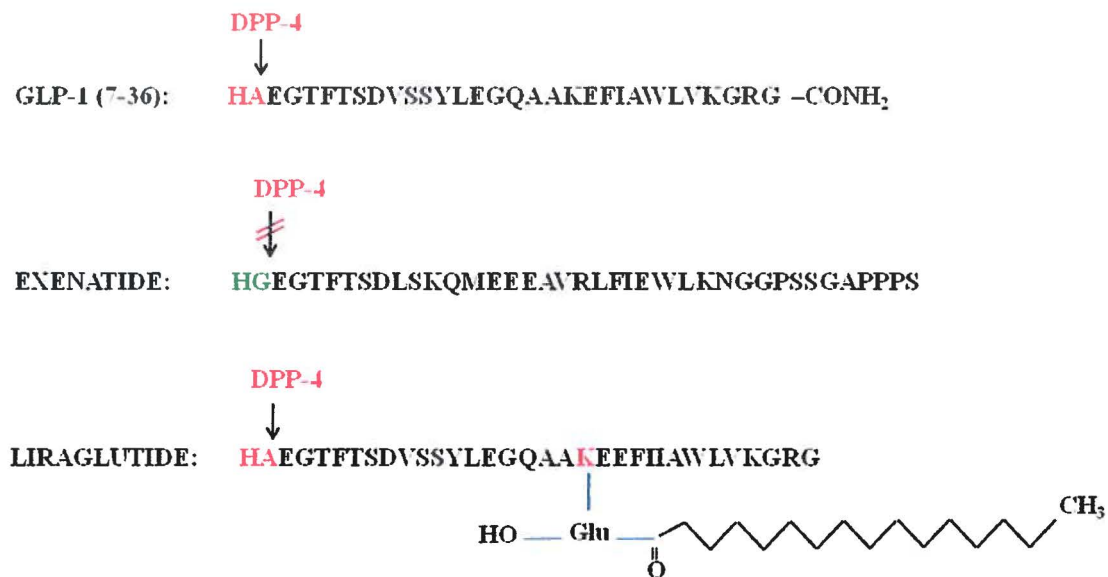
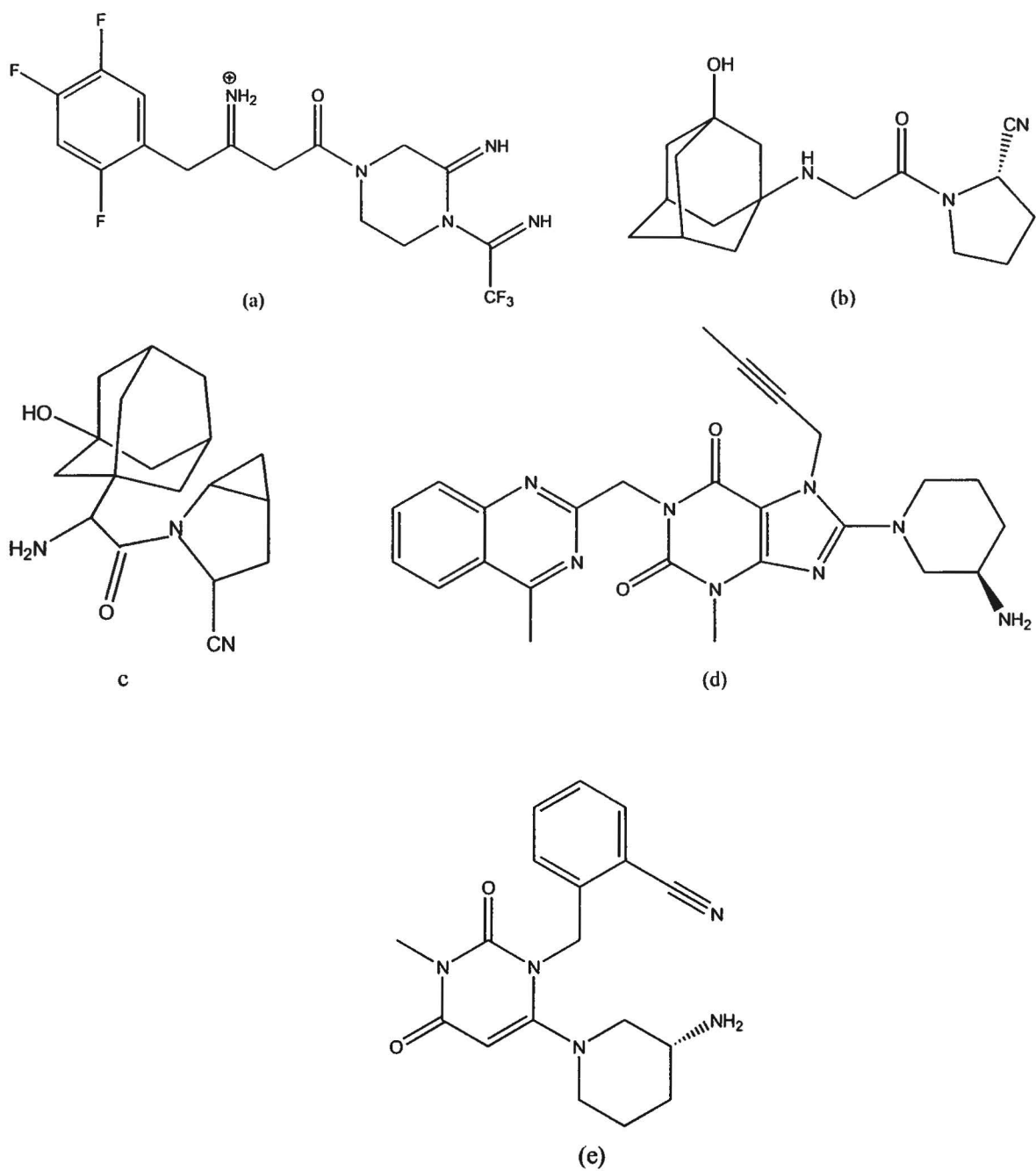


Figure 1-23. Structure of GLP-1(7-36)-CONH<sub>2</sub>, exenatide, and liraglutide

The first product, liraglutide (Victoza<sup>®</sup> in USA and Canada), is currently available in the United States, Canada and Europe. Liraglutide is a DPP-IV-resistant GLP-1 analog that achieves slowed absorption and increased half-life through the substitution of arginine for lysine at position 34 and the addition of a C16 fatty acid chain at position 26 allowing for reversible binding to albumin. The half-life of liraglutide is 11-15h resulting in continuous exposure with once-daily administration. Liraglutide significantly improves glycemic control with a low risk of hypoglycemia and significant weight loss compared with standard anti-diabetic therapies (Raun, von Voss et al. 2007). The second one (Bydureon<sup>®</sup>) is an extended-release version of exenatide that is also available in the United States and Europe. This extended-release formulation is administered once weekly. It is based on biodegradable polymeric microspheres, composed of exenatide in a poly-lactide-co-glycolide (PLG) polymeric matrix. This extended-release formulation, injected subcutaneously, slowly releases native exenatide into the subcutaneous space. Once absorbed, the general pharmacokinetic properties of exenatide are unchanged compared to exenatide.

#### *1.4.6. DPP-IV inhibitors*

DPP-IV inhibitors are approved in the United States and Canada for the treatment of type 2 diabetes. DPP-IV inhibition leads to elevated plasma concentrations of GLP-1, which in turn enhances GDIS and suppresses inappropriately elevated glucagon secretion. DPP-IV inhibitors are associated with a low risk of hypoglycemia and are weight neutral. They were reported to improve  $\beta$  cell function. Although DPP-IV inhibitors differ in terms of their chemistry (Fig. 1-24), they are all small molecules that are orally available.



**Figure 1-24. Structure of DPP-IV inhibitors: sitagliptin (a), vildagliptin (b), saxagliptin (c), linagliptin (d), and alogliptin (e)**

There are some differences between them in terms of their absorption, distribution, metabolism and elimination, as well as in their potency and duration of action, but their efficacy, both for inhibiting plasma DPP-IV activity and as anti-diabetic agents, appears to be similar. The first DPP-IV inhibitor, sitagliptin (Januvia<sup>®</sup>), was approved in 2006 by the Food and Drug Administration (FDA). Lately, vildagliptin, saxagliptin, alogliptin and linagliptin were approved either in the U.S. or in Europe. They were approved both as monotherapy as well as in combination with metformin, sulfonylurea, or thiazolidinedione (Triplitt, Cersosimo et al. 2010; Dicker 2011).

DPP-IV inhibitors have higher impact on portal vein GLP-1 concentrations than on concentrations in the peripheral circulation. Their use is linked though to a serious side effect as they may cause inflammation of the pancreas (pancreatitis) (Deacon 2012).

#### *1.4.7. Amylin receptor agonist*

Amylin is a peptide that is co-secreted with insulin from the pancreatic  $\beta$  cells. This hormone inhibits postprandial glucagon secretion and delays gastric emptying. Amylin receptors are located in an area of the brain that controls the vagus nerve, which in turn controls the pancreas and stomach. In T1DM and some T2DM diabetic individuals, amylin is deficient. Amylin replacement may therefore improve glycemic control in diabetes mellitus. However, human amylin exhibits physicochemical properties predisposing the peptide hormone to aggregate and form amyloid fibers, which makes it unsuitable for pharmacological use. A stable analog, pramlintide, with actions similar to that of the native peptide has therefore been developed (Table 1-2). Pramlintide is the first of a new class of amylinomimetic compounds. It was approved in March 2005 as a subcutaneous injected drug for the adjunctive treatment of patients who have type 1 or 2 diabetes mellitus. Clinical trials showed that this drug suppresses postmeal glucagon secretion, slows gastric emptying, reduces postprandial glucose levels, and improves glycemic control while managing weight loss. Pramlintide was also shown to decrease HbA1c, serum fructosamine, and total cholesterol levels.

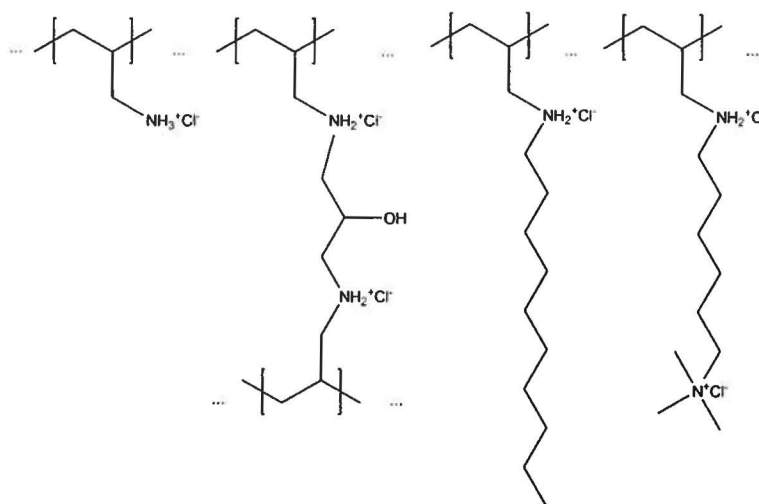
**Table 1-2. Human and rat amylin and the analog pramlintide**

Human amylin	KCNTATCATQRLANFLVHSSNFGAILSSTNVGSNTY-NH <sub>2</sub>
Rat amylin	KCNTATCATQRLANFLV'RSSNNLGPVLPPTNVGSNTY-NH <sub>2</sub>
Pramlintide	KCNTATCATQRLANFLVHSSNFGPILPPTNVGSNTY-NH <sub>2</sub>

It was associated with an increased risk of insulin-induced severe hypoglycemia. Also, other adverse events include nausea, anorexia, fatigue, and vomiting.

*1.4.8. Bile-acid sequestrant*

Bile acids promote bile formation and facilitate lipid absorption. Disturbance of the bile acid metabolism was observed in T2DM.



**Figure 1-25. Structure of colesevelam**

Bile acid sequestrants (BASs), which interrupt the enterohepatic circulation of bile acids and effectively reduce plasma cholesterol, support a link between bile acid and glucose metabolism. In lipid-lowering trials, bile acid sequestrants such as colesevelam (Fig. 1-25),

colestyramine (cholestyramine) and colestilan (colestimide), have also been shown to lower plasma glucose and glycosylated hemoglobin levels, suggesting the utility of these agents as a potential therapy for type 2 diabetes.

The bile acid sequestrants are a group of resins used to bind certain components of bile in the gastrointestinal tract. They disrupt the enterohepatic circulation of bile acids by combining with bile constituents and preventing their reabsorption from the gut. BASs were developed as lipid-lowering agents for the treatment of hypercholesterolemia. These agents, including colestyramine, colesevelam, colestilan, colestimide, and colestipol, significantly reduce elevated low-density lipoprotein (LDL) cholesterol levels and can be used as monotherapy or in combination with statins, fibrates, and/or cholesterol absorption inhibitors. Colestimide is approved in Japan, whereas colesevelam is approved in the U.S. and Canada for improving glycemic control in adults with type 2 diabetes. Furthermore, BASs may affect secretion of incretin hormones, particularly GLP-1 and glucose-dependent insulinotropic polypeptide (GIP).

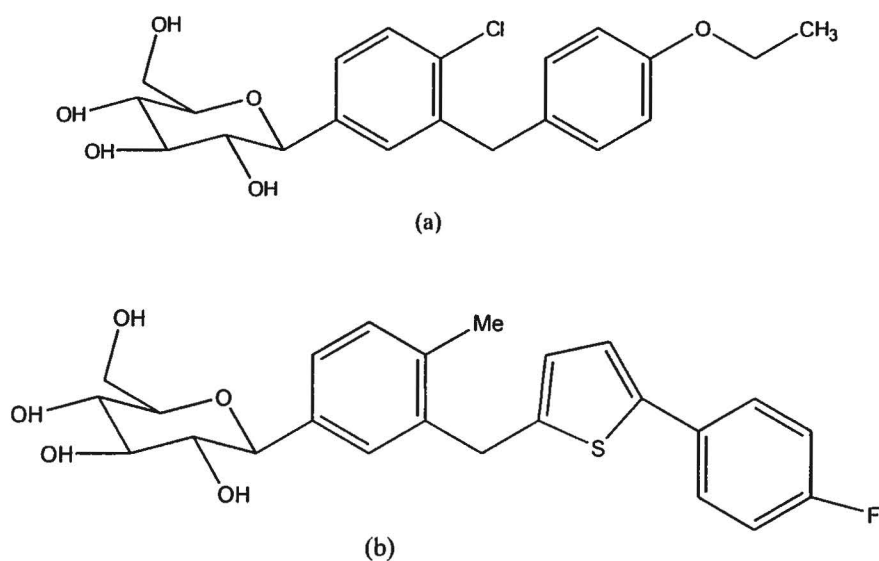
Potential mechanisms include effects on the farnesoid X receptor (the bile acid receptor) and TGR5 (a G protein-coupled receptor) within the intestine, as well as effects on the farnesoid X receptor within the liver, which may ultimately reduce endogenous glucose production (Handelsman 2011).

#### *1.4.9. Sodium/glucose co-transporter 2 (SGLT2) inhibitor*

Glucose enters the cells via two different types of membrane-associated carrier proteins, the glucose transporters (GLUTs) and the Na<sup>+</sup>-coupled glucose co-transporters (SGLTs). SGLTs couple the transport of glucose against a concentration gradient with the transport of Na<sup>+</sup>. Two SGLT isoforms, SGLT1 and SGLT2, have been identified. SGLT1 is located in the small intestine, the kidney and the heart. This isoform is a high-affinity, but low-capacity transporter and therefore accounts for only a small fraction of renal glucose reabsorption. In contrast, SGLT2 is a low-affinity, but high-capacity transporter. SGLT is located exclusively at the apical domain of the epithelial cells in the early proximal convoluted tubule.

Ninety per cent of filtered glucose is reabsorbed by this isoform (Idris and Donnelly 2009). Inhibition of SGLT2 leads to a reduction in blood glucose levels. T2DM diabetics also

express a significantly higher number of SGLT2 than healthy individuals. Therefore, SGLT2 inhibitors have potential use in the treatment of T2DM. Among inhibitors of SGLT2, phlorizin was first isolated in 1835 from the root bark of the apple tree. Although studies revealed that phlorizin administered orally to mice blunted the increase in blood glucose level, it was not further developed as a possible anti-diabetes therapy due to its non-selective capacity. Phlorizin also acts on SGLT1, which is mainly expressed in the gastrointestinal tract, to impair intestinal transport of glucose and results in gastrointestinal side effects, such as diarrhea.



**Figure 1-26. Structure of dapagliflozin (a) and canagliflozin (b)**

There are several SGLT2 inhibitors now in various stages of clinical development. Among them, dapagliflozin (Fig.1-26) (Forxiga<sup>®</sup>) has been approved in Europe in 2012. In the rat, dapagliflozin administration resulted in decreasing of fasting and postprandial glucose levels. In diabetic fatty rats, a doubling of urine glucose levels was noted, accompanied with the glycosuria. The presence of a C-aryl glucoside linkage of dapagliflozin confers resistance to degradation in the gastrointestinal tract by  $\beta$ -glucosidase enzymes. Another inhibitor, canagliflozin (Fig.1-26) was approved first in Europe in 2012 and afterwards in USA in March 2013. Canagliflozin decreased plasma glucose levels independent of food intake in high-fat,



hyperglycemic diet mice. In addition, ipragliflozin (ASP-1941), tofogliflozin, and empagliflozin (BI-10773) are now in Phase III clinical trials while remogliflozin etabonate is in phase IIb trials.

## 1.5. Other potential therapeutic targets for treatment of type 2 diabetes

For decades, ion channels have been successful targets for intervention of therapeutic agents. As an example, the modulators of a particular potassium channel,  $K_{ATP}$ , have been the mainstay of oral treatment for T2DM. In recent years, knowledge related to ion channel structures and the technologies for ion channel functional screening have significantly improved, which provides exciting opportunities for finding novel ion channel targets.

### *1.5.1. Ion channels as targets for the treatment of type 2 diabetes mellitus*

In  $\beta$  cells, the release of insulin can be associated to the activation of 3 types of potassium channels:  $K_{ATP}$ ,  $K_{Ca^{2+}}$ , and  $K_v$ . A  $K_{ATP}$  channel, in general, is a hetero-octameric complex of two different types of protein subunits: an inwardly rectifying  $K^+$  channel ( $K_{ir6.2}$ ), and a sulfonylurea receptor (SUR1).

$K_{ir6.x}$  belongs to the family of inwardly rectifying  $K^+$  ( $K_{ir}$ ) channels and assembles as a tetramer to form the channel pore. Binding of ATP to the intracellular domains of this subunit produces channel inhibition, causing membrane depolarization, leading to insulin exocytosis. SUR is a member of the ABC transporter family, with 17 transmembrane helices (TMs), arranged as one group of 5 TMs, and two repeats each of 6 TMs. More than one isoform exist for both  $K_{ir6.x}$  ( $K_{ir6.1}$ ,  $K_{ir6.2}$ ) and SUR (SUR1, SUR2A, SUR2B). In most tissues,  $K_{ir6.2}$  serves as the pore-forming subunit, but it associates with different SUR subunits. For example, it associates with SUR1 in the pancreas and brain; SUR2A in heart and skeletal muscle; and SUR2B in brain and smooth muscle. In vascular smooth muscle, the  $K_{ATP}$  channel is composed of  $K_{ir6.1}$  in association with SUR2B. Inhibitors of  $K_{ATP}$  channel activity fall into two groups: those that interact with  $K_{ir6.2}$  and those that interact with SUR. All drugs that block  $K_{ATP}$  channels stimulate insulin secretion, but only those that interact with the SUR subunit are used therapeutically to treat T2DM. In  $\beta$  cell type, binding of sulfonylureas to the cytoplasmic domains of SUR1

ultimately results in closure of the pore formed by  $K_{ir6.2}$ . The  $NH_2$ -terminus of  $K_{ir6.2}$  couples to the  $COOH$ -terminus of SUR1. Studies of recombinant  $K_{ATP}$  channels suggest that the sulfonylurea moiety interacts with residues in the TM 15-16 linker of SUR1, whereas the other (benzamido derivatives) may interact with the TM 5-6 linker in the  $NH_2$ -terminal part of the protein.  $K_{Ca^{2+}}$  channels are activated in response to a rise in intracellular  $Ca^{2+}$  concentrations. Although  $K_{Ca^{2+}}$  channels regulate the relationships between membrane potential and intracellular  $Ca^{2+}$  influx, which is the major determinant in exocytosis, the role of  $K_{Ca^{2+}}$  channels in GDIS remains controversial. However, at low concentrations, TEA and charybdotoxin, which inhibit  $K_{Ca^{2+}}$  channels, have no effect either on the membrane potential or insulin release (Hahn, 2010).

$K_v$  channels are activated in response to a change in membrane potential. These channels act to counter depolarizing influences on the cell and are important in repolarizing action potentials. Blocking of these channels prolong the duration of action potentials that maintain the plasma membrane in a depolarized state, and sustain the calcium influx through opened calcium channels, and enhance insulin secretion in a glucose-dependent manner. For these reasons,  $K_v$  channels have been considered as a potential target for the development of a novel therapy for T2DM, which might offer advantages over the currently used sulfonylureas. Increasing evidence has demonstrated that modulation of some of the voltage-gated  $K_v$  channels may yield anti-diabetic actions.  $K_v$  channels belong to the six-transmembrane family of  $K^+$  channels consisting of  $K_{v1}$  to  $K_{v11}$  subfamilies. They regulate cell membrane potential by controlling the rate of  $K^+$  exit from the cell. Inhibition of the  $K_v$  current prolongs the action potentials, sustains the opening of voltage-dependent  $Ca^{2+}$  channels, and thereby enhances GDIS. Such a therapeutic strategy would be expected to pose a lower risk for hypoglycemic events in comparison with sulfonylureas and  $K_{ATP}$  channel blockers. Thus, the  $K_v$  channel has attracted much attention as a potential therapeutic target for treatment of T2DM. Dominant-negative knockdown and pharmacological inhibition suggest that  $K_{v2}$  channels, in the  $\beta$  cells, account for 60% of delayed rectifier currents, whereas  $K_{v1}$  channels account for 25%. Moreover, inhibition of these  $K_v$  channels specifically enhances GDIS. It has been found that  $K_{v2.1}$  forms the predominant component of repolarizing currents in mouse and human  $\beta$  cells. Compared with controls,  $K_{v2.1}$  null mice have reduced fasting blood glucose levels and elevated serum insulin levels. In isolated islets, glucose tolerance is improved and insulin secretion is enhanced.

Dominant-negative “knockout” of  $K_{v2.1}$  decreases  $K^+$  current by 60-70% and enhances GDIS from rat islets. In isolated  $K_{v2.1}$  knockout  $\beta$  cells,  $K_v$  currents were decreased by 83% (MacDonald, Ha et al. 2001; Tamarina, Kuznetsov et al. 2005; Jacobson, Kuznetsov et al. 2007; Yoshida, Nakata et al. 2010). These data supports a role for specific enhancement of GDIS by  $K_{v2.1}$  blockers, which may provide a new opportunity for the treatment of T2DM.  $K_{v1.7}$  channels would also have this potential. In rodent islets,  $K_{v1.7}$  channel is expressed at high levels. Blocking of  $K_{v1.7}$  channel enhanced insulin secretion. Consequently, it is useful for the design of new pharmacological agents to control glucose homeostasis (Finol-Urdaneta, Remedi et al. 2012).

Insulin resistance is defined as inhibition of insulin stimulation of several metabolic pathways including glucose transport, glycogen synthesis and anti-lipolysis. Elevated plasma FFA levels can account for a large part of insulin resistance in obese patients with type 2 diabetes. Among potential ion channels,  $K_{v1.3}$  is expressed in a number of insulin sensitive tissues, including fat and skeletal muscle. Gene inactivation or pharmacological inhibition of  $K_{v1.3}$  increases peripheral glucose homeostasis and insulin sensitivity by stimulating glucose uptake in adipose tissue and skeletal muscle. Regarding the mechanism of this indication, it is thought that inhibition of  $K_{v1.3}$  facilitates the translocation of the glucose transporter GLUT4 to the plasma membrane, which increases the amount of GLUT4 at the plasma membrane. It is well known that GLUT4 is the major insulin responsive transporter that is predominantly restricted to adipose and skeletal muscle tissues. In addition, studies have confirmed that mutations in the  $K_{v1.3}$  gene exist in humans and are associated to alterations of glucose homeostasis. Therefore,  $K_{v1.3}$  is a promising target for the development of drugs for the improvement of insulin resistance in T2DM (Choi and Hahn 2010).

$Ca^{2+}$  channels play crucial roles in stimulus-secretion coupling in pancreatic  $\beta$  cells. In the voltage-gated L-type  $Ca^{2+}$  channel,  $\beta_3$  subunit is believed to play a key role in the assembly/expression of the channel complex and modulate  $Ca^{2+}$  currents through  $\alpha_1$  subunits. It has been well documented that inhibition of L-type  $Ca^{2+}$  channels reduces insulin secretion. But, surprisingly, knockout of L-type  $Ca^{2+}$  channel  $\beta_3$  subunit showed an increase of GDIS. The  $\beta_3$  subunit knockout mice appeared to have a more efficient glucose homeostasis compared to wild-type mice. It is thought that the removal of  $Ca^{2+}$  channel  $\beta_3$  subunit enhances  $Ca^{2+}$  oscillation frequency via a modulation of InsP3-induced  $Ca^{2+}$  release. It is known that an oscillatory

increase of free  $[Ca^{2+}]_i$  in pancreatic  $\beta$  cells is a key feature in GDIS. Since the increase in insulin release was manifested only at high glucose concentrations, blocking the  $\beta_3$  subunit in the  $\beta$  cells might constitute the basis for a novel diabetes therapy. Besides, the voltage-gated N-type  $Ca^{2+}$  channel is localized in the plasma membrane of  $\beta$  and  $\alpha$  cells in the pancreatic islets. Electrophysiological and pharmacological studies have shown that glucagon secretion from  $\alpha$  cells in the islets is a  $Ca^{2+}$ -dependent process, and a N-type  $Ca^{2+}$  channel blocker partially inhibits the  $Ca^{2+}$  influx in  $\alpha$  cells. Glucagon activates the glycogenolytic and gluconeogenic pathways, thereby increasing hepatic glucose production. The N-type  $Ca^{2+}$  knockout mice showed lower plasma glucagon and a higher glucose clearance rate in glucose tolerance tests. These results suggested that N-type  $Ca^{2+}$  channels play a role in glucagon release. Thus, N-type  $Ca^{2+}$  channel blockers might be candidate anti-diabetic agents that could treat type II diabetic patients via decrease of glucose production. In addition to these ion channels, others, which are involved in the insulin resistance, can be applied for T2DM.

### **1.5.2. GPCRs as targets for the treatment of type 2 diabetes mellitus**

Several G protein-coupled receptors (GPCRs) expressed in islet  $\beta$  cells are known to be involved in the regulation of islet functions, and therefore are potential therapeutic targets. This is evident from the success of GLP-1 mimetics, which promote activation of the GLP-1 receptor to stimulate insulin secretion and inhibit glucagon secretion, and also have the potential to increase  $\beta$  cell mass. Other islet  $\beta$  cell GPCRs that are involved in the regulation of islet functions include lipid GPCRs and the GIPR. FFAs have been shown to stimulate insulin secretion. The insulinotropic action of FFAs through GPCR was first proposed for GPR40, which is highly expressed in mouse, rat and human pancreatic  $\beta$  cells (Kebede, Alquier et al. 2009). The GPR40-deficient mice have impaired acute insulin secretory response to FFA, which enforces the importance of GPR40 in this respect. GPR40 has been suggested to mediate the majority of the FFA-potentiated GDIS (Steneberg, Rubins et al. 2005). GPR40 is coupled to  $G_{aq}$  with a subsequent increase in cytosolic  $Ca^{2+}$  concentration (Burant 2013), although a mechanism through activation of PLC has also been proposed. Long-term exposure of islets to FFA results in impaired GDIS through a lipotoxic action (Fujiwara, Maekawa et al. 2005; Graciano, Valle et al. 2013). This effect might be of importance for the long-term deterioration

of  $\beta$  cell functions in T2DM. The mechanism of the lipotoxic effects of FFAs in  $\beta$  cells has been shown to be complex and to involve both metabolic and genetic perturbations (Haber, Procopio et al. 2006). Interestingly, the GPR40-deficient mice were protected against the lipotoxic effects on glucose homeostasis caused by high-fat diet. This suggests that the effect by FFA, besides the stimulation of insulin secretion, may also be mediated by GPR40. This conclusion is corroborated by results in transgenic mice with  $\beta$  cell-specific overexpression of GPR40 (Steneberg, Rubins et al. 2005). These mice developed overt diabetes due to severely impaired insulin secretion, which is seen in association with perturbed expression of  $\beta$  cell genes in analogy with changes seen during lipotoxicity. Therefore, GPR40 is important for both FFA-induced potentiation of GDIS and the deleterious effects of fatty acids. In addition to the insulin secretion, FFAs also stimulate glucagon secretion, as demonstrated in isolated rat and mouse islets (Edfalk, Steneberg et al. 2008; Wang, Zhao et al. 2011). It remains to be established whether GPR40 mediates this effect. A study opened up this possibility because it was demonstrated that GPR40 receptors are identified in glucagon-producing clonal  $\alpha$  cells and in mouse  $\alpha$  cells (Flodgren, Olde et al. 2007). Hence, efforts have been made to produce small molecule GPR40 receptor agonists and antagonists to investigate their potential as drugs for type 2 diabetes (Bharate, Nemmani et al. 2009). In clonal  $\beta$  cells, insulin secretion could be potentiated by addition of a GPR40 agonist, suggesting that acute activation of GPR40 may be useful to stimulate insulin secretion (Briscoe et al., 2006). However, because the mouse model with transgenic overexpression of GPR40 exhibited impaired  $\beta$  cell functions and type 2 diabetes (Steneberg, Rubins et al., 2005), chronic activation of the receptor may cause deleterious effects. Therefore, a GPR40 antagonist may be a more efficient concept because patients with type 2 diabetes usually have elevated circulating FFAs (Bharate, Nemmani et al. 2009). Further studies are needed to evaluate whether GPR40 agonists or antagonists are suitable for anti-diabetic treatment.

GPR119 is highly expressed in pancreatic  $\beta$  cells and enteroendocrine cells. It directly promotes GDIS and indirectly increases GLP-1 levels thereby representing a promising target for the treatment of T2DM (Ha, Kim et al. 2013). Its agonists, HD0471042, a 3-isopropyl-1,2,4-oxadiazol-piperidine derivative, *in vitro*, significantly elevated insulin (Ha, Kim et al. 2013) and GLP-1 release (Zhang, Feng et al. 2013). Moreover, GPR119 agonists also increased

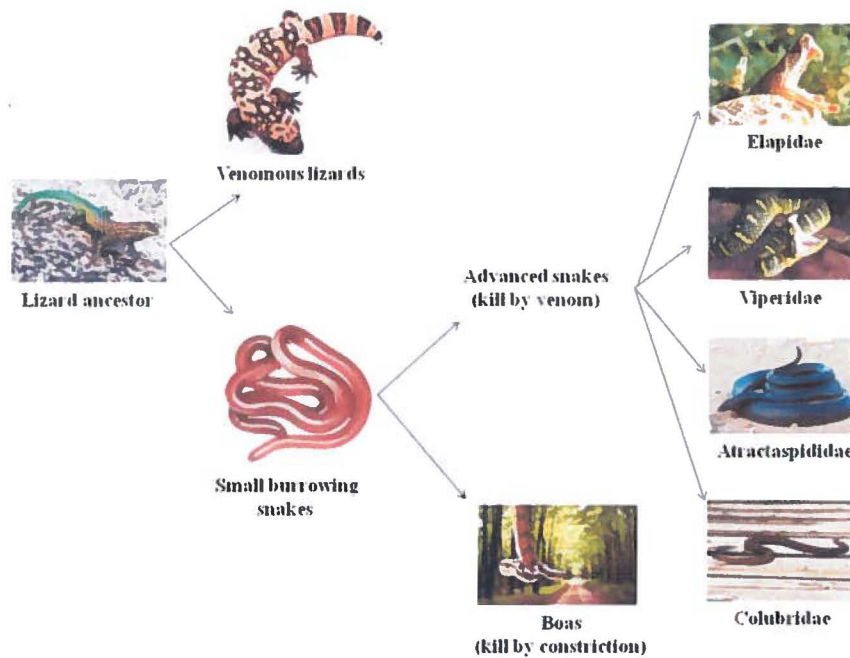
proglucagon (PG) gene expression that constitutes an essential first step in GLP-1 biosynthesis (Chepurny, Bertinetti et al. 2013). In *in vivo* experiments, GPR119 agonists improved glucose tolerance whereas insulin and GLP-1 levels were increased in a dose-dependent manner (Ha, Kim et al. 2013; Zhang, Feng et al. 2013). Furthermore, in the presence of DPP-IV inhibitor, GPR119 agonist stimulated  $\beta$  cell regeneration in diabetic mice (Chepurny, Bertinetti et al. 2013). These results demonstrated that GPR119 agonists represent a new type of anti-diabetes agent that could be highly potent for the treatment of type 2 diabetes.

Among the potential targets, the cholecystokinin (CCK) receptor must also be considered. CCK is a neuropeptide that is released from the gut in response to nutrients, such as lipids, to lower food intake. CCK is known as a regulator in the digestive tract and as a neurotransmitter in the nervous system. CCK interacts with the CCK<sub>A</sub> receptors located mainly on pancreatic acinar cells and CCK<sub>B</sub> receptors mostly in the brain and stomach. CCK-8, the biologically active form of CCK, lowers glucose production and increases  $\beta$  cell mass (Kuntz, Pinget et al. 2004; Cheung, Kokorovic et al. 2009). As well, the muscarinic 3-acetylcholine receptor (M3R) might be a key GPCR useful in the treatment of diabetes. M3Rs are largely distributed in the body, *e.g.*, smooth muscles, endocrine glands, exocrine glands, lungs, pancreas, and brain. In particular, these receptors are expressed in regions of the brain that regulate insulin homeostasis. They are also highly expressed on pancreatic  $\beta$  cells and are critical regulators of glucose homeostasis by modulating insulin secretion. The physiological role of M3R was explored in a study using  $\beta$  cell-specific M3R knockout and  $\beta$  cell-specific M3 over-expression in mice. It was found that mice with M3R knockout had reduced insulin secretion and impaired glucose tolerance, whereas M3 muscarinic transgenic mice had increased insulin secretion and glucose tolerance. Therefore, M3Rs are of profound importance for  $\beta$  cell functions, both as mediating the cholinergic neurotransmission, which is of crucial significance after meal ingestion, and also for the glucose competence of the  $\beta$  cells (Gautam, Ruiz de Azua et al. 2010). These data then suggest that CCKR and M3R activation could be targets for the treatment of T2DM.

## 2. Snake venom and their applications in biomedicine

### 2.1. The relationship between snake and Gila monster

About 200 million years ago, venomous lizards and first snakes (small burrowing creatures) evolved from a common lizard ancestor. Then 120 million years later, these first snakes split into constrictor-style snakes and more advanced snakes, which further divided into four families, including Elapidae, Viperidae, Atractaspidae and Colubridae, possessing venom glands. Sequencing of the mRNA from the cells that make up the venom glands was carried out and an evolutionary tree was constructed.



**Figure 1-27. Relationship between snakes and Gila monster**

Similarities between the mRNA of venom glands of the advanced snakes and venomous lizards suggested that these glands did not evolved independently and were in fact closely linked to those found in the abovementioned lizard ancestor. Hence, venom-producing cell types

evolved only once, in the common ancestor of snakes plus some other reptiles, including the green iguana and the Gila monster (Fry, Vidal et al. 2006).

## **2.2. Nature's Blockbusters of *Elapidae***

Taxonomic classification of the *Colubroidea* superfamily subdivides venomous snakes into five distinctive groups: *Atractaspididae*, *Colubridae*, *Elapidae*, *Hydrophiidae* and *Viperidae* (Underwood 1979). Similar to other venoms, snake venoms also contain proteins, peptides, enzymes and toxins that will vary among these different families. For example, snakes from the *Elapidae* family, comprising the black mamba and the king cobra, possess a highly neurotoxic venom as compared to the *Viperidae* venoms that rather present a high hemotoxic content with a little neurotoxic activity (Adukauskiene, Varanauskiene et al. 2011).

Biochemical characterizations demonstrated that venoms contain up to 300 bioactive molecules, each of them having a particular target and effect. Among all known venoms derived from plants and other animals, snake venoms are considered to be the most highly complex and developed. More precisely, these toxins can be related to cardiotoxins, myotoxic peptides, dendrotoxins, disintegrins, three-finger-structure toxins, PLA2 and hemorrhagins, but many other component activities remain unidentified. Acting independently, synergistically or antagonistically, their effects can be local (at the bite site) or systemic, reaching their specific target through the blood stream, thereby leading to many cumulative disruptions (Adukauskiene, Varanauskiene et al. 2011; Fox 2013; Vyas, Brahmabhatt et al. 2013).

## **2.3. Toxins affecting the central nervous system**

Neurotoxins in snake venom can block transmission of acetylcholine from nerve to muscle at the pre-synaptic junction or affect the activity of the muscle fiber at the post-synaptic junction, causing the prey paralysis. Pre-synaptic neurotoxins ( $\beta$ -neurotoxins), isolated from *Elapidae* and *Viperidae* snakes, such as cobrotoxin,  $\alpha$ -bungarotoxin, notexin or taipoxin provoke the disappearance of acetylcholine-containing vesicles. Alpha-bungarotoxin, the first pre-synaptically active toxin isolated from the elapid *Bungarus multicinctus*, possesses a phospholipase and a potassium channel-binding subunit that combined their effects to destroy



sensory and motor neurons. Crotoxin, a pre-synaptic neurotoxin with cytotoxic activity, is currently being used in phase I clinical trials on advanced cancer patients, as an anticancer agent that is believed to act through a novel mechanism of action (Jain and Kumar 2012). Several toxins found in snake venom are also able to bind competitively to the nicotinic acetylcholine receptor (nAChR) located at the post-synaptic membranes of skeletal muscles and neurons, preventing neuromuscular transmission. Also, muscarinic toxins such as MT1-7, isolated from the green mamba, can recognize selectively and with a high potency the mAChRs, thus representing a useful pharmacological tool for investigating the physiological roles of the muscarinic receptor subtypes (da Silva, de Medeiros et al. 2011; Servent, Blanchet et al. 2011). This particular type of toxin could be used in the treatment of Alzheimer's disease since the involvement of muscarinic receptors was elucidated using these subtype-specific mamba toxins (Piggott, Owens et al. 2003; Koh DC 2006 ).

#### **2.4. Toxins affecting the hemostatic system**

In addition to the cardiotoxins and their actions on the cardiovascular system, toxins affecting the hemostatic system, such as thrombin-like enzymes, prothrombin, platelet aggregation inhibitors and disintegrins, are also found in snake venom (Marsh and Williams 2005). Hence, many components in various snake venoms disrupt normal blood flow and normal blood clotting. They can either activate prothrombin or have a direct effect on fibrinogen and thus have a thrombin-like activity, such as crotalase, ancrod and batroxobin (Bell 1997; Henschen-Edman, Theodor et al. 1999; Rowe and Stegemann 2009; Vu, Stafford et al. 2013). Reptilian fibrinogenolytic enzymes, usually serine proteases or metalloproteinases, induce through a different factor (fibrin or fibrinogen) the destruction of fibrin-rich clots and prevent progression of the clot formation. Some of them, including atroxase and fibrinogenase, are being investigated for the development of blood-clotting inhibitors (Kini and Evans 1991; Tu, Baker et al. 1996; Lin, Qi et al. 2013). Disintegrins, potent inhibitors of various integrins, can either interact with RGD motif-dependent integrins, leukocyte integrin-binding disintegrins and the  $\alpha 1\beta 1$  integrin-binding disintegrins. The RGD-dependent integrins are involved in the pathophysiology of many diseases including thromboembolic disorders, some cancers (Allan, George et al. 2006; Eble and Haier 2006) and Alzheimer's disease (Denda and Reichardt 2007;

Wu and Reddy 2012). Disintegrin, by inhibition of the fibrinogen receptor  $\alpha$ IIb $\beta$ 3 integrin on the platelet, can modulate platelet aggregation. By blocking the platelet integrins and especially  $\alpha$ IIb $\beta$ 3, they can reduce tumor growth, inhibit angiogenesis, and block metastasis. Another mechanism explaining their crucial role in cancer therapy involved the induction of apoptosis in endothelial cells (Montenegro, Salla-Pontes et al. 2012). Finally, their actions on the fibronectin receptor,  $\alpha$ 5 $\beta$ 1 integrin, which is implicated in the regulation of angiogenesis, might have a pivotal role in several diseases including Alzheimer's. Disintegrins, specific for leukocyte integrins and the  $\alpha$ 1 $\beta$ 1 integrin-binding disintegrins, are also being investigated as new areas in angiogenesis pharmaceutical research (Ma, Xu et al. 2011; Marcinkiewicz 2013; Sampieri 2013). C-type lectins represent an important group of proteins that bind to a wide range of coagulation factors, von Willebrand factor or specific receptors such as GPIb,  $\alpha$ 2 $\beta$ 1 and GPVI. These C-type lectins are important in hemostasis and to platelet receptors and display both anti-coagulant and platelet-modulating activities (Jennings, Spearman et al. 2005; Arlinghaus and Eble 2012). Botrocetin, the first C-type lectin described, is able to activate platelets via GPIb and von Willebrand factor (Fukuda, Doggett et al. 2002; Yamamoto-Suzuki, Sakurai et al. 2012). Convulxin, flavocetin-A, RVV-X, EMS16, and CA-1, all isolated from various snake venoms, represent useful tools for elucidating the mechanisms involved in clotting and platelet activation, as well as providing new possibilities in diagnosis and treatment through their interaction with platelets, plasma and the vascular wall.

Myotoxins can severely damage skeletal muscle leading to tenderness, pain, muscle weakness, skeletal muscle breakdown and myoglobinuria. These myotoxins initiate cycles of degeneration and regeneration of skeletal muscle (Fernandez, Caccin et al. 2013). Myotoxin- $\alpha$ , one of the best-known myotoxins, is a small, basic protein devoid of enzymatic activity, which binds specifically to the sarcoplasmic reticulum of muscles, causing a change in ion permeability. This perturbation, which affects directly sarcoplasmic reticulum and muscle fibrils, induces severe damage of the skeletal muscle (Hirata, Nakahata et al. 1999; Koh DC 2006 ). PLA2, which can either have myotoxic, cardiotoxic or neurotoxic actions, triggers a cascade of inflammatory events characterized by increased microvascular permeability and edema formation, leukocyte recruitment into tissues, nociception and release of inflammatory mediators that mimic a number of systemic and local inflammatory disorders in humans. Ultimately, these

processes will induce edema, blister formation and local tissue necrosis. Of clinical importance, some PLA2, isolated from the snake venom, are potential anti-cancer drugs (Rodrigues, Izidoro et al. 2009; Arouri and Mouritsen 2012). However, snake venom also contains PLA2 inhibitors and the biotechnological potential of PLA2 inhibitors may provide therapeutic molecular models with antiophidian activity to supplement the conventional serum therapy against these multifunctional enzymes. Naturally occurring anti-toxic factors that neutralize PLA2 have been isolated from the blood of both venomous and non-venomous animals. Snake PLA2 inhibitors (PLIs) demonstrated specific affinities for various PLA2 enzymes but also exert some anti-enzymatic, anti-myotoxic, anti-edema-inducing, and anti-bacterial activities, which make them highly valuable as pharmacological tools (Soares and Giglio, 2003). For example, specific PLIs derived from animal sources show a high therapeutic value since they might block PLA2 inflammatory processes responsible for acute and chronic neurological disorders associated with neurodegenerative diseases, such as neural trauma, Alzheimer's and Parkinson's diseases (Mahalka and Kinnunen 2013).

## **2. 5. Therapeutic alternative from venom peptides**

Each year, several new natural toxins with highly specific actions are discovered, characterized and used both as pharmacological tools and as templates for drug design (Koh and Kini 2012; Cragg and Newman 2013). Toxins have evolved in plants, animals and microbes, as part of defensive and/or prey capture strategies. For instance, following envenomation, physiological signs and symptoms such as paralysis, myolysis, coagulopathy and hemorrhage, renal damage and failure, and cardiotoxicity are observed. These types of symptoms suggest that various systems, particularly the central nervous system and cardiovascular system, as well as the muscular and vascular systems, are affected (Rash and Hodgson 2002; Favreau and Stocklin 2009; Osipov and Utkin 2012; Quintero-Hernandez, Jimenez-Vargas et al. 2013). Venom, defined as a complex mixture of proteins, peptides, enzymes, toxins, lipids, carbohydrates, biogenic amines and trace amounts of non protein inclusions, is usually produced in specialized glands of arthropods and reptiles and is employed to paralyze their prey and cause its subsequent death (Rash and Hodgson 2002; Favreau and Stocklin 2009; Osipov and Utkin 2012; Quintero-Hernandez, Jimenez-Vargas et al. 2013). More particularly, toxins are homogenous structures

that are isolated, extracted, or derived from plant, animal, or microbial sources and have a specific locus of action. Non-peptide toxins such as ciguatoxins and brevetoxins are able to activate voltage-sensitive sodium channels and have proven to be invaluable research tools (Schlumberger, Mattei et al. 2010; McCall, Jacocks et al. 2012). Epibatidine, a potent antinociceptive, which probably activates some nicotinic acetylcholine receptors, is an example. Usually found in animal venom, which comprises a highly complex mixture of peptide toxins, they are often characterized by diverse and selective pharmacological compounds that represent a unique source of leads and structural templates for new therapeutic agents. This evolved biodiversity of peptides is able to target a wide variety of membrane bound proteins, specific ion channels, enzymes and G protein-coupled receptors (Adams, Callaghan et al. 2012; Insera, Kompella et al. 2013; Quintero-Hernandez, Jimenez-Vargas et al. 2013). Thus, several major diseases such as cancer, neurodegeneration, neuromuscular problems, inflammation, hematological disorders, autoimmune infection and cardiovascular problems have been targeted to evaluate a possible treatment via peptide toxin-based drugs (Piggott, Owens et al. 2003; Adams, Callaghan et al. 2012; Mizuno, Ito et al. 2012; Osipov and Utkin 2012).

### *2.5.1. Venom peptides and cardiovascular pathologies*

The first venom-based drug, named captopril, was discovered in 1975. This first oral angiotensin I-converting enzyme (ACE) inhibitor was identified based on the observation that the venom from a Brazilian viper (*Bothrops jararaca*) caused a sudden, massive drop in blood pressure. After several years of structure-activity relationship and biological studies to characterize this toxin as a potent ACE inhibitor, scientists from Squibb Pharmaceutical were able to create captopril, the first oral ACE inhibitor, opening new avenues for toxins as potential applications pertaining to the cardiovascular system. Cardiotoxins/Cytotoxins, isolated in the late 40's from Indian cobra venom, are direct lytic factors and membrane-active polypeptides mainly targeting excitable cells and they have been investigated as potential treatment for chronic circulatory insufficiency. These toxins, which are pore-forming agents, are defined as a single-chain, highly hydrophobic, basic, short polypeptides that cause depolarization and contracture of cardiac, skeletal and smooth muscles, as well as depolarization and loss of excitability of nerves (Wu, Chiu et al. 2012; Tsai, Chu et al. 2013). However, due to their high cytotoxicity,

cardiotoxins are also known to be membrane-active proteins that recognize the membrane proteoglycans. This compound shows therefore promising properties for cancer treatment (Lin, Chien et al. 2012; Tsai, Hsieh et al. 2012; Vyas, Brahmhatt et al. 2013; Yen, Liang et al. 2013).

### 2.5.2. *Venom peptides and cancer*

The search for biological antitumor agents has been pursued for over half a century. In 1933, Calmette reported an antitumor effect of the venom of *Naja sp.* on adenocarcinoma cells. Years later, a thrombin-like enzyme (crotalase) from the venom of *Crotalus adamanteus* demonstrated its ability to reduce *in vitro* and *in vivo* the growth of mouse B16 melanoma cells. Based on these results, the author suggested that crotalase significantly retarded tumor growth *in vivo* but did not present cytotoxic or cytostatic effects on normal cells *in vivo*.

Tumoral cells produce around them a microenvironment, composed of fibrin deposits from the nearby blood vessels, which will act as a protective shield against the responses of the immune system. To explain the reduced growth of cancer cells without cytotoxic activity, it was proposed that crotalase was able to destroy the fibrogenic microenvironment, leaving tumor cells unprotected. However, certain fractions isolated from snake venoms revealed a direct cytolytic activity on tumor cells (Lip 1995). Thus, it was hypothesized that rejection of tumor cells observed with different venom may be the result of one or more mechanisms. Atroporin and Kaotree are cancer cells inhibitors, isolated from snake venom, that have lost their toxicity (by chemical engineering) but retained cytotoxic anti-cancer activity. These proteins possess the unique property to selectively kill cancer cells without harming the normal cell population. Combination of synthetic Atroporin and Kaotree, which are selective for various types of cancer cells, can cause regression of tumors, depending upon their size. Actually, in such cases where surgery is inevitable, the combination of Atroporin and Kaotree can serve as a treatment to prevent recurrence of cancer. The introduction of such biologics for the treatment of cancer changed the practice of chemotherapy to biotherapy (Lipps 1995). A tumor is an aggregation of cancer cells due to the excessive rapid growth property of these particular cells. By the time the patient is diagnosed for cancer, it may have metastasized. Diagnosis generally leads to surgery to remove cancer growth or tumor followed by chemotherapy. Precision is paramount in operations to remove tumors, when cancerous cells can be missed and left behind. It is especially important

when dealing with the brain, where some 80% of malignant cancers return at the edge of surgical sites and where surrounding neurons must not be damaged. Currently, surgeons use color, texture and blood supply to distinguish cancerous tissues from healthy ones. Furthermore, chlorotoxin, a substance derived from scorpion venom, possesses the ability to bind cancer cells. Joined to a fluorescent marker, Cy5.5, this toxin becomes a molecular beacon that emits photons in the infrared spectrum, illuminating whole tumors and even clusters of only a few hundred cancerous cells. The probe marks tumors with at least 500 times more sensitivity than a magnetic resonance imaging scan, which will only work if more than a million cancer cells are present. This new type of “painting” agent is able to illuminate brain tumors in mice as small as 1mm in diameter or detect 200 prostate cancer cells travelling through a mouse’s lymph system. As such, chlorotoxin-Cy5.5 could be used as a non-invasive screening tool for the early detection of skin, cervical, esophageal, colon and lung cancers, and may help identify positive lymph nodes in patients with breast, prostate and testicular cancers (Jiang, Zhou et al. 2013; Kovar, Curtis et al. 2013).

### *2.5.3. Venom peptides and neuropathic pathologies*

Neuropathic disease, defined as a central nervous system damage, is correlated to an excessive stimulation of the neurotransmitter glutamate. Pathological states, ranging from cerebral ischemia to neuropathological conditions, including motor neuron disease such as amyotrophic lateral sclerosis (ALS), Alzheimer’s, Parkinson’s disease and epilepsy, are often characterized by modifications of synaptic junctions and an improper balance between glutamate and the  $\gamma$ -aminobutyric acid (GABA) neurotransmitter system. The glutamate-induced response at the post-synaptic junction is mediated by metabotropic and ionotropic receptors. The action of metabotropic receptors occurs via a GTP-binding protein resulting in calcium mobilization and the ionotropic receptor activation (N-methyl-D-aspartic acid (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate) that will ultimately lead to calcium, sodium, potassium and chloride channel permeability (Nimmrich and Ebert 2009; Kwak, Hideyama et al. 2010; Field, Walker et al. 2011). Strategies, involving modulation of sodium channels, repetitive discharge or blockade of voltage-dependent calcium channels or glutamate

receptors, are being investigated for neuroprotective purposes and potential treatment of neurodegenerative disorders.

Identification of new neuroactive peptides from venomous species has become extremely attractive due to the potential high selectivity of these molecules for a wide variety of ion/receptor channel subtypes. Although channel activators are typically toxic, subtype-selective inhibitors might have considerable therapeutic potential as research tools or to modulate channel functions. Given their crucial role in the central nervous system, it is not surprising that a number of venoms from spiders (Mourao, Oliveira et al. 2013; Palagi, Koh et al. 2013), sea anemone (Frazao, Vasconcelos et al. 2012), scorpions (Pedraza Escalona and Possani 2013), cone snails (Peigneur, Van Der Haegen et al. 2013) and snakes (Barber, Isbister et al. 2013) have evolved to target various receptors and channels. The use of peptide toxins, demonstrating different specificities and selectivities, has shown clinical relevance in neuroprotection and helped disclose the mechanisms underlying the pathogenesis of several diseases like Alzheimer's, ALS, myasthenia gravis and Parkinson's. Omega-conotoxin, an N-type calcium channel antagonist isolated from a cone snail, exerts some neuroprotective activity attributed to the inhibition of the release of excitatory neurotransmitters (Richard and McIntosh 2006). In the same way, cone snail toxin peptides, conantokin G, a potent NMDA receptor antagonist, has demonstrated the ability to alter sodium channel gating. This toxin selectively inhibits NR2B subunits of N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx in central nervous system neurons. Conantokin G induces sleep-like symptoms in young mice and hyperactivity in older mice. This compound is under phase II clinical trials under the name CGX-1007 by Cognetix Inc. It has been shown to be an effective antiepileptic agent in several animal models of seizure. Kalitoxin, charybdotoxin and margatoxin represent highly selective blockers of potassium channels that might be useful in the treatment of neurological diseases (Abbas 2007). Some of these toxins might also hold new avenues for the treatment of ALS, Alzheimer's and Parkinson's diseases. Two spider toxins, the *Joro* spider toxin and the agatoxin, have demonstrated some activities in studies related to ALS and calcium permeability. Toxins targeting neuronal nicotinic and/muscarinic acetylcholine receptors are critically important in several neuronal disorders, including Alzheimer's and Parkinson's, which are characterized by a down regulation of several cholinergic functions and a reduced number of nicotinic and muscarinic acetylcholine receptors

(nAChRs/mAChRs). Indeed, this observation was made possible by the use of  $\alpha$ -conotoxin MII,  $\alpha$ - or  $\kappa$ -bungarotoxin, as selective markers. These toxins, directed against specific acetylcholine receptor subtypes, may be useful in evaluating their precise role in the pathogenesis of diseases like ALS, Alzheimer's or Parkinson's and should help to develop optimal strategies for new therapeutic treatments. As a consequence of their high selectivity, venom peptides have proved to be particularly useful *in vitro* and *in vivo*. Considering their highly conserved primary and secondary features, their exquisite subtype selectivity and the wide range of biological and physiological targets, toxins isolated from venomous animals have opened up new research avenues. Using these toxins as lead compounds might help the development of new therapeutic treatments, as well as diagnostic and pharmacological tools for medical researchers.

#### 2.5.4. *Venom peptides and diabetes*

According to the World Health Organization, every year in the world, there are about 4.6 million deaths (or 6% of world mortality) by diabetes. Moreover, as mentioned before, by 2030, unless preventive measures are taken, 552 million people worldwide will have diabetes, with the largest increase occurring in developing countries. In healthy nondiabetic subjects, oral administration of glucose produces a substantial enhancement of insulin response compared with intravenous administration of glucose. This discrepancy has been called the *incretin effect*, and it is diminished in people with type 2 diabetes. Subsequent studies identified two primary gut-derived hormones that are responsible for most of the incretin effect: GIP and GLP-1. GLP-1 is a better therapeutic target and has accordingly been studied to a greater extent. It inhibits postprandial glucagon release and stimulates GDIS. Unfortunately, GLP-1 has an *in vivo* half-life of less than 2 minutes, which prevented its use as a therapeutic compound. However, exendin-4, a 39-amino acid peptide (53% structural homology to GLP-1) that was first isolated from the salivary secretions of the Gila monster lizard (*Heloderma suspectum*), shares many of the glucoregulatory actions with GLP-1 and has aroused great attention for its potential for the treatment of diabetes. The only currently available incretin mimetic, which involves GLP-1 agonists or analogs that are resistant to DPP-IV-mediated degradation, is exenatide (Byetta<sup>®</sup>), a GLP-1 agonist that was approved by the FDA in 2005 and by Health Canada in 2012, as adjunctive therapy for patients with type 2 diabetes. Exenatide is a synthetic analog of exendin-4



that binds to and activates the human GLP-1 receptor (Triplitt 2007). Exenatide provokes a sustained reduction in hemoglobin *A1c* (a good measure of diabetes care), probably related to its effects on pancreatic  $\beta$  cells, accompanied by a dose-dependent weight loss. Consistent with the known activities of GLP-1, exenatide promotes  $\beta$  cell proliferation and differentiation in animal models and also enhances  $\beta$  cell functions (Tschen, Georgia et al. 2011; Derosa and Maffioli 2012; Stolovich-Rain, Hija et al. 2012). Improvements in other cardiovascular risk factors, including increases in high-density lipoprotein and decreases in diastolic blood pressure, were also observed (Blonde, Klein et al. 2006). Exenatide enhances endogenous insulin secretion in a glucose-dependent manner. Another toxin,  $\alpha$ -latrotoxin ( $\alpha$ -LTX) induces exocytosis of small synaptic vesicles in neuronal cells both by a calcium-independent mechanism and by opening cation-permeable pores. This toxin also increased insulin exocytosis in pancreatic  $\beta$  cells INS-1E and in the derived mouse insulinoma cell line MIN6, in the absence of extracellular calcium. Studies showed that  $\alpha$ -LTX binds to latrophilin, a member of the GPCR family. Sensitivity to  $\alpha$ -LTX correlated with expression of latrophilin and transient expression of latrophilin in these cells produced  $\alpha$ -LTX-induced exocytosis. Consequently, direct stimulation of exocytosis by a GPCR mediates the  $\text{Ca}^{2+}$ -independent insulinotropic effects of  $\alpha$ -LTX in the absence of altered ion fluxes (Lang, Ushkaryov et al. 1998). In islet  $\beta$  cells, when the plasma membrane is depolarized,  $\text{K}_v$  channels become activated, which causes a membrane repolarization that results in the closure of the VDCC, leading to a cessation of insulin secretion. Thus, inhibition of  $\text{K}_v$  currents should broaden the duration of action potentials that maintain the plasma membrane in a depolarized state and sustain the calcium influx through opened calcium channels, and enhance insulin secretion in a glucose-dependent manner. For these reasons, the  $\text{K}_v$  channels have been considered as a potential target for the development of a novel type 2 diabetes therapy, which might have many advantages over the currently used sulfonylureas. In nature, a variety of peptide toxins isolated from the venom of spiders, scorpions, and cone snails, among others, functionally inhibit  $\text{K}_v$  channels and have proven to be valuable pharmacological tools for evaluating specific channel characteristics. Of the  $\text{K}_v$  channel subtypes that are expressed in islet  $\beta$  cells,  $\text{K}_{v2.1}$  is the dominant isoform, which constitutes over 60% of the currents mediated by the  $\text{K}_v$  channels. Disruption of the  $\text{K}_{v2.1}$  gene alters the glucose-induced islet electrical activity and enhances insulin secretion. This indicates that  $\text{K}_{v2.1}$  mediates the majority of repolarizing

delayed rectifier current in rat  $\beta$  cells and antagonism of  $K_{v2.1}$  may prove to be a novel glucose-dependent therapeutic treatment for type 2 diabetes. Hanatoxin (HaTx) is a polypeptide toxin isolated from *Phrixotrichus spatulata* spider venoms. HaTx inhibits the voltage-gated potassium channel  $K_{v2.1}$  potently with nanomolar affinities. HaTx binds to the surface of the  $K_{v2.1}$  channel at four equivalent sites within voltage-sensor domains and shifts channel opening to more depolarized voltages. HaTx is able to increase the glucose-induced elevation of  $[Ca^{2+}]_i$  in human islets, therefore enhances GDIS (Tamarina, Kuznetsov et al. 2005). HaTx thus becomes a specific pharmacological probe in the regulation of insulin secretion in T2DM. Similarly, guangxitoxin 1E (GxTX-1E), a neurotoxin isolated from the venom of the *Plesiophrictus guangxiensis* spider, is able to interact with the voltage sensors in  $K_{v2.1}$  channels to inhibit almost 90% of  $K_v$  current. By shifting the voltage dependence of channel activation to more depolarized potentials, GxTX-1E broadens the  $\beta$  cell action potential, enhances glucose-stimulated intracellular calcium oscillations, and enhances insulin secretion from mouse pancreatic islets. This feature points to the advantages of applying  $K_{v2.1}$  channel blocker therapies for the treatment of type 2 diabetes. Likewise, SGTx1, a peptide toxin isolated from the venom of the *Scodra griseipes* spider and exhibiting an amino acid sequence highly (76%) homologous with that of HaTx, inhibits  $K^+$  currents in oocytes expressing  $K_{v2.1}$  channels and shifts the activation of the channel to more depolarized voltages. This toxin also has the same surface profile as HaTx. However, the differences in the charge distribution of SGTx1 revealed differences in binding affinity and conformational homogeneity. Because of that, SGTx1 appeared as a good substitute for HaTx (Lee, Kim et al. 2004). Jingzhaotoxin-I (JZTX-I) is a potent neurotoxin from a tarantula (*Chilobrachys jingzhao*) venom that inhibits both sodium and potassium channels. JZTX-I is able to inhibit activation of the potassium channel subtype  $K_{v2.1}$ . Using Ala-scanning mutagenesis strategy, it has been demonstrated that mutations I273A, F274A, E277A, and K280A in S3b-S4 reduced toxin binding affinity by 6-, 10-, 8-, and 7-fold, respectively. These data suggest that JZTX-I inhibits  $K_{v2.1}$  activation by targeting its binding site in S3b-S4, which is formed by I273, F274, E277, and K280 (Tao, Wu et al. 2013).

Resistance of peripheral tissues to insulin also characterizes type 2 diabetes mellitus. Indeed, the effects of insulin, insulin deficiency and insulin resistance vary according to the physiological function of the tissues and organs concerned, and their dependence on insulin for

metabolic processes. Those tissues defined as insulin-dependent, based on intracellular glucose transport, are principally adipose tissues and muscles. Glucose uptake into those cells is essentially insulin-dependent via GLUT 4 (Wilcox 2005). Mechanistic studies showed that  $K_{v1.3}$  gene deletion and channel inhibition enhanced peripheral insulin sensitivity by increasing the amount of glucose transporter 4 (GLUT4) at the plasma membrane and the uptake of glucose in skeletal muscle and adipose tissue via intracellular  $Ca^{2+}$  signaling (Li, Wang et al. 2006). Therefore, these results indicated that  $K_{v1.3}$  may play a critical role in insulin action by controlling peripheral insulin sensitivity (Choi and Hahn 2010). Genetic ablation of the voltage-gated potassium channel  $K_{v1.3}$  improves insulin sensitivity and increases metabolic rate in mice. Inhibition of  $K_{v1.3}$  in mouse adipose and skeletal muscle is reported to increase glucose uptake through increased GLUT4 translocation. Toxin peptides from venomous animals comprise the largest families of ion channel blockers, and they are becoming valuable sources of new drugs for channelopathies. With respect to the  $K_{v1.3}$  channel, there are many structurally diverse peptide toxins. Among the many  $K_{v1.3}$  blockers, margatoxin (MgTX), a peptide of 39 amino acids, stimulated glucose uptake in adipose tissue and skeletal muscle and its effect on glucose transport was additive to that of insulin (Li, Wang et al. 2006).

Blockers of ion channels have significant therapeutic potential in the management of diseases affecting excitable tissues. It is well established that ion channels are also required for the proper physiological functions of classically nonexcitable cells such as lymphocytes. In this sense, a key step in the generation of an efficient immune response and protection of the organism against various infectious agents is the antigen-induced activation and proliferation of T cells that can be inhibited by blockers of plasma membrane  $K^+$  channels. Autoimmune diseases are characterized by the generation of autoreactive T cell clones that react to self-antigens, thereby leading to the destruction of specific tissues, such as myelinated neurons in multiple sclerosis or insulin-producing pancreatic  $\beta$  cells in type I diabetes mellitus. The proliferation of the autoreactive T cells mediating tissue damage can be specifically and persistently inhibited by blockers of  $K_{v1.3}$  potassium channels. This underlines the therapeutic potential of high-affinity and high-specificity  $K_{v1.3}$  inhibitors. Among others, Bmp02, a 28-amino acid peptide purified from the venom of the Chinese scorpion *Buthus martensii*, which had been demonstrated to be a potassium channel blocker, displays affinity on insect and mammalian  $K^+$

channels expressed in *Xenopus* oocytes. BmP02 inhibited currents of  $K_{v1.3}$  with an  $IC_{50}$  of 7 nM. This toxin blocks  $K_{v1.3}$  in a weak voltage-dependent manner and slightly shifts the current activation curve to positive potentials. Lys16 of this toxin was found to be important in the contact with the  $K_{v1.3}$ . Substitution within this toxin might give rise to improved blocking properties for  $K_{v1.3}$  (Zhu, Gao et al. 2012). Kbot1, a scorpion toxin with 28 amino acid residues, is the shortest toxin sequenced in *Buthus occitanus* scorpion. Three disulfide bridges stabilize the structure and its primary structure is 93% identical to that of BmP02. Kbot1 blocks  $K_{v1.3}$  currents with an  $IC_{50}$  of 15 nM (Mahjoubi-Boubaker, Crest et al. 2004). This toxin exhibited a low neurotoxicity in mice after intracerebroventricular injection. Similarly, Ctri9577, a 39-amino acid peptide including six cysteines, is the first neurotoxin of the *Chaerilidae* family that was cloned from the venom of the scorpion *Chaerilus tricostatus* through constructing its cDNA library. Ctri9577 selectively inhibited the  $K_{v1.3}$  channel current with an  $IC_{50}$  of 0.49 nM. This finding presents a novel potential drug candidate targeting  $K_{v1.3}$  channels for the therapy of autoimmune diseases, and diabetes as well (Xie, Feng et al. 2012). Also, the BmKTX toxin, isolated from *Buthus martensii* and belonging to the scorpion  $K^+$  channel-inhibitor family, shows over 80% sequence identity with members of the kaliotoxin group. BmKTX blocks the cloned voltage-gated  $K_{v1.3}$  from rat brain, expressed in *Xenopus* oocytes ( $IC_{50}$  of 0.6-1.6 nM) (RomiLebrun, Lebrun et al. 1997). Similarly, the  $K^+$  channel blocker MeuKTX, isolated from *Mesobuthus eupeus*, an orthologue of BmKTX, potently blocks  $hK_{v1.3}$  channels with an  $IC_{50}$  of 171 pM. MeuKTX and BmKTX have the same channel spectrum and pharmacological potency. Finally, analysis of the structure-function relationships of the  $\alpha$ -KTx3 subfamily toxins revealed several key sites that are useful for designing toxins with improved activity on  $hK_{v1.3}$ , an attractive target for T cell-mediated autoimmune diseases (Gao, Peigneur et al. 2010). Thus, OSK1 ( $\alpha$ -KTx3.7), a 38-residue toxin cross-linked by three disulfide bridges, initially isolated from the venom of the scorpion *Orthochirus scrobiculosus*, blocks potently the  $K_{v1.1}$ ,  $K_{v1.2}$  and  $K_{v1.3}$  channels with  $IC_{50}$  values of 0.6, 5.4 and 0.014 nM, respectively. Moreover, the OSK1 analogue,  $[K^{16}, D^{20}]$ -OSK1 (OSK1 with Glu<sup>16</sup> replaced with Lys<sup>16</sup> and Lys<sup>20</sup> substituted with Asp<sup>20</sup>), shows an increased potency on  $K_{v1.3}$  channel, with an  $IC_{50}$  value of 0.003 nM. These data suggest that OSK1, as well as its analog  $[K^{16}, D^{20}]$ -OSK1, could serve as leads for the design and production of new immunosuppressive drugs.

GSIS from pancreatic  $\beta$  cells is regulated by a series of electrogenic events leading to insulin secretion. Coordinated electrical activity allows pancreatic  $\beta$  cells to respond to secretagogues with  $\text{Ca}^{2+}$  entry via specific  $\text{Ca}^{2+}$  channels, followed by insulin secretion. In the case of glucose stimulation, this activity is organized into slow depolarizing waves with a plateau from which action potentials (AP) rapidly fire. The resulting depolarization activates voltage-gated channels, including the L-type  $\text{Ca}^{2+}$  channel, which leads to the rising phase of the AP. Continued depolarization activates  $\text{K}_v$  channels, allowing membrane repolarization and the falling phase of the action potential via  $\text{K}^+$  efflux from the  $\beta$  cell.  $\text{Ca}^{2+}$  entry and elevated  $[\text{Ca}^{2+}]_i$  additionally regulate  $\text{K}_{\text{Ca}^{2+}}$  channels, which affects AP repolarization and the duration of the slow wave. As such,  $\text{K}_{\text{Ca}^{2+}}$  channels influence also  $\beta$  cell APs and thereby influence insulin secretion.  $\text{K}_{\text{Ca}^{2+}}$  channels were isolated from  $\beta$  cells and they include big-conductance  $\text{K}^+$  channel (BK), and small-conductance  $\text{K}^+$  channel (SK). Jacobson reported that human  $\beta$  cell AP amplitude had been regulated by 200 nM siltoxin, a BK specific inhibitor, whereas isopimaric acid (10  $\mu\text{M}$ ), a BK specific activator, causes a significant reduction in human  $\beta$  cell AP. Fast  $\text{Ca}^{2+}$  fluctuation responses, with similar changes in amplitude, were also observed (Jacobson, Mendez et al. 2010).

Also, the SK channel inhibitor apamin caused AP firing at 500 nM. Therefore, activation of SK channels can regulate  $\beta$  cell electrical activity. These data demonstrate that  $\text{K}_{\text{Ca}^{2+}}$  channels can modulate islet AP amplitude, thus resulting in a calcium influx and identifying their role in  $\beta$  cells (Jacobson, Mendez et al. 2010).

As described above, many toxins isolated from spider venoms have potential applications in diabetes, but there has been no similar research with snake venom in relation to this disease. Especially through a long-time Vietnamese collaboration focusing on the therapeutic potential of snake peptides, this opened an avenue for us to verify if components of snake venom can be used for the treatment of diabetes.

## **HYPOTHESIS**

Many studies showed that snake venoms are cocktails of pharmacologically active proteins and peptides such as neurotoxins, cardiotoxins, cytotoxins, nerve growth factors, lectins, disintegrins and hemorrhagins. These compounds target various physiological pathways that are involved in many human diseases and therefore, some of them could be used to develop therapeutic agents for the treatment or prevention of numerous human diseases, including diabetes (Camargo, Ianzer et al. 2012). Marshall McCue observed that snakes could survive up to two years without food. Accordingly, he reported that snakes can reduce their metabolic rate by up to over 70% (McCue 2007), thus demonstrating that these animals possess certain factors essential for this process. In addition, snakes are classified within the same family as the Gila monster, the lizard in which was discovered the peptide exendin-4 that exhibits a potent insulinotropic activity. Consequently, it was hypothesized that snakes, that are also animals able to control their metabolism, possess biological components showing potent insulinotropic properties.

## **OBJECTIVES**

**Objective 1.** The search for insulinotropic compounds in the venom of the Vietnamese cobra snake *Naja kaouthia* and determination of their targets and mode of action.

**Objective 2.** The synthesis of analogs of promising lead compounds and the study of their biological activities, as well as their cell signaling pathways.

## **CHAPTER II. RESULTS**

## PUBLICATION 1

### Cardiotoxin-I: An Unexpectedly Potent Insulinotropic Agent



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**Contribution of the authors:** A large part of the work carried out in the laboratory was achieved by Thi Tuyet Nhung Nguyen (TTNN). David Chatenet (DC), Myriam Létourneau (ML), Nam Hai Truong (NHT) and Alain Fournier (AF) participated in the choice of the scientific orientations of the research program focussing on peptide toxins from snake venom. DC and ML supervised the work related to the peptide isolation, as well as the peptide biochemical and pharmacological characterizations. Benjamin Folch (BF) and Nicolas Doucet (ND) were responsible for the structural analysis of cardiotoxin-I. TTNN wrote the first draft of the manuscript while DC and AF revised and finalized the paper. It was published in *ChemBioChem* (13:1805-1812, [2012] – Impact factor: 3,740).



## PUBLICATION 2

### Design of a truncated cardiotoxin-I analog with potent insulinotropic activity



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**Contribution of the authors:** A large part of the work was carried out jointly by Thi Tuyet Nhung Nguyen (TTNN) and Benjamin Folch (BF). TTNN was responsible for the design and synthesis of the peptides, as well as their biochemical and pharmacological characterizations, whereas BF and his supervisor, Nicolas Doucet (ND), performed the structural analysis of cardiotoxin-I and its derivatives. David Chatenet (DC), Myriam Létourneau (ML), Nam Hai Truong (NHT) and Alain Fournier (AF) participated in the choice of the scientific orientations of the research program focussing on peptide toxins from snake venom. DC and ML supervised the work related to the peptide synthesis, as well as the peptide biochemical and pharmacological characterizations. TTNN and BF wrote the first draft of the manuscript while DC, ML, ND and AF revised and finalized the paper. It was published in *Journal of Medicinal Chemistry* (Impact factor in 2012: 5,614).

§ Authors equally contributed to the studies

## **CHAPTER III. GENERAL DISCUSSION AND PERSPECTIVES**

Medicine has come a long way in a very short time. Huge leaps in the field of medicine have opened the door for new and interesting drugs, among which toxins from venomous animals created exciting new avenues. Venomous animals represent a vast and largely untapped source of potent biologically active molecules that can offer better treatment options for consumers because of their unique potency and effectiveness. The venoms themselves are natural compounds including proteins, peptides, enzymes and other active agents. They are products of millions of years of evolution, through natural selection, that makes them highly potent. These molecules serve the dual purpose of prey capture and digestion and/or defense against predators. In particular, the venomous peptides are directed against a wide variety of pharmacological targets, making them an invaluable source of ligands for studying the properties of these targets in different experimental paradigms. Moreover, a few venom-based drugs were developed from observed biological actions of protein or peptide toxins and are currently on the market. For instance, following envenomation, effects on the cardiovascular system and blood circulation are observed for various venoms. Accordingly, Capoten<sup>®</sup> (captopril), an oral drug and a member of a class of drugs called angiotensin converting enzyme (ACE) inhibitors, was the first venom-based drug approved by FDA in 1981. This medicine has been used in the treatment of hypertension and congestive heart failure. Also Integrilin<sup>®</sup> (eptifibatide) and Aggrastat<sup>®</sup> (tirofiban), two antiplatelet drugs of the glycoprotein IIb/IIIa inhibitor class, which were approved in 1998 and 1999, respectively, have been used to reduce the risk of acute cardiac ischemic events. The  $\omega$ -conotoxins produced by piscivorous cone snails presently remain amongst the most selective antagonists of N-type VDCC and, for that reason, are being pursued as drug candidates for the management of neuropathic pain. Prialt<sup>®</sup> (ziconotide) is the synthetic form of an  $\omega$ -conotoxin peptide of the cone snail species, *Conus magus*. Ziconotide acts as a selective N-type VDCC blocker. It inhibits the release of pro-nociceptive neurochemicals such as glutamate, the peptide substance P and calcitonin gene-related peptide (CGRP), in the brain and spinal cord, resulting in pain relief.

Among venoms, snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. They exhibit their actions via specific receptors, ion channels or plasma proteins and interfere in the prey's physiological processes. The toxicity of these proteins

is one of the main reasons for our fascination with snakes. In addition to the abovementioned examples, the discovery of nerve growth factor (NGF), dendrotoxin, cobra venom factor (CVF), convulxin, and botrocetin strongly supports that snake venom possesses a tremendous potential as research tools. However, while many venoms from different animals have been reported to be able to stimulate insulin release (Zhou, Li et al. 2009; Baptista-Saidemberg, Saidemberg et al. 2012; Finol-Urdaneta, Remedi et al. 2012), there is still no report indicating that snake venoms contain any agent that could be useful for the development of a therapeutic compound targeting diabetes. Physiologically, a snake can survive up to two years without food and its metabolic rate can be reduced by more than 70% (Mccue 2007), suggesting that its body contains factors involved in this process. In addition, snakes share an evolutionary relationship with the Gila monster (Fry, Vidal et al. 2006), from which was isolated exendin 4, a peptide participating to the reduction of the metabolic rate of the animal. Consequently, the potent insulinotropic activity of this compound was the basis of the hypothesis that snakes might possess similar components related to their unique metabolism.

### **3. 1. Outlook of actual type 2 diabetes treatments**

Diabetes is increasing at an alarming rate. It will be affecting over 350 million individuals worldwide in 2015, and it is expected to spread to approximately 550 million by 2030. T2DM is the most prevalent form, accounting for 90-95% of worldwide cases of diabetes. T2DM is a complex endocrine and metabolic disorder. The interaction between several genetic and environmental factors results in a heterogeneous and progressive disorder with variable degrees of insulin resistance and pancreatic  $\beta$  cell dysfunction. Over time, declining  $\beta$  cell function and insulin resistance lead to the inability to maintain normoglycemia, and hyperglycemia ensues. T2DM usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce it. Insulin resistance is a major contributor to progression of the disease and to complications of diabetes. There are numerous non-pharmacologic and pharmacologic treatment modalities to achieve and maintain normoglycemia. Non-pharmacological approaches include diet modification, weight control and regular exercise. Pharmacological approaches will be applied when the blood glucose level cannot be controlled with diet and exercise. Lifestyle

interventions are effective; however, long-term adherence is typically poor. Furthermore, lifestyle interventions have a modest durability in the face of worsening glycaemia over time. Therefore, most patients with T2DM will require one or more drug therapies to manage their glycaemia.

Available pharmacological agents include biguanides, sulfonylureas, thiazolidinediones, insulin, glucagon-like peptide I analogs,  $\alpha$ -glucosidase inhibitors, nonsulfonylurea insulin secretagogues, amylin agonists and dipeptidyl peptidase-IV inhibitors. Because of the variable and progressive pathophysiological changes associated with T2DM, differently acting pharmacological compounds are needed at different stages of the disease to complement lifestyle change benefits, which can be effective but difficult to maintain. Pharmacological compounds, however, have several limitations. Most of the initial improvements in glycaemia are not sustained because of continued  $\beta$  cell dysfunction (Mudaliar, Chang et al. 2003; Alivannis, Giannikouris et al. 2006; Todd and Bloom 2007; Aquilante 2010). Furthermore, many of these treatments have side effects: hypoglycemia, weight gain, gastrointestinal disturbances, peripheral edema, and potential cardiovascular effects. Therefore, new agents need to be developed to sustain glycemic control, prevent the decline in  $\beta$  cell function, assist with weight loss, improve insulin action, and have a favorable effect on cardiovascular disease.

### **3. 2. Characteristics related to the insulinotropic effect of CTX-I isolated from Vietnamese *Naja kaouthia* snake venom**

Examination of the literature has revealed that regarding the amino acid sequence, cardiotoxins constitute a family of homogeneous compounds (Chang, Huang et al. 2000) exhibiting various pharmacological functions. Although characteristics such as hemolysis (Kao, Lin et al. 2010), cytotoxicity (Lin, Su et al. 2010), and depolarization (Fletcher, Tripolitis et al. 1993) are often described for CTXs, none of them has been reported to be able to stimulate insulin release from  $\beta$  cells. In this project, we first demonstrated that CTX-I, which was isolated from the Vietnamese *Naja kaouthia* cobra snake, was able to stimulate insulin secretion from the rat  $\beta$  cell line, INS-1E. Starting from two grams of crude lyophilized venom, 22 fractions corresponding to peptides with a molecular mass ranging from 3kDa to 10kDa were collected. After screening their insulinotropic activity, four peptide fractions identified as NTTN-16,

NTTN-18, NTTN-20, and NTTN-21 were found to be able to significantly enhance insulin secretion in absence of glucose. However, only the NTTN-16 fraction had no cytotoxic or cytolytic activity against INS-1E. Moreover, the addition of glucose further enhanced the insulinotropic activity of NTTN-16. Amino acid sequencing of NTTN-16 showed that it contained 60 amino acids (Fig. 3-1). Also, the analysis revealed a 100% sequence identity with CTX-I, a molecule previously isolated from the Taiwanese *Naja kaouthia* snake venom.



**Figure 3-1. Amino acid sequence and disulfide bridge connectivity of *Naja kaouthia* CTX-I**

Following the identification of the sequence, the peptide was synthesized in our laboratory. Similar to native peptide, the synthetic version of CTX-I produced a significant stimulation of insulin release in both the absence and presence of glucose in a concentration-dependent manner. The lack of cytotoxic effect of CTX-I indicated that the insulin-releasing activity cannot be simply attributed to cell lysis or toxicity. This indicates clearly the involvement of regulated secretory pathway(s) in this insulinotropic action. Therefore, one can ask "How CTX-I triggers the insulin secretion from INS-1E cells?". It is known that an increase of free  $[Ca^{2+}]_i$  is a key trigger for insulin release (Rutter, Tsuboi et al. 2006). Also, a significant reduction in CTX-I insulinotropic effect was observed in the presence of nifedipine, a L-type  $Ca^{2+}$  channel blocker, thus suggesting that CTX-I-induced insulin stimulation was  $Ca^{2+}$ -dependent. Similarly, mastoparan, a peptide toxin isolated from wasp venom, was shown to stimulate the release of insulin by enhancing  $iCa^{2+}$  release (Baptista-Saidemberg, Saidemberg et al. 2012). Extracellular  $Ca^{2+}$  can enter cells through L-VDCCs activated by membrane depolarization, resulting in  $iCa^{2+}$  increase. Nonetheless,  $Ca^{2+}$  can be released from intracellular stores and endoplasmic reticulum (ER) (Rorsman, Eliasson et al. 2011). So far, thapsigargin, an inhibitor of  $Ca^{2+}$ -ATPase found on the ER (Noguchi, Takada et al. 2008), did not affect the CTX-I-induced  $iCa^{2+}$  increase, thereby suggesting that the  $iCa^{2+}$  enhancing action of the peptide was produced by  $Ca^{2+}$  influx via L-VDCCs. Furthermore, the stability of CTX-I-stimulated

insulin exocytosis in the presence of thapsigargin indicated that the peptide insulinotropic effect was not related to mobilization of  $\text{Ca}^{2+}$  from ER.

In  $\beta$  cells, many signals such as closing the  $\text{K}_{\text{ATP}}$  channels, activating GPCRs, opening  $\text{Ca}^{2+}$  channels, and blocking  $\text{K}^+$  and  $\text{Na}^+$  channels can activate the increase of  $[\text{Ca}^{2+}]_i$ , which eventually augment insulin exocytosis (Rutter, Tsuboi et al. 2006; Yamagata, Senokuchi et al. 2011; Schmidt, Jakab et al. 2013; Tao, Wu et al. 2013; Yao, Chen et al. 2013). Among them,  $\text{K}_{\text{ATP}}$  channels that are present in  $\beta$  cells play a key role in insulin secretion. However, CTX-I can stimulate insulin release in cells, even in absence of glucose, a condition that stops the production of ADP from ATP, thus ruling out the involvement of  $\text{K}_{\text{ATP}}$  channels in CTX-I-associated insulin exocytosis. Indeed, these channels close in presence of ADP and this allows the entry of  $i\text{Ca}^{2+}$  into the cells to induce insulin release. Some venom peptides were shown to affect  $\beta$  cell function and a few mechanisms of insulinotropic action have been characterized (Eddlestone, Komatsu et al. 1995; Deng, Kuang et al. 2009; Aguilar, Perez-Reyes et al. 2010; Baptista-Saidemberg, Saidemberg et al. 2012; Finol-Urdaneta, Remedi et al. 2012; Yao, Chen et al. 2013). Among them and similarly to CTX-I, *Agelaia* MP-I, a 14-amino acid peptide, was found to be able to enhance insulin secretion without interacting with  $\text{K}_{\text{ATP}}$  channels (Abdel-Wahab, Power et al. 2008; Baptista-Saidemberg, Saidemberg et al. 2012). These results suggested a different mechanism for those peptides, possibly by a G protein interaction or  $\text{K}^+$  channel inactivation (Abdel-Wahab, Power et al. 2008; Baptista-Saidemberg, Saidemberg et al. 2012). Due to the structural and physicochemical similarities of this peptide with mastoparan-X, a very well described G protein-interacting peptide, the authors suggested a possible mechanism for this peptide that would involve a G protein interaction (Baptista-Saidemberg, Saidemberg et al. 2012). So, could CTX-I act also *via* a GPCR ?

We know that the unique insulin secretagogue exenatide is working through GLP-1R, which are also expressed on INS-1E cells (Luo, Kong et al. 2013). Nonetheless, CTX-I did not bind to this receptor, pointing out that its insulinotropic action was not produced through GLP-1R signaling. It has been also reported that activation of  $\beta$  adrenergic receptor triggers adenylyl cyclase (AC), consequently resulting in an elevation of intracellular cAMP levels, and eventually in an increase in PKA activity (Doyle and Egan 2007). The observation that CTX-I can stimulate insulin release in the presence of either an AC inhibitor (2',5'-dideoxyadenosine) or a PKA

inhibitor (H89), ruled out the involvement of this receptor in the CTX-I mechanism of insulin exocytosis.

In  $\beta$  cells, inhibition of  $K^+$  currents should broaden the duration of action potentials that maintain the plasma membrane in a depolarized state, sustain the  $Ca^{2+}$  influx through opened  $Ca^{2+}$  channels, and consequently enhance GDIS (Wulff, Castle et al. 2009). In addition,  $iCa^{2+}$  increase will activate large-conductance  $Ca^{2+}$ -activated  $K^+$  channels (BK channels). The role of these channels in regulating  $\beta$  cell electrical activities is controversial. An earlier study of BK channels suggested that they do not play a role in regulating the electrical activity of the  $\beta$  cells (M. Kukuljan 1991). However, more recent studies demonstrated that BK channels controlled the amplitude of  $\beta$  cell action potentials (Houamed, Sweet et al. 2010; Jacobson, Mendez et al. 2010). Although inhibition of BK channels modulates cell electrical activity, these channels appear to play a negligible role in insulin exocytosis (Jacobson, Kuznetsov et al. 2007; Rorsman, Eliasson et al. 2011). Since CTX-I enhances  $iCa^{2+}$ , which is coupled to insulin secretion, this strongly suggests that the CTX-I action is probably not through these channels.

While the role of  $Ca^{2+}$ -activated  $K^+$  channels is controversial, voltage-gated  $K_v$  channels were proved to play a significant role in insulin release. Many toxins isolated from venomous organisms are able to inhibit these channels (Pennington, Harunur Rashid et al. 2012; Tao, Wu et al. 2013), thereby enhancing insulin exocytosis (Herrington, Zhou et al. 2006; Finol-Urdaneta, Remedi et al. 2012). For instance, guangxitoxin-1 inhibits 90% of delayed-rectifier voltage-gated potassium ( $K_v$ ) currents. Accordingly, guangxitoxin-1 is able to broaden  $\beta$  cell action potential, modulate  $iCa^{2+}$  oscillations, and enhance insulin secretion (Herrington, Zhou et al. 2006).

### **3. 3. Potential ability of CTX-I to inhibit $K_v$ channels**

Although CTXs are known to damage cells by interacting with lipid components in membranes (Kao, Lin et al. 2009), they can also target proteins (Lin, Lin et al. 2004). The primary toxic effect of CTXs is associated with a direct action on heart (Chien, Chiang et al. 1994; Kini and Doley 2010). CTXs were found to modulate ion channels and to cause depolarization of muscle cells (Kini and Doley 2010). Moreover, potassium channel-interacting proteins (KChIPs) can specifically bind to the cytoplasmic N-terminus of  $K_{v4}$   $\alpha$ -subunits and

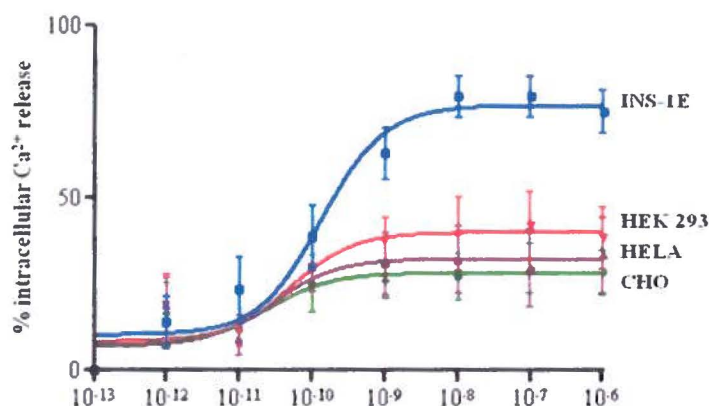


regulate the ion-current of  $K_v$  channels. Taiwanese cobra cardiotoxin 3 (CTX-3) was able to enhance the interaction between KChIP1 and  $K_v$  channels (Lin, Lin et al. 2004). These results suggested that CTX-I might modulate  $K_v$  channels. In support of this hypothesis, CTX-I exhibits structural homologies to toxins acting on potassium channels. Especially, its core domain, which incorporates loops II and III, folds into a short  $\alpha$ -helix motif that stacks against an analogous 3-strand  $\beta$ -sheet, in a similar fashion than do other toxins acting on potassium channels. Furthermore, the overlay of some functionally important residues such as Lys<sup>23</sup>, Lys<sup>50</sup> and Asn<sup>55</sup> in CTX-I, with Lys<sup>27</sup> Arg<sup>25</sup> and Asn<sup>30</sup> in some  $K_v$  blocker toxins, further suggested that CTX-I could act as a  $K_v$  blocker through a very similar mechanism.

### **3. 4. Biological features of CTX-I and the proposed mechanism of action**

CTX-I actions are cell type-dependent, as demonstrated by its  $iCa^{2+}$  stimulating ability in different cell lines, including INS-1E, HEK293T, HeLa, and CHO (Fig. 3-2). As we know,  $Ca^{2+}$  entry via L-VDCCs activated by membrane depolarization, and  $Ca^{2+}$  release from inositol-1,4,5-trisphosphate- (IP3) and ryanodine-sensitive intracellular  $Ca^{2+}$  stores, are the major sources of  $iCa^{2+}$ . Nonetheless, in  $\beta$  cells,  $Ca^{2+}$  channels appear to be the main component for  $iCa^{2+}$ . Among the tested cell lines, the maximum effect was observed in INS-1E, while the minimum was found in CHO cells. In non-excitabile cells, these  $iCa^{2+}$  signals are usually generated by  $Ca^{2+}$  release from the intracellular  $Ca^{2+}$  stores, which can be triggered by a variety of intracellular messengers. We observed that CTX-I action did not implicate  $Ca^{2+}$  from the endoplasmic reticulum (ER) because in the presence of thapsigargin, the  $Ca^{2+}$  increase effect was not influenced. Since both HEK293T and HeLa cells store  $Ca^{2+}$  into the endoplasmic reticulum (ER), the calcium increase induced by CTX-I in these cells could have been caused by a putative lytic action of CTX-I. However, our data showed that such a pore-forming activity that could lead to a drastic disruption of the plasma membrane and cell necrosis was not involved in the increase in intracellular  $Ca^{2+}$  of INS-1E cells. Hence, the difference of CTX-I-induced  $iCa^{2+}$  release observed with the cell lines could then be explained by either an absence of specificity of CTX-I for a subtype of  $K_v$  channel or a variable expression of CTX-I-targeted  $K_v$  channels. Because changes in free cytosolic  $Ca^{2+}$  concentration is the key activation signal for many

physiological processes, the finding that CTX-I can trigger calcium release makes it an interesting potential pharmacological tool.

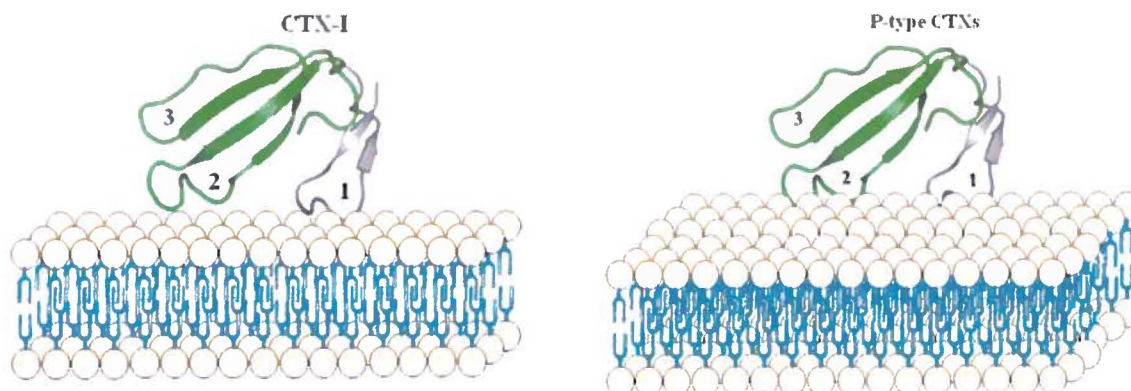


**Figure 3-2. Ca<sup>2+</sup> release effect of CTX-I in different cell lines**

The efficiency of excitation-contraction coupling in vascular smooth muscle (VSM) is controlled by the  $iCa^{2+}$  concentration (Kwan, Kwan et al. 2002). CTX-I was able to augment  $iCa^{2+}$  in INS-1E cells, but the vasoconstrictive activity of CTX-I on isolated rat aorta rings was very weak. Human urotensin II (hUII), the most potent mammalian vasoconstrictor, was used as positive control. As expected, hUII induced a concentration-dependent contraction whereas CTX-I, at 3  $\mu$ M, produced only 10% of the maximum contraction observed with hUII. In fact even at 10  $\mu$ M, CTX-I provoked only 50% of the KCl-induced contraction (Nguyen, Folch et al. 2012). This result further supports the hypothesis that CTX-I exhibits different biological effects in function of cell type.

Cardiotoxins (CTXs) are members of a group of common venom polypeptides of around 60 amino acid residues present in abundance in the *Elapid* snake family. They exert various biological effects such as cytotoxic activity including lysis of erythrocytes, and depolarization and contraction of muscular cells (Kao, Lin et al. 2010). Despite their similar sequences, CTXs display different cytotoxic potencies and hemolytic activities. CTX-I itself belongs to the S-type CTXs because of the presence of a serine residue at position 29. In agreement with studies

reporting that S-type CTXs are generally less potent hemolytic molecules than the P-type, which are characterized by the presence in their sequence of a proline moiety at position 31 (Chien, Chiang et al. 1994), CTX-I, up to  $10^{-6}$  M, was unable to induce hemolysis of human erythrocytes, which were found to be the most sensitive among mammalian red blood cells (Nguyen, Folch et al. 2012). Similar assays carried out with CTX-II and CTX-IV, which are classified in the same S-type group, were able to lyse human erythrocytes. However, the examined concentration was 200 times higher than that of CTX-I (Jang, Krishnaswamy et al. 1997). In comparison to CTX-I, CTX-II and CTX-IV, CTX-III, a P-type CTX, was able to lyse very potently most erythrocytes isolated from several mammalian, avian and reptilian species (Kao, Lin et al. 2010). The variance in erythrocyte lytic activities of these CTXs is attributed to a difference in the distribution of the positively charged residues in their structures. Thus, S-type CTXs bind to phospholipid membranes only through their lipid-binding site in loop 1, whereas P-type CTXs bind to membranes by anchoring their two lipid binding sites found in loop 1 and loop 2. This feature brings P-type CTX molecules closer to the membrane surface, which probably allows a deeper penetration of the polypeptide into the phospholipid bilayers (Fig. 3-3).



**Figure 3-3. Mode of binding of S-type CTXs and P-type CTXs**

Hence P-type CTXs bind more strongly and therefore, they induce more leakage and aggregation/fusion of phospholipids than S-type CTXs. Thus, CTX-I should be associated to a S-type character in terms of hemolytic effect. However, it is important to remain careful about this conclusion because the CTX-I concentrations that were used were ten times lower than those

used in other studies (Troiano, Gould et al. 2006; Kao, Lin et al. 2010). CTXs are also known as cytolytins, direct lytic factors and membrane active polypeptides. Cytolytins are widely used myotoxic agents (Harris 2003). In agreement with this feature, we evaluated CTX-I effects on the murine myogenic cell line C2C12. No significant increase in the percentage of early apoptotic cells was observed but a slight increase in late apoptotic cells was recorded (Nguyen, Folch et al. 2012).

Various biological activities were associated to cardiotoxins but, to the best of our knowledge, never an insulinotropic effect was reported for this class of compounds. Hence, in this project, we first demonstrated the insulin stimulating action of CTX-I, a 60-amino acid polypeptide isolated from the Vietnamese cobra *Naja kaouthia*. Although it is a complex molecule containing 4 disulfide bridges, we succeeded in synthesizing this peptide, as shown by the physicochemical characterizations. Moreover, we used this synthetic material to demonstrate the capacity of CTX-I to induce insulin production from INS-1E cells, both in the presence and absence of glucose. The effect appeared to be  $\text{Ca}^{2+}$ - and cell type-dependent. Indeed, in excitable INS-1E cells, the  $\text{Ca}^{2+}$  stimulating effect of CTX-I is probably triggered by blocking  $\text{K}^+$  channels, whereas in non-excitable cells, such as HEK-293, HeLa, and CHO, its action might be caused by a cytolytic/ necrotic ability. This character of CTX-I might open a new therapeutic avenue for T2DM. As a matter of fact, among the medications for T2DM, many insulin secretagogues stimulate the pancreas to produce more insulin, even in low plasma glucose concentrations, which can lead to hypoglycemia. This undesired effect results in cognitive impairment, unconsciousness, seizures, and even death. By contrast, hyperglycemia also causes devastating complications, including kidney failure, blindness, nerve damage, amputation, heart attack, stroke, and pregnancy-related complications. In this context, the properties of CTX-I, a new insulin-releasing compound, deserves to be further studied because it can enhance insulin release, as well in the absence as in the presence of glucose.

### **3. 5. Truncated CTX-I analogs and their biological characteristics**

The biological activities of CTXs are spreaded within three loops (Kini 2011). In particular, CTX-I, with its key loops II and III containing from 25 to 40 residues, show structural

homologies with  $K_v$  channel blockers. While loops I and II are known to target lipid bilayers of cell membranes, which consequently causes the leakage of cytosol and leads to cell death (Ma, Armugam et al. 2002), loop III is capable of depolarizing muscle cells, a process that usually involves calcium mobilization. CTX-I is able to trigger insulin release from INS-1E cells via calcium mobilization. These observations brought us to synthesize truncated CTX-I analogs with either (1) loops I and II or (2) only loop III, based on the hypothesis that these shorter peptides, containing key CTX structural features, would maintain some of the biological activities, such as the insulintropic effect. Using an orthogonal strategy, in which each cysteine pair was protected differently, we synthesized first the fragment 41-60 of CTX-I, containing the disulfide bridges in their native configuration (Fig. 3-4).



**Figure 3-4. Primary structure of CTX-I<sub>41-60</sub>**

On one hand, the fragment CTX-I<sub>41-60</sub>, which includes 20 amino acids, exerts an insulintropic effect on INS-1E cells, particularly in the presence of glucose. Similarly to CTX-I, this fragment stimulates insulin release *via* a  $Ca^{2+}$ -dependent mechanism, without implicating  $K_{A1P}$ . Interestingly, in the CTXs, residues at positions 44, 46 and 50 of loop III are important for exhibiting a depolarization activity (Chang, Huang et al. 2000). Hence, CTX-I<sub>41-60</sub> might depolarize the INS-1E cells, thereby leading to the opening of the  $Ca^{2+}$  channels. The ensuing augmentation of  $iCa^{2+}$  produces the insulin secretion. Up to  $10^{-6}$ M, CTX-I<sub>41-60</sub> was also not affecting the viability of INS-1E cells. Therefore, from the CTX-I lead compound, which contains 60 amino acids, we were successful to generate a 20-amino acid derivative still exhibiting a significant insulintropic action. As a follow-up of this finding, a systematic SAR study of this fragment, such as an alanine scan, would be highly informative and would facilitate the design of analogs showing better affinity and insulintropic activity. On the other hand, the fragment 1-39 (Fig. 3-5), containing the first two loops, was also investigated. This peptide, at  $10^{-6}$ M, was clearly cytotoxic, as it caused about 50% of cell death, compared to the control. It thus possesses the ability to penetrate into the phospholipid bilayers of the cell membranes. In fact, surprisingly, CTX-I<sub>1-39</sub> was much more toxic than the whole CTX-I molecule, which was

almost devoid of cytotoxicity in the INS-1E cells. This observation is in agreement with the literature that indicates that in CTXs, modifications of the residues localized on the surface of either loop I or II reduce dramatically the cytotoxic effect (Kini and Doley 2010).

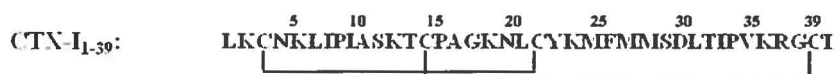


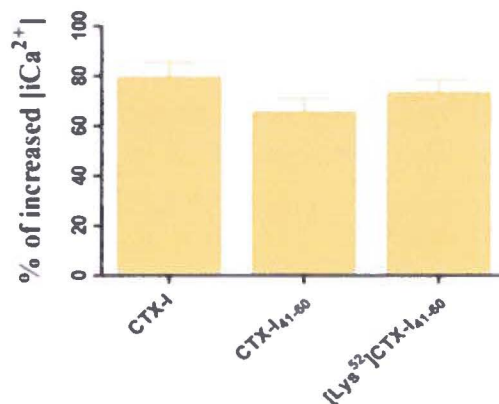
Figure 3-5. Primary structure of CTX-I<sub>1-39</sub>

### 3. 6. [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> and its advantages

Comparisons of K<sup>+</sup> channel toxins show that three positions are occupied by identical amino acids and/or residues with similar lateral chains pointing in the same direction: 1) a strictly conserved lysine in the center of the toxin, 2) an asparagine or polar residue at one end of the molecule and 3) an arginine or positively charged residue at the other end. Following structural homology studies of CTX-I with these toxins, it was noticed that Val<sup>52</sup> had a dominant and accessible position at the polypeptide surface and therefore, its replacement with a lysine residue could in fact mimic a central lysine side chain found in some K<sup>+</sup> channel blockers. Accordingly, a Lys substitution was introduced in the CTX-I C-terminal fragment 41-60 to produce the truncated analog [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>. As native CTX-I and its C-terminal fragment, this new derivative was practically devoid of any cytotoxic activity on INS-1E cells and was a potent promoter of the iCa<sup>2+</sup> influx (Fig. 3-6). This analog stimulated insulin secretion in the absence of glucose. Moreover, in the presence of glucose, its action was potentialized. The insulinotropic activity was iCa<sup>2+</sup>-dependent. As shown in Figure 3-6, this analog was as potent as CTX-I in the calcium release assay, and slightly more potent than the non-substituted fragment 41-60.

As previously mentioned, the substitution of Val<sup>52</sup> with Lys appeared to position favorably this residue that is thought to penetrate directly into the tunnel of the K<sub>v</sub> channel, a condition producing a blockade of the conduit. An <sup>86</sup>Rb<sup>+</sup> efflux assay, in which the radionuclide

acts as a tracer of potassium movement across the cell membrane, revealed that at 10 nM, [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> reduced by 25% the activity of potassium channels.

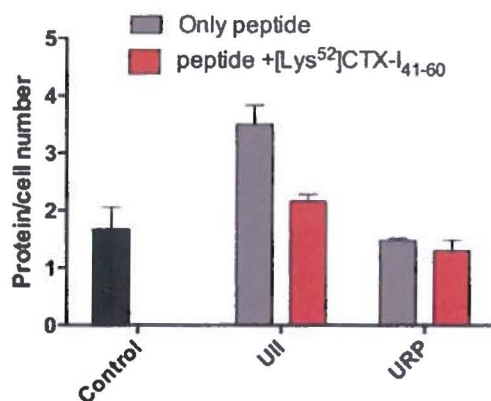


**Figure 3-6. Effects of CTX-I and its truncated derivatives on Ca<sup>2+</sup> release**

The partial blocking effect of the truncated analog could be attributed to blockade of a specific subtype of K<sub>v</sub> channels on INS-1E cells. Nevertheless, results with [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> strongly supports an important role for the positively charged residue in K<sub>v</sub> channels blocking toxins. In  $\beta$  cells, the blocking of K<sub>v</sub> channels extends the open state of the Ca<sup>2+</sup> channels, which increases  $iCa^{2+}$ , thereby leading to insulin exocytosis.

The similarity of key residues such as Lys<sup>52</sup>, Asn<sup>45</sup>, and Arg<sup>58</sup> in [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> to those present in K<sub>v1.3</sub> blockers suggests that this truncated CTX-I analog might target the same K<sub>v</sub> channel subtype. Moreover, [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> was unable to stimulate vasoconstriction of rat aorta, a phenomenon that is triggered by an increase of Ca<sup>2+</sup> through BK channels. Then, although it is not an absolute proof, this result ruled out an involvement of BK channels in the Ca<sup>2+</sup> mobilization effect. Also, it was reported that a hypertrophic phenotype could result from hyperglycemia-induced cell damages, a condition related to the progression of downstream diabetic complications (Satriano 2007). Consequently, we verified the capacity of a [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> treatment to prevent the UII-induced hypertrophy of H9C2 cells (Fig.3-7). We do not know yet what is the precise mechanism responsible for the inhibitory effect on cell hypertrophy. Nonetheless, because of its similarity to K<sub>v1.3</sub> blocking toxins, [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> might block the K<sub>v1.3</sub> channels present in the CHO cells. Alike, K<sub>v1.3</sub> channels are expressed on fat and liver cells (Choi and Hahn 2010) and therefore, through the inhibition of these voltage-gated channels,

[Lys<sup>52</sup>]CTX-I<sub>41-60</sub> could stimulate the trafficking of GLUT4 to the plasma membrane, a process known to be regulated by K<sub>v1.3</sub> channels and that can be modulated with the K<sub>v1.3</sub> blocker margatoxin (Li, Wang et al. 2006; Choi and Hahn 2010).



**Figure 3-7. Effects of [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> on urotensin-II- and URP-induced hypertrophy on H9C2 cells**

This increase of GLUT4 at the membrane favors the glucose entry into the cells and decreases the insulin resistance, thereby reducing the destruction of the  $\beta$  cells. Because the non-cytotoxic [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> is able to induce insulin secretion and prevent hypertrophy, thus limiting cardiovascular complications that are often found in type 2 diabetes, this toxin-derived peptide is an appealing lead compound for the development of a new strategy aimed at glycemic control, and prevention or delay of appearance of microvascular and macrovascular complications.

### 3.7. Perspectives

Screening biological substances to find new therapeutic compounds is a relatively new pharmacological trend. A few successful experimental works were reported with native toxins but often the selectivity or the mode of production was not optimal for therapeutic applications. Hence, structure-activity relationship studies can support the development of more potent and attractive drug molecules. BmKTX is an example. Indeed, this 38-amino acid toxin peptide



isolated from the scorpion *Buthus martensii* is a potent  $K_{v1.3}$  blocker with a  $K_d$  value of 0.2 nM. Three residues (Gly<sup>11</sup>, Ile<sup>28</sup>, and Asp<sup>33</sup>) of this peptide were substituted with Arg<sup>11</sup>, Thr<sup>28</sup>, and His<sup>33</sup>, resulting in a new analog, named ADWX-1 (Han, Yi et al. 2008). The ADWX-1 peptide blocked  $K_{v1.3}$  with picomolar affinity ( $IC_{50}$ , 1.89 pM), showing a 100-fold increase in activity compared with the native BmKTX toxin. Each substitution partially contributed to this improvement. As a matter of fact, the substitution of Asp<sup>33</sup> with His<sup>33</sup> helped BmKTX to eliminate a strong electrostatic repulsion found between Asp<sup>33</sup> and a conserved Asp residue localized in the channel that impeded the side chain of the conserved Lys<sup>26</sup> to block the pore. Also, to help the side chain of Lys<sup>26</sup> to align easily into the channel conduit, the polar Thr<sup>28</sup> residue was used to create a more favorable interaction between polar residues of the toxin and  $K_{v1.3}$ . Finally, Arg<sup>11</sup> was introduced to induce salt-bridge type interactions with four negatively charged residues contained in the tunnel structure of  $K_{v1.3}$ .



Figure 3-8. Sequence alignment of BmKTX and [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, using ClustalW

(\*) single, fully conserved residue; (:.) conservation between groups of strongly similar properties;  
 (.) conservation between groups of weakly similar properties.

The successful design of the highly potent ADWX-1 derivative of BmKTX brings us to propose an analogous SAR study with [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, in which similar substitutions to those introduced in ADWX-1 will be applied. Sequence alignment of [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> and BmKTX (Fig. 3-8) shows that Ile<sup>28</sup> and Asp<sup>33</sup> found in BmKTX correspond to Asn<sup>55</sup> and Asn<sup>60</sup>, in the truncated CTX-I analog, whereas the Gly<sup>11</sup> residue of BmKTX is at position 0 in the analog. Considering the tremendous improvement in affinity obtained with ADWX-1, which in fact is [Arg<sup>11</sup>, Thr<sup>28</sup>, His<sup>33</sup>]BmKTX, we propose as a SAR follow-up, to introduce equivalent changes in [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> and in CTX-I<sub>41-60</sub>. Single and multiple substitutions will be performed to establish the contribution of the residues (for instance [Lys<sup>52</sup>, Thr<sup>55</sup>]CTX-I<sub>41-60</sub>; [Thr<sup>55</sup>]CTX-I<sub>41-60</sub>; [Lys<sup>52</sup>, His<sup>60</sup>]CTX-I<sub>41-60</sub>; [Lys<sup>52</sup>, Thr<sup>55</sup>, His<sup>60</sup>]CTX-I<sub>41-60</sub>, etc.). Also, analogs with an Arg residue at position 0 of the CTX-I fragment (for instance, [Arg<sup>0</sup>, Lys<sup>52</sup>]CTX-I<sub>41-60</sub>; [Arg<sup>0</sup>]CTX-

I<sub>41-60</sub>; [Arg<sup>0</sup>, Lys<sup>52</sup>, Thr<sup>55</sup>, His<sup>60</sup>]CTX-I<sub>41-60</sub>, etc.) will be synthesized and evaluated. Finally, for this series of compounds, the effect of a permutation of the Lys<sup>52</sup> residue in [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> will be explored. Indeed, in BmKTX/ADWX-1, the central lysine is one residue further, a condition that might influence the affinity. Therefore, we will produce the analog [Cys<sup>52</sup>, Lys<sup>53</sup>]CTX-I<sub>41-60</sub> and check if this new configuration improves the biological properties of the CTX-I fragment.

*Which subtype(s) of K<sup>+</sup> channel is/are the target of [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> ?*

We showed that [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> is able to induce insulin secretion by blocking K<sup>+</sup> currents. Peptide-derived specific K<sup>+</sup> channel blockers, such as  $\alpha$ -dendrotoxin (blocker of K<sub>v1.1</sub>, K<sub>v1.2</sub>, and K<sub>v1.6</sub>) and charybotoxin (blocker of K<sub>v1.1</sub> and K<sub>v1.3</sub>), radiolabeled with 125-iodine, will be used in binding experiments (displacement of the radiolabeled channel blocker) to estimate the specificity of [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>. As a next step, electrophysiological experiments using a whole cell voltage clamp technique, with oocytes expressing a specific subtype of potassium channel (Orts, Peigneur et al. 2013) should allow the identification of the channel subtype(s) that is/are involved in the action of the truncated CTX-I derivative in  $\beta$  cells. Concentration-response curves will indicate the potency and selectivity of the peptide fragment and evaluation of the current-voltage relationships will help us to determine if the analog [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> acts as a gating modifier or physically blocks the channel pore.

*Does [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> stimulate the synthesis of insulin ?*

During the synthesis of insulin, insulin mRNA is translated first as a single chain precursor called preproinsulin, and the subsequent removal of its signal peptide in the endoplasmic reticulum generates proinsulin (Leibiger, Wahlander et al. 2000). Therefore, to investigate if [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> can activate the insulin synthesis, INS-1E cells will be treated with the truncated analog of CTX-I and relative expression levels of preproinsulin mRNA will be evaluated by real-time PCR. To avoid, as observed with some proteins that the mRNA level correlates poorly with the protein expression level, evaluation of translated proteins will

complement the study. Hence, levels of proinsulin will be detected using a Mercodia AB Mouse/Rat Proinsulin Elisa Kit (Chevenne, Deghmoun et al. 2011).

Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle, known as the C peptide. To generate the mature form of insulin, the C-peptide is cleaved from proinsulin in about equal amounts to insulin. Thus, the C-peptide can also be used as a marker of insulin production. So, by employing the Mercodia AB Rat C-peptide-Elisa Kit, we will further improve our evaluation of the effect of [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> on the synthesis of insulin (Asanghanwa, van Genderen et al. 2012).

*Does [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> stimulate the proliferation of  $\beta$  cells ?*

A decrease in functional  $\beta$  cell mass is a key feature of T2DM, although it is also sometimes observed with T1D patients. Consequently, an increase of  $\beta$  cell mass would benefit both type I and type II diabetics. Therefore, as an additional follow-up, cell cycle analysis will be performed on INS-1E cells treated or not with [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>. To do so, the DNA of cells will be stained with a fluorescent dye, such as propidium iodide, and then analyzed using flow cytometry to determine the ratio of cells in the G2/M phase of the cycle, a condition representing cells undergoing mitosis. Furthermore, it is reported that the increase in  $\beta$  cell proliferation is linked to upregulation of cyclins D1, D2, D3, and A, and downregulation of p21 (Velazquez-Garcia, Valle et al. 2011). Hence, to further validate our findings, using quantitative RT-PCR, cyclins D1, D2, D3, A and p21 mRNA expression will be evaluated.

*Does [Lys<sup>52</sup>]CTX-I<sub>41-60</sub> stimulate translocation of GLUT-4 ?*

Increase of the expression of GLUT4 at the plasma membrane facilitates the uptake of glucose in skeletal muscle and adipose tissue, which consequently enhances peripheral insulin sensitivity. Cell lines such as 3T3-L1 (Konstantopoulos and Molero-Navajas 2009) and C2C12 (Takazawa, Noguchi et al. 2008) can be used as model of adipose and skeletal tissues, respectively. The translocation of GLUT4 from the cytoplasm to the plasma membrane, after treatment of cells with [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, will be visualized using immunofluorescence microscopy with GLUT4-specific antibodies. To better evaluate cellular localization, fluorescent

markers of cell membrane and nuclei, such as the CellMask Plasma Membrane Stain from Life Technologies and the live-cell DNA stain Draq5 from Biostatus, will be used concomitantly.

*Does [Lys52]CTX-I41-60 stimulate insulin secretion in diabetic animals*

Goto-Kakizaki rats model was created by repetitive breeding of Wistar rats with the poorest glucose tolerance. This model is characterized by glucose intolerance and defective glucose-induced insulin secretion (Tourrel, Bailbe et al. 2002). With this model, insulin resistance was not the main initiator of hyperglycaemia, and the defective glucose metabolism is caused by aberrant beta cell mass. For that reason, this model will be chosen for our tests.

A characteristic of type 2 diabetes is the formation of amyloid within the islet tissue, which derives from islet amyloid polypeptide (IAPP). Rodent IAPP is not amyloidgenic, and thus, rodents normally do not model this aspect of the disease. However, transgenic mice have been created to express human IAPP (hIAPP) under the insulin promoter, which can form amyloid within the islets (Matveyenko and Butler 2006). A variety of hIAPP models have been created, and it has been demonstrated that increasing the expression of hIAPP increases beta cell toxicity. In addition, replicating beta cells are more susceptible to hIAPP toxicity, and thus, beta cell adaption to increased insulin demand in this model is restricted. Because of that, hIAPP mice can be used as another choice.

## **CONCLUSION**

Natural venoms provide many candidate peptides for designing potential drug leads targeting ion channels. However, strategies for improving the selectivity and potency of these peptides still remain a significant challenge. K<sub>v</sub> channels are an attractive target for the treatment

of T2D (Herrington et al. 2006), because their blockers increase the duration of glucose-induced action potentials and augment insulin release. Through the screening of peptides isolated from the venom of the *Naja kaouthia* snake, CTX-I was identified as a new insulin-releasing peptide. Moreover, structure-activity relationship analyses allowed the design of a shorter peptide, [Lys<sup>52</sup>]CTX-I<sub>41-60</sub>, still possessing potent insulin-releasing activity and devoid of cytotoxicity. Although future studies are needed to fully characterize this new analog in terms of insulintropic activity, this study constitutes the basis for the development of a new peptide-derived drug for the treatment of T2D.

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# BIO

## Supporting Information

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### **Cardiotoxin-I: An Unexpectedly Potent Insulinotropic Agent**

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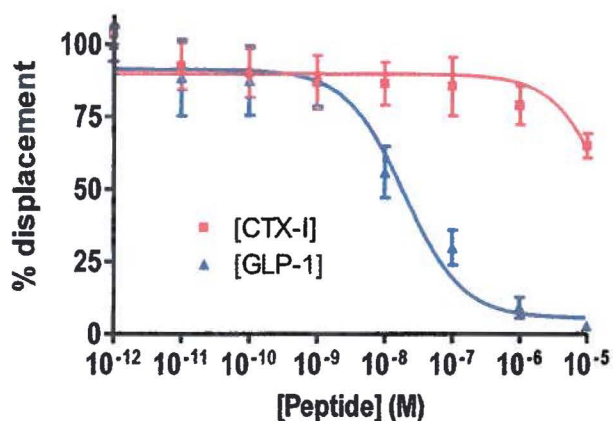
**Table S1.** Mass spectrometry analysis of isolated *Naja kaouthia* snake venom fractions.

Code number	Molecular weight (Da)	Code number	Molecular weight (Da)
NTTN-1	6956	NTTN-12	7819
NTTN-2	6842 / 6957	NTTN-13	3365 / 7628 / 7801
NTTN-3	6969	NTTN-14	6754 / 7625
NTTN-4	6934	NTTN-15	4421 / 7345 / 7602
NTTN-5	4098	NTTN-16	6695
NTTN-6	3231	NTTN-17	4086 / 6719
NTTN-7	3074 / 3132 / 3231	NTTN-18	6703
NTTN-8	3073 / 3215 / 4636 / 4705	NTTN-19	6638 / 6730
NTTN-9	4605 / 7810	NTTN-20	6637 / 6730 / 6977
NTTN-10	7811	NTTN-21	6724
NTTN-11	6380 / 7816	NTTN-22	6685/6731

**Table S2.** Identification of NTTN-16 as cardiotoxin-I (CTX-I)

Name	Species	Mass	Score	Sequence	% recovery	Identified peptide
CTX-I	<i>Naja kaouthia</i>	6696	131	LKCNKLIPIASKTCPAGKNL CYK <b>KMFMSDLTIPVKR</b> GCID VCPKNSLLVKYVCCNTDRCN	35%	K <b>MFMMSDLTIPVKR</b> K <b>MFMMSDLTIPVKR</b> G K <b>RGCIDVCPK</b> N

**Figure S1.** Binding affinity of CTX-I for GLP-1 receptors expressed on INS-1E cells



**Figure S2.** Effect of CTX-I ( $10^{-6}$  M) on intracellular calcium mobilization in INS-1E cells.

