PEPTIDE TOxin BIOENGINEERING - ADVANCEMENT OF FLUORESCENT PROBE DESIGN FOR TARGETING HUMAN K⁺ CHANNELS.

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DEDICATION

The following dissertation is dedicated to the influential people in my life, and to those who have helped to shape the person which I have become today:

• To my parents, Jeffrey and Lori Bergeron, who have provided every opportunity a child could dream for. Whose commitment to, and sacrifice for their children created a loving, nurturing environment which fostered happiness and success.

• My sister, Haley, who has showed me unwavering love and continued support, despite my shortcomings. Who has always supported my quest for knowledge and ambitions in life.

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GENERAL ABSTRACT

Transmembrane receptor proteins such as Potassium (K⁺) Channels are paramount in cellular excitability, signal transduction pathways and various pathophysiological conditions. Fundamental understanding of channel proteins, such as the Large Conductance Ca²⁺-activated Potassium (K⁺) Channel (BK), may have implications in elucidating and treating a myriad of disease states such as cancer, and those that effect electrical conduction. Classically, patch clamp analysis has been used to characterize transmembrane receptors pharmacologically, while antibodies have been utilized in varying fashions (Immunohistochemistry, Western Blots etc.) to demonstrate anatomical localization of protein expression. Fluorescent bioengineered peptide-toxins represent a paradigm shift from the current probe technology used to investigate channel expression, and pending confirmation of in vivo target specificity, could potentially be reformulated for the treatment of advanced disease states such as the primary brain tumors associated with glioblastoma multiforme (GBM). The primary objectives of the investigation are: (i) To use Solid Phase Peptide Synthesis (SPPS) to efficiently bioengineer two unique isoforms of the BK-specific scorpion venom peptide, Iberiotoxin, with a key non-native amino acid substitution to allow direct bioconjugation of a fluorophore, while retaining biological activity and pharmacological specificity. (ii) To utilize well established patch clamp techniques to determine the kinetic parameters of IbTx[D19K] and IbTx[D19K]-LC-FAM. A comparison of the Fluorometric Imaging Plate Reader (FLIPR) Membrane Potential (FMP) assay can be used to quantify dose response activity of IbTx[D19K] and IbTx[D19K]-FITC by measuring concentration induced changes in membrane potential (Ω: Ohms). A comprehensive analysis of the two techniques will establish the superior approach amenable to the current investigation. (iii) The fluorescent probe IbTx[D19K]-FITC can be used to successfully study the expression and trafficking of Ca²⁺ activated K⁺ channel (BK) in a human cell line. Successful completion of these objectives will illustrate that the development of a comprehensive evaluation platform would enhance our ability to study ion-channel physiology in real-time.
This platform could serve as a bioengineering template for the assembly of a comprehensive molecular toolbox of peptide probes, facilitating the investigation of a wide range of clinically significant ion channels. Domestic sources of peptides represent a reservoir of ready-made molecules that can be harvested for peptides amenable to the bioengineering process. In this study, local Hawaiian Conus species have been used to demonstrate effective approaches to peptide prospecting for expanding probe target distribution. These techniques, when implemented correctly, will expedite the development of potential therapies based on new found understanding of receptor physiology, and minimize adverse drug reactions during the advancement of investigational new drugs.
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**LIST OF ABBREVIATIONS**

- \(\mu g\)  
  micro gram

- AA  
  amino acid

- AChR  
  Acetylcholine receptors

- AIDS  
  acquired immunodeficiency syndrome

- BK  
  \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channel

- BuTX  
  Butantoxin

- ChTx  
  Charybdotoxin

- CTX  
  Chlorotoxin

- Da  
  dalton

- FDA  
  Food and Drug Administration

- hERG  
  Ether-a-go-go Related Gene

- g  
  gram

- HgTx  
  Hongotoxin

- HIV  
  human immunodeficiency virus

- IC\(_{50}\)  
  inhibitory constant of 50% sample

- IbTx  
  Iberiotoxin

- \(K_{2p}\)  
  Two-pore \(\text{K}^+\) channels

- \(K_a\)  
  association constant

- \(K_d\)  
  dissociation constant

- \(K_{Ca}\)  
  \(\text{Ca}^{2+}\)-regulated \(\text{K}^+\) channel

- KCN  
  Potassium (\(\text{K}^+\)) channel

- \(K_{ir}\)  
  inward rectifying \(\text{K}^+\) channel

- KTX  
  kaliotoxin

- \(K_V\)  
  voltage-gated \(\text{K}^+\) channel

- LcKTx  
  long chain KCN toxins

- LQTS  
  Long QT Syndrome

- MALDI  
  Matrix Assisted Laser Desorption Ionization

- MgTX  
  Margatoxin

- moka1  
  Mokatoxin-1

- MTX  
  Maurotoxin

- NIRF  
  near infrared fluorophore

- NMR  
  nuclear magnetic resonance

- NTX  
  Noxiustoxin

- PKPD  
  pharmacokinetic and pharmacodynamic

- PTM  
  pots-translational modification

- RP-HPLC  
  Reverse Phase-High Performance liquid chromatography

- ScyTx  
  Scyllatoxin

- SPPS  
  solid-phase peptide synthesis

- TOF  
  Time of Flight

- TsKapa  
  TsKapa
CHAPTER 1

SCORPION TOXINS SPECIFIC FOR POTASSIUM (K⁺) CHANNELS: A HISTORICAL OVERVIEW OF PEPTIDE BIOENGINEERING.

1.1. ABSTRACT

Scorpion toxins have been central to the investigation and understanding of the physiological role of potassium (K⁺) channels and their expansive function in membrane biophysics. As highly specific probes, toxins have revealed a great deal about channel structure and the correlation between mutations, altered regulation and a number of human pathologies. Radio- and fluorescently-labeled toxin isoforms have contributed to localization studies of channel subtypes in expressing cells, and have been further used in competitive displacement assays for the identification of additional novel ligands for use in research and medicine. Chimeric toxins have been designed from multiple peptide scaffolds to probe channel isoform specificity, while advanced epitope chimerization has aided in the development of novel molecular therapeutics. Peptide backbone cyclization has been utilized to enhance therapeutic efficiency by augmenting serum stability and toxin half-life in vivo as a number of K⁺-channel isoforms have been identified with essential roles in disease states ranging from HIV, T-cell mediated autoimmune disease and hypertension to various cardiac arrhythmias and Malaria. Bioengineered scorpion toxins have been monumental to the evolution of ion channel science, and are now serving as templates for the development of invaluable experimental molecular therapeutics.
1.2. AN INTRODUCTION TO POTASSIUM (K⁺) CHANNELS (KCN)

1.2.1. Transmembrane Receptor Proteins

Four main types of transmembrane receptor proteins exist, including channel-linked, enzyme-linked, intracellular and G-protein coupled receptors (Figure 1). These proteins are responsible for the initiation of physiological events or the transport of various biological species across, or through lipid bilayers or plasma membranes. Of these, channel-linked receptors, or ion-channels, comprise several categories of pore forming proteins which are found in the plasma membrane, mitochondria, nucleus, golgi-complex, and endoplasmic reticulum whereby they facilitate the flow of various ions which function to regulate ionic homeostasis, and are known to be involved in numerous cellular processes including electrolyte transport across epithelia and the propagation of neuronal action potentials (1-3). They are classified based on three factors; first, they facilitate the flow of one of four ions, Potassium (K⁺), Sodium (Na⁺), Calcium (Ca²⁺) or Chloride (Cl⁻), and second, they preferentially exhibit selectivity for at least one ion specifically. Last, their function is governed by gating which can be regulated by changes in voltage, a specific ligand, or a combination of the two (1, 4).
1.2.2. Potassium Channels, General Background

One such category of channel linked receptors, the potassium channels (KCN), are in essence, classified into four main groups based on structural characteristics, primarily the transmembrane spanning domain or alpha subunit, and subsequently broken down into families based on architectural similarities and phylogenetic relationships (6). KCNs are structurally characterized by a pore which runs the central length of the channel and consists of an essential part of all ion-channels known as the selectivity filter, a stretch of 5 amino acids which impart the rigid ion selectivity which epitomizes this type of transmembrane receptor protein (7).
The selectivity filter, observed at the channel pores most narrow choke point, is comprised of a ring of carbonyl oxygen atoms which mimic the coordination of K$^+$ ions in water and ensure that virtually no other ion species may pass (Figure 2-Right). Serving as temporary binding sites, the oxygen atoms facilitate transient dehydration of K$^+$ ions, a process that is energetically unfavorable for other ions, and thus contributes to ion specificity. There are two entry positions where the hydration shell is removed from the K$^+$ ion, four internal sites within the selectivity filter region in which K$^+$ ions are held in single file, and one final arrangement as the ions exit the channel pore where the hydration shell reforms, a process which further aids permeability and augments the rate of ion conduction (Figure 2-Left) (7, 8).

**Figure 2.** Potassium channel cross section illustrating the pore region and selectivity filter. (Left) The channel pore showing the transition of K$^+$ ions through the selectivity filter (yellow) and rehydration as they exit through the channel pore. (Right) A close up of the selectivity filter where oxygen atoms coordinate the arrangement of K$^+$ ions as they pass through the gating pore. Adapted from MacKinnon et al., 2003 (8).

Functionally, potassium (K$^+$) Channels (KCN) are extremely diverse and play an important physiological role in the secretion of various hormones and neurotransmitters,
regulating heart rate, neuronal excitation, Ca\(^{2+}\) signaling, cell volume regulation, as well as cell proliferation and migration (8-12).

1.2.3. **KCN Channel Groups & Nomenclature**

According to the International Union of Pharmacology, and the nomenclature system proposed by Gutman *et al.* 2003 (6), there are four distinct groups of potassium (K\(^+\)) channels (KCN) which are identified as K\(_V\), K\(_{Ca}\), K\(_{ir}\) or K\(_{2P}\) channels. These groups are classified based on their molecular structure, orientation within the lipid bilayer or plasma membrane and their function. In most cases, these groups are broken down further into distinct sub-groups, and finally into channel families which often have numerous members expressed as tissue specific isoforms which are differentiated by their varied composition of subunit assemblies and ligand preferences.

1.2.3.1. **Voltage-gated K\(^+\) Channels - K\(_V\)**

The first KCN group represents the voltage-gated potassium (K\(^+\)) channels which structurally consist of multiple subunits with six transmembrane spanning domains (TM) and a single pore, 6TM/1P (Figure 3) (13). This group can be broken down into three "K\(_V\)" sub-groups (Figure 3-A). The largest of the sub-groups comprises the K\(_{V1}\)-K\(_{V6}\) and K\(_{V8}\)-K\(_{V9}\) families. The second sub-grouping is small and distinct from the other K\(_V\) channels containing only the K\(_{V7}\) family, while the final K\(_V\) sub-group is made up of the K\(_{V10}\)-K\(_{V12}\) channel families.
1.2.3.2. Calcium (Ca^{2+})-activated K^+ Channels - K_{Ca}

The second group of Potassium (K^+) channels are recognized for dual regulation by both voltage and the presence of calcium (Ca^{2+}), and are known as the Calcium (Ca^{2+})-activated K^+ channels, or K_{Ca}. Structurally, K_{Ca} channels consist of six (or in rare cases seven) TM domains and form one pore, 6(7)TM/1P (Figure 4) (13). Represented by K_{Ca1}-K_{Ca5}, and named in accordance with the order of their discovery, this family can be further subdivided into two distinct phylogenetic sub-groups (Figure 4-A). One sub-group includes K_{Ca2}-K_{Ca3}, while the other comprises the K_{Ca1} and K_{Ca4} - K_{Ca5} families (6). K_{Ca} voltage gating is mediated by a voltage sensor located in the membrane spanning domain, and a cofactor, in this case Ca^{2+} which binds to the metal binding site of the cytosolic domain (15).
1.2.3.3. Inward Rectifier Channels - $K_{ir}$

The third group, known as inward rectifier ($K_{ir}$) channels, play a critical role in cardiac excitability and mutations of channels in this group have been linked to numerous cardiac abnormalities (Figure 5) (16). Channels have two TM spanning domains and form a single pore, 2TM/1P (13). $K_{ir}$ channels produce a small outward current and are crucial for terminal repolarization of the cardiac action potential (17).
1.2.3.4. Two Pore Channels - K$_{2P}$

The final group of KCN channels differ from the previously outlined groups as a result of an additional pore opening (13). Structurally it has four TM domains and two pores, 4TM/2P, making it unique amongst KCNs (Figure 6). Known as the two-pore channel or K$_{2P}$, this KCN group has a non-systematic naming system and is missing the K$_{2P}8$, 11 and 14 subfamilies (6). K$_{2P}$ channels play a physiological role in resetting the resting membrane potential as they are responsible for background leak K$^+$ currents in excitable cells (18). They have been further implicated in signal transduction mechanisms, mediated by neurotransmitters and peptide-hormones, sensory transduction, ion transport, cell volume regulation, metabolic regulation and even apoptosis (19, 20).
1.2.4. The Role of KCN in the Manifestation of Human Disease

Potassium (K\(^+\)) channels have been implicated in a diverse range of human pathologies which manifest through a number of varying mechanisms including gene mutations known as channelopathies, transcriptional abnormalities, post-translational modifications, misfolded proteins, misconfigured α/β subunit assemblies, artificially up- or down-regulated expression and trafficking defects (21-23). These alterations in native KCN expression and activity have been linked to neonatal convulsions, ataxia, asthma, T-cell mediated autoimmune disease, hypertension, various cardiac arrhythmias, epilepsy and even cancer (13, 24-29).

Typically congenital, disease states can also be drug-induced, both illicit and prescribed, as is the case with acquired long-QT syndrome (Figure 4) (30-32). As a pharmaceutical target, KCN isoforms show great promise for the development of novel drugs, ligands and molecular therapeutics (13, 24, 33). Of particular interest to this work are the Calcium (Ca\(^{2+}\))-activated potassium (K\(^+\)) channels, K\(_{Ca}\), which can be divided into two distinct subfamilies, the first consisting of intermediate (IK) and large conductance (BK)
channels, and the latter comprising small conductance (SK) types (34). Expressed ubiquitously in multi-celled organisms, K_{Ca} are present in a stunning variety of tissue types and are involved in a broad range of physiological process including hyperpolarization, neurosecretion, smooth muscle tone as well as action potential shape and frequency (34, 35).

![Figure 7. The role of the KV7.1 (IKS) and KV11.1 hERG potassium channels in the regulation of the cardiac action potential and their role in the manifestation of various cardiac arrhythmias including of Long and Short-QT Syndromes. Adapted from Wulff et al., 2009 (13).](image)

1.3. **CLINICAL IMPACT**

1.3.1. **Potassium Channels as Clinical Targets**

As previously stated, potassium channels have been implicated in a number of human pathologies such as Asthma (36), Cardiac Arrhythmia (37), T-cell mediated autoimmune disease (37, 38), immune response to infection and inflammation (39), and Hypertension (40). The bioengineering of scorpion peptides represents a valuable area of research, and a potential pool of bioactive scaffolds for the development of novel diagnostic and therapeutic tools, used in the study and treatment of the following disease states.
1.3.1.1. **Asthma**

According to the Center for Disease Control and Prevention (CDC) over 18 million people (~8% of the U.S. population) are affected by asthma. It is one of the most common long-term diseases in children, affecting ~9% of all children (roughly 7 million). Usually treated with corticoid steroids, this approach is becoming ineffective in a growing number of those afflicted.

Two potassium channels (K\(_V\)1.3 & K\(_{Ca}\)3.1), expressed in non-excitable cell types (K\(_V\)1.3: T-cells; K\(_{Ca}\)3.1: mast cells, macrophages, airway smooth muscle cells, fibroblasts and epithelial cells), have emerged as potential therapeutic targets. These KCN isoforms are widely distributed in immune and structural airway cells, playing a key role in cellular activation, proliferation and migration, major factors in asthma pathophysiology (36). As novel targets for the treatment of asthma, there are a number of known scorpion peptides that naturally interact with the described KCN isoforms, potentially making them an attractive therapeutic treatment options. Margatoxin (MgTX; *Centruroides margaritatus*) inhibits K\(_V\)1.3 with a \( K_d = 110\) pM (41), while Charybdotoxin (ChTx: *Leiurus quinquestriatus hebraeus*) and Maurotoxin (MTX; *Scorpio maurus palmatus*) inhibit K\(_{Ca}\)3.1 with respective \( K_d \)'s of 5 nM and 1 nM (36, 42). The sequences for all peptide toxins discussed in this chapter have been summated in Table 5. Non-specific binding of these toxins to K\(_{Ca}\)1.1 and K\(_V\)1.3 (ChTx) and K\(_V\)1.2 (MTX) pose a problem, as does administration.

Chimeric modeling (Section 1.7.1) may resolve issues relating to specificity, while cyclization (Section 1.7.3) could potentially improve oral bioavailability. This hypothesis developed based on the orally active K\(_V\)1.3 blocker ICA-17043 (Senicapoc), (Icagen Inc., Durham, N.C.), which inhibits late airway response and the development of bronchial hyperresponsiveness in sheep-asthma models (43).

1.3.1.2. **Cardiac Arrhythmia**

A host of KCN isoforms including K\(_V\)1.5, K\(_V\)4.2, K\(_V\)4.3, K\(_V\)7.1, and K\(_V\)11.1 have been implicated for their involvement in a number of cardiac arrhythmias such as atrial fibrillation (44), Long QT syndrome (45) and torsade de pointes (37, 46). Generically
defined as any irregularity in the normal activation sequence of the myocardium, arrhythmias and resulting sudden cardiac death are responsible for 600,000 deaths each year in the United States alone (47). Unfortunately, many currently available prescription drugs also elicit side effects that result in varying arrhythmic physiological states. In light of this, the United States Food and Drug Administration (FDA) requires pharmaceutical companies to perform cardiovascular toxicity testing on all investigational new drugs (48).

A recent survey of 29 pharmaceutical companies showed that 93% based their assessment of ventricular repolarization risk solely on a Kv11.1 competitive binding assay (49). A highly selective scorpion toxin, BeKm-1 (Buthus eupeus), is known to bind the outer vestibule of Kv11.1, and could potentially be bioengineered as a valuable probe for the investigation of hERG associated cardiac arrhythmia and a potential screen for investigational new drugs (50).

### 1.3.1.3. T-Cell Mediated Autoimmune Diseases

T-cell mediated autoimmune diseases include Multiple Sclerosis, Type-1 diabetes, rheumatoid arthritis, and psoriasis (37). Investigators in the Merck Laboratories used the scorpion peptide, Margatoxin (MgTX: Centruroides margaritatus) to show that Kv1.3 blockade can inhibit immune response in vivo by depolarizing the T-cell membrane, effectively reducing the driving force for the entry of Ca²⁺ through the calcium-release activated Ca²⁺ channel. Kv1.3 channel blockers also selectively inhibit Ca²⁺ signaling, proliferation, and in vivo migration of CCR7⁻ effector memory T-cells and therefore act as immunomodulators as opposed to immunosuppressants (which have their own obvious drawbacks). Interestingly, blockade of Kv1.3 by MgTX is the impetus for the translocation of the glucose transporter, GLUT4, to the plasma membrane which functionally improves sensitivity to insulin (51). Some inherent problems with bioengineering scorpion toxins for the treatment of Kv1.3 channelopathies include (i) the short half-life of scorpion toxins—which could potentially be overcome by peptide-backbone cyclization, or addition of stabilizing side chain functionality; (ii) the differential expression of KCN isoforms (human vs. mouse)—which could be resolved
using chimeric toxins in the well established mouse model of autoimmune disease; and (iii) toxin-channel specificity (37).

The α-KTx family of scorpion toxins represents an attractive collection of compounds, which target Kv1.3. This family can be broken down into two distinct groups, A and B, where group B toxins have a truncated N-termini and two site specific residues (His\(^9\) and Asp\(^{33}\)) which account for the functional divergence between these groups (38). These scorpion toxins could potentially serve as structural templates used to bioengineer novel molecular therapies for the treatment of Kv1.3 channelopathies such as T-cell mediated autoimmune disease.

1.3.1.4. Immune Response to Infection and Inflammation

The voltage-gated ion channels Kv1.3 and Kv1.5, which constitute the main KCN assemblage in macrophages, are involved in the activation and proliferation of leukocytes. Proliferation and activation of which trigger an induction of the outward K\(^+\) current that is under transcriptional, translational, and posttranslational control (39). Macrophages are phagocytes that serve also as antigen-presenting cells producing inflammatory and immunoactive substances that modulate the immune response, a process that is functionally stimulated by hormones and cytokines. KCN blockade by specific antagonists decreases macrophage production and proliferation thereby reducing the activation of cytokines and reducing the presentation of antigens to T-lymphocytes (52).

The major KCN isoform expressed in macrophages is a tetrameric Kv1.3/Kv1.5 hybrid. Currently, no hybrid Kv1.3/Kv1.5 toxin is known to exist, therefore molecular therapies would require the design of chimeric toxin based on Kv1.3/1.5 pore characteristics. The toxin Vm24 (Vaejovis mexicanus smithi), could potentially be utilized as a base scaffold due to its extreme affinity for Kv1.3 (\(K_d = 2.9\) pM) (53).

1.3.1.5. Hypertension

The Ca\(^{2+}\)-activated K\(^+\) channel, KCa1.1 (BK), from the gene slo, functionally regulates smooth muscle tone in pulmonary airways and vascular beds (40, 54). This is
accomplished by facilitating feedback regulation against the rise of intracellular Ca\(^{2+}\), membrane depolarization and vasoconstriction that in turn promotes outward K\(^{+}\) current, initiating membrane hyperpolarization (55). Due to this regulatory role in vascular tone, KCa1.1 has been identified as a molecular target for the treatment of hypertension.

Scorpion toxins have been classified which act on KCa1.1, as first demonstrated by ChTx(56). Unfortunately ChTx displays promiscuous behavior, cross reacting with other KCN isoforms, making it less than ideal as a therapeutic candidate. Interestingly, two short chain scorpion toxins, Iberiotoxin (IbTx: *Buthus tamulus*) (54), and BmTx3B (*Buthus martensi Karsch*) (57), in addition to one long chain scorpion toxin BmP09 (*Buthus martensi Karsch*) are well documented (58), selective inhibitors of KCa1.1. While most long chain scorpion toxins are known to be specific for Na\(^{+}\) channels, the sulfoxide produce by Met\(^{66}\) in BmP09 induces a drastic shift in target selectivity (58). Chimeric bioengineering could elucidate structural characteristics responsible for this shift in specificity towards KCa1.1, creating a highly specific, and potent toxin scaffold that could hypothetically be cyclized for therapeutic application.

Other human pathologies have been associated with varying KCN isoforms including links between KV1.1/2 and episodic ataxia, partial seizures and myokymia disorders (59), KV3.4 and Alzheimer’s (60), KV7.2–7.5 and epilepsy (61), as well as KV7.2–7.5 and the treatment of neuropathic pain (62), but are beyond the scope of this review. The link between these diseases and KCNs remains an important area of research and treatments are being investigated. Using some of the bioengineering techniques highlighted here, various scorpion toxins such as BTK-2 (*Mesobuthus tamulus*) and OdK1 (*Odonthobuthus doriae*) as well as BmTX3 (*Buthus martensi*) could prove invaluable as molecular scaffolds for the design and development of peptide therapeutics for the treatment of Episodic Ataxia (63, 64), and Alzheimer’s (65) respectively, two debilitating human pathologies. A number K\(^{+}\) channels have been identified and extensively documented as a possible therapeutic targets for various human pathologies. Using advanced techniques in peptide bioengineering, as outlined above, scorpion toxins could potentially play a major role of the development of future diagnostic and therapeutic biopharmaceuticals.
1.4. MEDICALLY RELEVANT PEPTIDE TOXINS

1.4.1. Scorpion Toxins - General Background

Scorpion toxins represent a vast bio-cache (~100,000) of pharmacologically relevant peptides that have provided an important foundation for advancing the study and understanding of various Na\(^+\), K\(^+\), Cl\(^-\) and Ca\(^{2+}\) ion channels, as well as their associated ion-channel associated diseases, or channelopathies (66). Potassium channels (KCN) in particular, have been identified as a therapeutic target for the treatment of a myriad of channelopathies ranging from asthma (36) diabetes, angina, cardiac ischemia and hypertension (67) to chronic inflammation, autoimmune disease and even cancer (9).

From their preliminary use as ligands to study the basis and selectivity of ion-channel electrophysiology, scorpion toxins have steadily evolved into a versatile bioengineering platform for designing functional probes, with a diverse range of applications including the localization of ion channels in cellular models (54), purification of novel ion-channels (56), discerning isoform selectivity (68), serving as molecular therapeutics (69), and even advancing visualization of tumor foci for improved detection and intraoperative resection of life threatening malignancies (70). These efforts are leading to an increased value in their therapeutic development and application, which serves to augment and diversify our present understanding of ion-channel pathophysiologies.
Table 1. A comprehensive listing of scorpion toxins with natural specificity for various Potassium (K+) channel targets. Adapted from Rodriguez de la Vega, et al., 2003 (71).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Channels tested</th>
<th>(k_d) (mm)</th>
<th>Toxin</th>
<th>Channels tested</th>
<th>(k_d) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charybdotoxin/o-KTx 1.1</td>
<td>Shaker (d)</td>
<td>120</td>
<td>Mauurotoxin/o-KTx 6.2</td>
<td>Shaker (d)</td>
<td>2.4</td>
</tr>
<tr>
<td>Kc,1,3 (r)</td>
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<td>Kc,1,2 (m)</td>
<td>0.12</td>
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<td></td>
</tr>
<tr>
<td>Kc,1,1 (r)</td>
<td>2.1</td>
<td>Kc,1,3 (h)</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kc,2,1 (h)</td>
<td>5</td>
<td>Kc,2,2 (h)</td>
<td>High*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iberotoxin/o-KTx 1.3</td>
<td>Kc,4,1 (d)</td>
<td>1.7</td>
<td>Kc,2,3 (h)</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Noxilatoxin/o-KTx 2.1</td>
<td>Shaker (d)</td>
<td>160</td>
<td>Kc,4,1 (h)</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Kc,1,1 (m)</td>
<td>24</td>
<td>P20/o-KTx 7.1</td>
<td>Kc,1,1 (d)</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Kc,1,3 (r)</td>
<td>0.31</td>
<td>Kc,1,3 (h)</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kc,4,1 (r)</td>
<td>480</td>
<td>P01/o-KTx 8.1</td>
<td>Kc,2,2 (h)</td>
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<td></td>
</tr>
<tr>
<td>Mangatoxin/o-KTx 2.2</td>
<td>Shaker (d)</td>
<td>160</td>
<td>Kc,2,3 (h)</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Kc,1,3 (h)</td>
<td>0.03</td>
<td>BmP20/o-KTx 9.1</td>
<td>Kc,2,3 (h)</td>
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</tr>
<tr>
<td>Kc,1,3 (r)</td>
<td>0.41</td>
<td>Cobatoxin/o-KTx 10.1</td>
<td>Shaker (d)</td>
<td>700</td>
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</tr>
<tr>
<td>Agitoxin 2/o-KTx 3.2</td>
<td>Shaker (d)</td>
<td>0.16</td>
<td>Kc,1,1 (h)</td>
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</tr>
<tr>
<td>Kc,1,1 (r)</td>
<td>0.044</td>
<td>PbTx1/1-o-KTx 11.1</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>Kc,1,3 (r)</td>
<td>0.004</td>
<td>Butanolatoxin/o-KTx 12.1</td>
<td>Shaker (d)</td>
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<td></td>
</tr>
<tr>
<td>TsKai/o-KTx 4.1</td>
<td>Kc,2,2 (h)</td>
<td>80</td>
<td>OtK2/o-KTx 13.2</td>
<td>Kc,1,1 (r)</td>
<td>High</td>
</tr>
<tr>
<td>Kc,2,3 (h)</td>
<td>197</td>
<td>Kc,1,2 (h)</td>
<td>97</td>
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<td></td>
</tr>
<tr>
<td>Kc,2,2 (h)</td>
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<td>BmKXK/1-o-KTx 14.1</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>Kc,2,3 (h)</td>
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<td>Aa1/o-KTx 15.1</td>
<td>Shaker (d)</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>P08/o-KTx 5.2</td>
<td>Kc,2,3 (h)</td>
<td>22</td>
<td>IpX (r)</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Kc,2,2 (h)</td>
<td>25</td>
<td>Marentoxin/o-KTx 16.2</td>
<td>Kc,1,1 (r)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Tamapin/o-KTx 6.4</td>
<td>Kc,2,1 (h)</td>
<td>42</td>
<td>TXKs/04/o-KTx 17.1</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Kc,2,2 (r)</td>
<td>0.02</td>
<td>Te32/1-o-KTx 18.1</td>
<td>Shaker (d)</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Kc,2,3 (r)</td>
<td>1.7</td>
<td>IcX</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P31/o-KTx 6.1</td>
<td>Shaker (d)</td>
<td>32</td>
<td>Engtourny-TK 1.1</td>
<td>IaX</td>
<td>16</td>
</tr>
<tr>
<td>Kc,1,2 (h)</td>
<td>11</td>
<td>Kc,1,1 (h)</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kc,2,2 (h)</td>
<td>100</td>
<td>BmK+/-o-KTx 2.1</td>
<td>Kc,1,1 (h)</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Kc,2,3 (h)</td>
<td>250</td>
<td>CnEng/k1-y-KTx 3.1</td>
<td>IaX</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td>Kc,2,3 (h)</td>
<td>200</td>
<td>Ca3Eng/k1-y-KTx 4.1</td>
<td>IaX</td>
<td>239</td>
<td></td>
</tr>
</tbody>
</table>

Here we focus on the process of scorpion toxin bioengineering and illustrate key developments in their evolution and application as KCN probes. To do this we provide a basic overview of scorpion biology, illustrate the use of scorpion toxins to investigate KCN; then advance to the study of peptide-toxin structure and its importance in establishing structure activity relationships, finally illustrating the transition via bioengineering to advance scorpion toxin analogues as biopharmaceuticals and therapeutic tools.

### 1.4.2. Scorpion Biology

There are approximately 1,500 individual scorpion species, most of which toxinologically represent little danger to humans (72). There are however several species (~25) which are known to be capable of causing human fatalities (73), the majority of which belong to the “old world” family Buthidae, widely distributed in the afrotropical and palaeartic ecozones (74). The most poignant example of these being the Indian Red Scorpion.
(Hottentotta tamulus), generally recognized as the most lethal of all scorpion species (75).

Physiologically, scorpions display characteristic features, but have changed little over the millennia. Appearing in the fossil record nearly 450 million years ago during the middle Silurian period, scorpions are segmented animals displaying fundamental physiology including a head (prosoma), abdomen (mesosoma) and tail (metasoma). Major appendages include chelate pedipalps (pinchers), chelicerae (morphologically related to mandibles), pectines (contact chemosensors), eight legs (arranged in four sets of two), and a telson (for venom delivery in predation and defense) at the apex of the tail (76); see Figure 8.

![Figure 8. Basic Scorpion morphology. The body is divided into three major sections, the tail or metasome (mt); the abdomen or mesosoma (ms); and the head region or prosoma (pr); Distinct structures are also highlighted including pinchers or pedipalp (pd); mandibles or chelicerae (ch); contact chemosensors (pectines - pt), and the venom apparatus or telson (t). Adapted from Weber et al. 2012 (77).](image)

The geographic distribution of scorpions is immense, with known colonization of every landmass short of Antarctica. Acclimatization to all non-boreal habitats has occurred including desert, savannah, grasslands, temperate forests, tropical forests, rain forests and intertidal zones; from as high as 5500 m in elevation, to 800 m below ground (78). Ecologically speaking, in many habitats scorpions have become fundamental in terms of predation, density, diversity and standing biomass (73).

Scorpions are the athletes of the arachnid world, dexterous in defense and prey capture. Known to be voracious eaters, their diet includes insects, spiders and other scorpions,
extending to snakes, lizards and even rodents. Amazingly, even flying insects are little match for the lightning quick pedipalps of these predators. In order to determine the strategy required for successful prey capture, scorpions are capable of analyzing the predator to prey size ratio, and evaluate how one should engage in mortal combat. Generally, small prey is crushed with the pedipalps, while larger or unsecured prey receives a sting and subsequent envenomation. Adult scorpions and those species with large pedipalps tend to crush their prey, while smaller scorpions and those with small pedipalps, sting and utilize venom in order to subdue, which may require multiple injections. Prey is often oriented head first and consumed. Interestingly arachnids, including scorpions, are thought to be the first to implement the use of toxins in prey capture/defense indicating a high degree of evolution resulting in toxin-receptor isoform selectivity (79).

Upon prey impalement, the scorpion injects a venom comprised of phyla specific neurotoxins (toxins), which are known to cause up to 5000 human fatalities a year (66, 80). Initially, with the isolation of Scorpamins in 1961 (81), the resulting biologically active toxins were thought to be multimeric proteins. In 1967, Rochat et al. found that the bioactive toxins were actually composed of single polypeptide chains, 63 to 64 amino acids (AAs) in length, held in a specific three-dimensional conformation through internal-disulfide bridging (82). Subsequent analysis has revealed the complexity of the venom which consists of a cocktail of low molecular weight proteins, oligopeptides, free AAs, nucleotides, low molecular weight salts and organic compounds (83).

1.4.3. The Use of Scorpion Toxins to Investigate Potassium Channels

The foundation for our current knowledge regarding the pharmacological action of scorpion toxins was generated mainly through electrophysiological experiments on isolated muscle and nerve cells using voltage clamping (or later, patch-clamp). Inspired by the work of Cole in 1949 (84), Hodgkin and Huxley first described ground breaking experiments, for which they would later win the Nobel Prize, whereby ionic current (in the form of resistance) was measured across the membrane of an excitable cell or tissue type, in their case, giant squid axon (Loligo vulgaris) (85). This foundational work now provides us the basis of modern electrophysiology, where we now understand that ion
channels control the flux of ions that cause the generation and propagation of nerve impulses and action potentials. Molecular dissection of ion channels using scorpion peptide toxins, has lead to direct evidence that specific mutations, phenotypically expressed as channel up/down regulation, can be correlated to changes, sometimes fatal, in the propagation and conduction of electrical impulses in the body. As evidenced by sudden death heart attacks manifested by underlying Long QT Syndrome (LQTS), a channelopathy associated with the regulation and expression of the potassium channel $K_{V}11.1$ from the human Ether-a-go-go Related Gene (hERG) (86).

The first reported interaction between scorpion venom and KCNs was in 1982, by Carbone et al. when venom preparation obtained from the Mexican scorpion *Centruroides noxius* was applied to a giant squid axon, while monitoring by means of voltage-clamp (87). The single constituent responsible for the observed activity was Noxiustoxin (NTX: *Centruroides noxius*), a 39 AA peptide purified from homogenized crude venom extract, and separated by Sephadex G-50 chromatography, followed by ion-exchange (88). Although isolated and purified, the multi-faceted potential of the toxin was not yet realized. Over the next few years, several additional scorpion toxins were reported with distinct KCN activity. With advances in chromatographic technology, combined with the establishment of single channel recordings, the use of this new class of toxins was expanded in 1985, when Miller et al. first used ChTx to identify and pharmacologically characterize a novel $Ca^{2+}$ regulated KCN, now known as $K_{Ca}1.1$, MaxiK or BK (56, 89, 90).

As the decade progressed, the number of selective peptide toxins increased. These receptor specific research probe discoveries were augmented by scorpion diversity, intraspecial variation and the introduction of novel purification techniques such as Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (91). The high-pressure approach had many advantages over traditional gravity based (low pressure) separation technologies including increased limits of detection and purity (89). The primary approach to defining and characterizing purified peptides at the time was through the use of N-terminal Edman degradation, an approach with various limitations centered around the lack of recognition for most post-translational modifications (PTMs), which
are now known to functionally augment potency and isoform selectivity of scorpion toxins (92, 93). An example of this limitation is seen in IbTx, where pyroglutamic acid exists as the N-terminal AA, a residue that is functionally blocked at its \( \alpha \)-amino function by a cyclized R-chain group.

The expanded number of ion channel probes combined with an advanced understanding of toxin-receptor interactions, facilitated the development of a technique known as pore mapping (94, 95) or molecular footprinting (96), which stimulated elucidation of structural determinants in isoform selectivity. Also used as immunogens, toxins can be utilized to produce site-directed anti-peptide antibodies. These in turn can neutralize the toxin-induced lethal effects \textit{in vivo}—an imperative aspect of anti-scorpionic serotherapy in the treatment of human envenomation. The expansion of scorpion toxin discovery represents the establishment of a novel set of tools to study medically significant KCN isoforms, and further, has stimulated the incorporation of receptor physiology in disease models (97).

1.4.4. Peptide Toxin Structure

KCN scorpion toxins vary in length from 23 to 64 AAs with estimated molecular weights usually less than 4000 Da. As highly constrained polypeptides they adhere to either the inhibitor cysteine knot or disulfide-directed \( \beta \)-hairpin folding motif (98). Extensive research was required in initial toxin studies to determine primary AA sequence, pharmacological target and establish three-dimensional structure. Proteolytic enzymes have been used to assist in defining critical disulfide bridging, with Edman degradation providing the location of these bonding pairs, a major undertaking with any multiple disulfide bond-containing constituent. This was only possible once purified target materials were available, as achieved by paper and/or column chromatography (99).

Initially, X-Ray Crystallography was one of the few techniques available for establishing intricate details regarding molecular conformation. Results were slow, as crystallization techniques required specialized training, together with large amounts of pure material (100, 101). Studies have shown that using racemic mixtures of L- and D-peptides has enhanced the ability to crystallize peptide toxins, improving the quality of molecular
models (102). Soon after, the power of NMR spectroscopy was realized beyond the standard small-molecule/organic analysis, and its application in peptide structure determination was embraced (103). Using multi-dimensional NMR spectroscopy (500 MHz) it was determined that most scorpion peptide toxins adhere to a generalized α/β (scaffold) structural conformation that includes a characteristic number and location of α-helices and β-sheets (104-107). Interestingly, using MTX, Fajloun et al. showed that point mutations could be made which shifted the disulfide bridge framework, without altering the overall α/β scaffold of the toxin (42). This revealed that the conserved α/β scaffold conformation was independent of toxin chain length, primary sequence and ion channel specificity, but importantly, that disulfide bridge patterns were paramount in tertiary structure stabilization and therefore pharmacological activity (42, 108, 109).

This was followed by the first computer based modeling, which was rudimentarily effective at predicting three dimensional structures by comparing homology sequences with previously defined toxin structures. Using graphics programs (i.e., FRODO), the quality of analysis was a reflection on the power of the hardware/software of the time, and not necessarily the data (101). Advancement in both computer systems and modeling software has significantly impacted the quality of current models (110), which can now incorporate electrostatic distribution and structural constraints (See Figure 9), characteristics of paramount importance in receptor docking (64). The modeling of bi-molecular interactions between toxin and receptor proves invaluable in establishing peptide templates for advanced probe bioengineering (111-113).

Figure 9. Computer models illustrating electrostatic distribution for three K+ channel (KCN) scorpion toxins. Adapted from Kumar et al. 2011 (64).
First applied in the study of fundamental AAs and proteins in the late 1950’s (114) and early 60’s (115, 116), mass spectrometric analysis of scorpion venoms remained undocumented until 1993 (117). This technique revolutionized peptide research by providing instant mass analysis and sequence determination, while simultaneously minimizing sample requirements. Adding to this early work, advancements in mass spectrometry (i.e., Matrix Assisted Laser Desorption Ionization, MALDI-; Time of Flight, TOF-) have mirrored the exponential nature of toxin discovery by aiding in their isolation and identification. This is limited only by scaling parameters (signal to signal ratio) of the physical equipment being employed (118). These approaches have advanced the structural determination of folding motif and the characteristic cysteine frameworks that help infer channel isoform selectivity (119). Yet care must be taken in their analysis, as with these high-energy techniques, side chain functionality and PTMs are known to be susceptible to degradation under stringent conditions (120, 121).

1.5. PEPTIDE PRODUCTION

1.5.1. Synthetic Toxin Production

Major advancements in scorpion toxin development have been dependent on chemically synthesized toxins. Significant benefits of chemical assembly include the production of non-native analogs (122, 123), truncated (124-126) or point mutated toxins (54, 127), chimeric compounds (69, 128), biotinylated molecules (54, 129), toxins labeled with fluorescent moieties (130, 131), pseudo peptides (38, 132, 133), or those containing D-amino acids (102, 134), compounds which would prove difficult or impossible to produce using recombinant technology (135). These approaches enhance functionality, utility and breadth of scorpion toxins in biomedical research.

The well-established synthetic approach pioneered by Merrifield, known as Solid Phase Peptide Synthesis (SPPS) (136), was first applied to a scorpion toxin with KCN activity in 1989. This resulted in the production of native-like material and truncated versions of NTX (126). The biologically active, synthetic peptide was desirable for two reasons, (i) a large number of scorpions (~1000) would need to be milked in order to obtain quantities of material suitable for pharmacological experimentation (1 mg), as NTX represents only
a fraction of the total protein (~1%) in the crude venom extract (88), and (ii) truncated versions of the peptide do not occur naturally (126). NTX and the truncated terminal peptide segments were assayed and their pharmacological activity compared. Interestingly, NTX1-9 was toxic to mice through various routes of administration (intraperitoneal, subcutaneous or intraventricular), although necessitating application at higher doses (200 μg/10 g mouse weight) compared to native NTX (40 μg/20 g mouse weight) to achieve a parallel pharmacological response.

Regardless of concentration, data showed that synthetic peptides retained biological activity and that their epitopes were capable of eliciting the desired pharmacological response, a concept currently being applied in cyclotide-epitope grafting for the production of novel molecular therapeutics (137). Simultaneously, both the solid-phase and solution-phase synthesis of ChTx were undertaken and completed (89, 138). SPPS inherently had direct advantages over solution-phase in terms of scheme complexity, use of less stringent reagents, and a single deprotection step (138). SPPS exploits the ability to control the quality of peptide back-bone elongation by monitoring coupling yields using Ninhydrin following the addition of each successive AA residue (139). This was not possible for the solution-phase synthesis as 5 segments were assembled individually and then ligated using water-soluble carbodiimide and one of two triazoles, either HOBt or HOOBt (138).

Additional advantages of SPPS become apparent when considering the incorporation of functional PTMs, which can be inserted into sequences thereby inferring native conformations, and resulting in the retention of biological activity (see below). There are however limitations to the synthetic production of peptides which become apparent when dealing with lengthy (>40 AAs), or extremely hydrophobic sequences, as seen in many scorpion toxins which act on Na\(^+\) channels. Long-chain synthetic scorpion toxins are difficult to fold due to the insolubility of the reduced form which leads to aggregation and the formation of polymeric material (135).

The initial KCN toxin studies focused on highly folded, tricyclic structures containing three disulfide bonds, with basic AAs arranged in clusters on the topical surface of the toxin (89). The chemical synthesis of a new class of KCN toxins with four disulfide
bridges was initiated when MTX was produced which displayed wide ranging activity on Kv1.1, Kv1.2 and Kv1.3 isoforms (140). Solid-phase strategies simplified sequential point mutation studies, otherwise known as Alanine scanning, which are commonly used to investigate key structural components of pharmacological activity. In other cases, peptide toxin yields have been increased through the incorporation of chemically selective measures to direct disulfide bond formation using specially protected AAs (i.e., Cysteine-ACM; acetamidomethyl) and the application of native chemical ligation, potentiating the use of synthetic peptide toxins in a diverse range of research areas.

1.5.2. Recombinant Toxin Production

An alternate method for the production of peptide toxins is through the use of genetic engineering and recombinant technology. Typically this utilizes a modified bacterial expression system, with modified antibiotic resistance, used to promote the selection of replicating bacteria expressing the desired target peptide. Recombinant products obtained by genetic engineering have continued to improve our knowledge in the field of scorpion toxins and ion channels; however limitations exist mainly with low yields and at present, the lack of flexibility in the incorporation of non-native side chain functionality.

Parallel to the initial KCN scorpion peptide assembly via SPPS, the first synthetic scorpion toxin gene was created, and transfected into common gram-negative bacteria, Escherichia coli (141). The difficulties encountered in this early work included amino acid codon bias between mammalian and bacterial systems (organism specificity), and problems pertaining to the establishment of 3-Dimensional conformation, or correct disulfide bond connectivity (142). Codon bias has been somewhat circumvented with the utilization of advanced E. coli cell lines (i.e., BL21-CodonPlus(DE3)-RIPL; Agilent Technologies) infused with increased copies of specialized tRNAs (142, 143), while challenges in disulfide bond formation have been overcome, to some degree, by the inclusion of protein disulfide isomerase to assist in folding (134, 142, 144).

The most significant hurdle experienced when using recombinant technology for the production of synthetic toxins is the inclusion of PTM’s, often critical components which influence folding, and establish toxin specificity and efficacy. Documented functionality
of PTM’s in toxins is diverse, ranging from N-terminal cyclization, C-terminal amidation, carboxylation, phosphorylation and glycosylation to sulfonation, bromonation and hydroxylation (142, 145, 146). Beyond C-terminal amidation, little is known about the extent and diversity of PTM’s in scorpion toxins with documented KCN activity. This area warrants additional investigation based on the potential to exploit side chain functionality in diversifying activity and application of prospective synthetic analogs for clinical use.

This problem is being addressed presently through manipulation of genetic techniques to allow incorporation of non-coded AAs that display considerable chemical diversity (147-151). Using synthetic tRNA’s, non-coded AAs have been incorporated with wide ranging functional prosthetic groups from redox-active AAs which modulate electron transfer (152, 153), residues with ketone or azide functionality which aid in chemoselective bioconjugation (154, 155), photocaged or photoisomerizable AAs which can photoregulate biological processes (156-158), metal binding AAs utilized in catalysis (149), fluorescent side chains which aid in localization and the study of channel dynamics (159-161), as well as native post translational modifications including hydroxylated and sulfonated residues (147, 162). Presently these approaches are absent in scorpion toxin production.

Assembly and purification of recombinant systems and scorpion toxins is lengthy and complex having a direct impact on rate of production, cost and yield. At present, SPPS represents a more flexible option in terms of creating complex toxins in large quantities, however, the advances made in the incorporation of non-native AAs, genetic engineering is developing momentum that will undoubtedly augment scorpion toxin research.

1.6. PROBE DESIGN

1.6.1. Structure Activity Relationship (SAR)

Defining a toxin’s 3-Dimensional conformation, and further, the biologically active binding interface is an important part in understanding the structure activity relationship (SAR) between toxin and receptor. This information becomes paramount when considering advanced peptide modifications and toxin bioengineering. Fundamentally,
Peptide toxins are short, single chain proteins interconnected by multiple disulfide bonds. The specific configuration of these disulfide bridges is indicative of compact folding and extraordinary molecular stability giving rise to target isoform selectivity and potency (105, 109, 132, 163). Several factors influence pharmacological activity in scorpion peptide toxins, these include: (i) primary sequence (encompassing charge and hydrophobic nature of residues); (ii) number and spacing of disulfide bridges; (iii) geometrical orientation of α-helices and β-sheets, and (iv) electrostatic and dipole orientation.

![α helix and β sheet structures](image)

*Figure 10. Examples of secondary structures, α-helices and β-sheets. Basic motifs found in peptide toxins. Adapted from Cooper and Hausman, 2007 (164).*

Initial structure studies examined single point mutants, created by scanning the primary sequence (excluding the cysteine framework) with the small, inert (in terms of R-group functionality) AA alanine, in an effort to isolate binding determinants while retaining native folding and structure characteristics. Each of the resulting alanine mutations can then be examined for pharmacological impact in terms of changes to $K_d$, $K_a$ or IC$_{50}$. The first alanine scan through a KCN scorpion peptide was performed by Park and Miller (1992) on ChTx (165). That investigation highlighted the importance of several surface reactive residues, most notable was Lys$^{27}$, a highly conserved AA later identified as a
critical pore-occluding characteristic in numerous KCN toxins (165-168). These studies advanced the mapping of bimolecular interaction and demonstrated the powerful insights provided by the implementation of computer modeling (111, 169). At present there is a correlation (amongst the scorpion toxins) between dipole orientation and channel isoform specificity, while observed electrostatic features present in the contact surface influence toxin potency (64, 170). This phenomenon can be observed in Figure 9 which demonstrate the capability of current computer modeling techniques and also illustrates electrostatic patterns which characterize KCN scorpion toxins.

Correlation between disulfide framework pattern, amino acid spacing and molecular target has been observed in scorpion peptides (122, 123, 132), as well as other classes of toxins from various natural sources (i.e., Conus - (171)). KCN scorpion toxins do not deviate from this trend, with a wide range of disulfide framework patterns falling into five main classes as seen in Table 2. The manipulation of differential AA spacing and PTMs within these established KCN scorpion toxin familial disulfide frameworks should be undertaken based on the documented potential to harness this information for the bioengineering and production of synthetic templates for prospective molecular therapies of various human pathologies (68, 105, 132). This approach has been broached in the production of a fully synthetic, successful peptide, Mokatoxin-1 (moka1), specific for Kv1.3 and active in the regulation of immune T-cells (133). As well as a disulfide variant of MTX (MTXp11) whose disulfide bridge pattern has been altered without effecting the overall conformation of the peptide (108). Interestingly, target affinity of the toxin was altered, despite the lack of change in the secondary structure.
Table 2. Established disulfide frameworks recognized within scorpion peptides with demonstrated KCN activity.

<table>
<thead>
<tr>
<th>Class</th>
<th>Example</th>
<th>Disulfide Connectivity</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>KTX</td>
<td>GVEINVKCSGSPQCLPKDAGMRFGKCMNKRCCHCTP</td>
<td>$K_{Ca}$</td>
<td>(163)</td>
</tr>
<tr>
<td>II</td>
<td>CnERG1</td>
<td>DRDSCVDKSRCAKYGYYQECQDCCKNAGHNGGTCMFFKCKCA</td>
<td>$K_{V11}$</td>
<td>(172)</td>
</tr>
<tr>
<td>III</td>
<td>MTX</td>
<td>V5CTGSKDYPACRKGPTCNACINKCSCKCYS$^*$</td>
<td>$K_{V1}$, $K_{Ca2}$, $K_{Ca3.1}$</td>
<td>(108)</td>
</tr>
<tr>
<td>IV</td>
<td>HsTx1</td>
<td>ASCRTPKDADPRKETGCPTYKCMNKRCNKCNRC$^*$</td>
<td>$K_{V1.1}$, $K_{V1.3}$</td>
<td>(42)</td>
</tr>
<tr>
<td>V</td>
<td>TtBut-Tx</td>
<td>WCSTCLDLAGSRECYDPCFKAFRAHGCNKMKRCNCTY</td>
<td>Shaker B</td>
<td>(173)</td>
</tr>
</tbody>
</table>

The above outlined disulfide frameworks further represent a potential starting point for the design of novel, isoform specific diagnostic and therapeutic peptides which target directed channel isoforms. As diagnostic probes, scorpion toxins have many advantages over primary antibody based detection systems, including specificity, and the ability to bind non-fixed tissue and cell samples. The inherent structure of KCN scorpion toxins makes them ideal candidates to be used as molecular probes for the multivaried investigation of ion channel function and physiology. Advanced bioengineering has further illuminated the potential clinical application of therapeutic KCN scorpion peptides for the treatment of various human pathologies.

1.6.2. Radiolabeled Scorpion Toxins and Receptor Localization

The development of anatomical channel localization studies and pharmacological displacement assays were facilitated by the development and production of radiolabeled derivatives of KCN scorpion toxins. Understanding both primary and secondary structure when designing and bioengineering diagnostic KCN probes from scorpion toxin scaffolds is vital in order to maintain native conformation and in turn biological activity.

The first radiolabeled scorpion toxin developed was Iodinated ChTx, used to investigate the $K_{Ca1.1}$ (MaxiK; BK) (174). Native ChTx was isolated from crude venom and labeled
with $[^{125}\text{I}]\text{NaI}$ using the IODO-GEN method (175) to produce $[^{125}\text{I}]\text{ChTx}$, where monoidination occurred at the C-terminal Tyrosine residue (Tyr$^{36}$). This novel bioengineered peptide toxin probe demonstrated how chemical modifications can be made to the primary structure while maintaining topology of the secondary structure and retaining biological activity, thus expanding their applicability in research. Subsequently, the ability to radiolabel a synthetic peptide was demonstrated when Scyllatoxin, also known as Leiurotoxin-1 (ScyTx; Leiurus quinquestriatus hebraeus) was radiolabeled at His$^{31}$ (IODO-GEN method) and used for autoradiographic investigation of Small conductance Ca$^{2+}$ activated K$^+$ channel (SK) in rat brain sections (176).

The binding of the scorpion peptide IbTx to $K_{Ca1.1}$ depends on interaction of Tyr$^{36}$ with the channel pore, resulting in the inability to utilize this commonly targeted AA for derivatization. Recombinant IbTx with a single point mutation, D19C, was iodinated using sulphydryl reactive $N$-[${}^3\text{H}$]ethylmaleimide at the post-oxidative free thiol (177). A two-point mutant (D19Y/Y36F) was also devised and produced recombinantly and the expressed peptide, IbTx-D19Y/Y36F, was iodinated at position Tyr$^{19}$ ($[^{125}\text{I}]\text{Na}$ in the presence of Enzymobeads; Bio-Rad) (178). The latter probe was successfully used to investigate the expression of $K_{Ca1.1}$ in bovine tracheal smooth muscle sarcolemmal membranes.

As technology progressed, so too did the production of other radiolabeled KCN toxin-probes. They have been used to investigate the expression and distribution of a number of KCN isoforms in a wide range of cell types, contributing uniquely to understanding the role of KCN in membrane bioenergetics and their subsequent effects on human physiology. Table 3 illustrates the usefulness, diversity and progression of radiolabeled KCN probes, and in some cases the extent of bioengineering undertaken to achieve their potential as effective molecular calipers.
Table 3. List of radiolabeled KCN probes used in the investigation of receptor tissue localization/expression as well as electrophysiology in terms of binding kinetics.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Modification</th>
<th>Name</th>
<th>Label</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChTx</td>
<td>native</td>
<td>$[^{125}\text{I}]$ChTX</td>
<td>$[^{125}\text{I}]-\text{Y36}$</td>
<td>K$<em>{Ca}$, K$</em>{V}$</td>
<td>(174)</td>
</tr>
<tr>
<td></td>
<td>R19C</td>
<td>$[^{3}\text{H}]$-ChTx-R19C</td>
<td>$[^{3}\text{H}]-$C19</td>
<td>K$<em>{Ca}$, K$</em>{V}$</td>
<td>(179)</td>
</tr>
<tr>
<td>ScyTx</td>
<td>F2Y</td>
<td>$[^{125}\text{I}]$-[Tyr2]ScyTx</td>
<td>$[^{125}\text{I}]-\text{Y2}$</td>
<td>K$_{Ca}$</td>
<td>(180)</td>
</tr>
<tr>
<td>MgTX</td>
<td>native</td>
<td>$[^{125}\text{I}]$MgTX</td>
<td>$[^{125}\text{I}]-\text{Y37}$</td>
<td>K$_{V}1.2, 1.3$</td>
<td>(41)</td>
</tr>
<tr>
<td></td>
<td>V5Y, Y36F</td>
<td>[mono-iodo-Tyr5, Phe36]-IbTx</td>
<td>$[^{125}\text{I}]-\text{Y5}$</td>
<td>K$_{Ca}1.1$(BK)</td>
<td>(181)</td>
</tr>
<tr>
<td>IbTx</td>
<td>D19C</td>
<td>$[^{3}\text{H}]$-IbTx-D19C</td>
<td>$[^{3}\text{H}]-$C19</td>
<td>K$_{Ca}1.1$(BK)</td>
<td>(177)</td>
</tr>
<tr>
<td></td>
<td>D19Y/Y36F</td>
<td>$[^{125}\text{I}]$IbTx-(D19Y/Y36F)</td>
<td>$[^{125}\text{I}]-\text{Y19}$</td>
<td>K$_{Ca}1.1$(BK)</td>
<td>(178)</td>
</tr>
<tr>
<td>BeKm-1</td>
<td>native</td>
<td>$[^{127}\text{I}]$-BeKm-1</td>
<td>$[^{127}\text{I}]-\text{Y11}$</td>
<td>K$_{V}11.1$(hERG)</td>
<td>(182)</td>
</tr>
<tr>
<td>HgTx</td>
<td>A19Y/Y37F</td>
<td>$[^{125}\text{I}]$HgTx$_{1}$-A19Y/Y37F</td>
<td>$[^{125}\text{I}]-\text{Y19}$</td>
<td>K$_{V}1.1/2/3/6$</td>
<td>(183)</td>
</tr>
<tr>
<td>BmTX3</td>
<td>native</td>
<td>$[^{125}\text{I}]$-sBmTX3</td>
<td>$[^{125}\text{I}]-\text{Y37}$</td>
<td>A-type current</td>
<td>(184)</td>
</tr>
<tr>
<td>NTX</td>
<td>native</td>
<td>$[^{125}\text{I}]$Noxiustoxin</td>
<td>$[^{125}\text{I}]-\text{Y37}$</td>
<td>K$_{V}1.1$</td>
<td>(185)</td>
</tr>
<tr>
<td>KTX</td>
<td>KTX(1-37)</td>
<td>$[^{125}\text{I}]-\text{KTX}(1-37)$</td>
<td>$[^{125}\text{I}]-\text{H34}$</td>
<td>K$_{Ca}$</td>
<td>(186)</td>
</tr>
<tr>
<td>Lqh III</td>
<td>native</td>
<td>$[^{125}\text{I}]$Leuirutoxin III</td>
<td>$[^{125}\text{I}]-\text{Y8}$</td>
<td>K$_{V}1.1$</td>
<td>(185)</td>
</tr>
</tbody>
</table>

1.6.3. Bioconjugation (Fluorescence)

In recent years there has been a paradigm shift moving away from the use of radiolabeled toxins due to several factors including the inherent difficulties and dangers associated with employing radioactivity, and the need for a more versatile probe platform. The need persists however, to study and understand ion channel physiology in order to incorporate innovative mechanisms into human pathological disease models, potentially elucidating novel treatment options and routes of therapeutic intervention. In turn, this has led to major advancements in the design and chemical synthesis of fluorescently bioengineered toxin-probes that have alleviated many of the issues experienced when working with radiolabeled toxins.

Stimulated by the introduction of novel chemical strategies for the attachment of varying prosthetic groups, a process referred to as bioconjugation, as well as a growing selection of available fluorophores, covalent and ionic (biotin-streptavidin complexes) techniques have been developed to bioconjugate fluorescent moieties to KCN scorpion toxins for the classical purpose of detecting and localizing ion channel isoforms in vivo, as illustrated by ChTx-biotin (129). Initially, basic strategies developed with standard N-terminal derivatization, and advanced to modification of the ε-amino functionality of introduced...
lysine residues (amine chemistry) (187-189). Alternatively, yet with its own unique set of conditions and difficulties, the use of thiols as targeted conjugation sites have gained popularity (thiol chemistry) (130, 179, 190). Another approach currently being developed for fluorescent incorporation is phosphate-azide “click” chemistry (191-193).

In 1993, Robitaille et al. employed modified bioconjugation strategies previously applied to the ω-conotoxin GV1A (194, 195) to produce the first biotinylated scorpion toxin with directed KCN activity (129). Prepared via SPPS, ChTx-biotin was created via direct chemical conjugation of Succinimidyl-6-(biotinamido)hexanoate to the exposed N-terminal (129). The ChTx-biotin probe was used to investigate the distribution of Ca\(^{2+}\)-gated K\(^+\) channels in relation to Ca\(^{2+}\) channels and release sites of transmitters at the neuromuscular junction. Shimony et al. (1994), used a bacterial expression system to produce a folded biologically active mutant of ChTx (i.e., ChTx[R19C]). This novel bioengineered peptide retained an unpaired “spinster cysteine” for derivatization based on thiol directed chemistry (179). The point of the insertion was determined by in-depth structural analysis, and ChTx[R19C] was produced with the strong nucleophilic thiolate mutation made at a location far from the interactive surface, again in order to retain functional activity of the toxin during the bioconjugation of fluorescent labels.

Attributed partly to the entrenched use of primary antibodies in the scientific culture, combined with potential difficulties in constructing and purifying fluorescent bioengineered peptide probes, most have been used recently in the periphery in ion channel research. Recently however, limitations in antibody technology, in terms of dealing with live-cell processes, are appearing which represent obstacles in advancing live-cell visualization. There is a growing desire for advanced imaging and localization technology, this requirement has emerged specifically from the increased need to investigate real-time physiological processes in vivo, including channel protein translation, trafficking and surface expression.

In 2002, a fluorescent version of Hongotoxin (HgTx; Centruroides limbatus) was produced synthetically (130), and subsequently used in receptor localization experiments utilizing ultrasensitive microscopy known as the single-dye tracing technique (196). A number of different fluorescent moieties were successfully conjugated to the bioactive
toxin while simultaneously maintaining potency, namely Cy3, Alexa488 and Alexa546, although a number of other dyes were also conjugated with varying degrees of success. From this study, it was determined that both positioning within the toxin as well as selection of fluorophore are crucial to producing successful fluorescent-toxin probes. The resulting fluorescent toxin was proficient in localizing Kv1.3 in Rat brain sections (130), as well as Jurkat cells at the single molecule level (196).

Refuting common conception that amine modification of lysine destroys pharmacological activity of scorpion toxins targeting K+ channels (129), Bingham et al. (2006) synthesized a biotin derivative of IbTx by replacing aspartic acid (Asp19), with the non-native AA N-ε-(d-biotin-6-amidocaproate)-L-lysine (Anaspec; IbTxD19K-LC-Biotin), incorporating a spacing component, or “linker” which provided steric clearance from the peptide backbone. This eliminated perturbation of the three-dimensional tertiary structure of the toxin, which was essentially locked-in by the disulfide framework. Coupled with the secondary fluorophore, Alexa488-Streptavidin (Molecular Probes), the probe enabled visualization of BK (hslo) channel in stably transfected HEK293 using standard epifluorescent microscopy (54). Akcan et al. (2012) further substantiated this bioengineering approach with the bioconjugation of a near infrared fluorophore (NIRF) to lysine 27 of Chlorotoxin (CTX; Leiurus quinquestriatus quinquestriatus) which dramatically increased serum half-life when assayed (189). From this groundwork, in vivo channel localization and visualization can be advanced exponentially with the application of superior imaging techniques (i.e., confocal microscopy; see Figure 3). Use of such bioengineered toxins allows for the visualization of cellular surface details, including banding and patch like clustering of ion channels in expressing cells (54, 129).
To demonstrate the utility of fluorescent KCN probes the most compatible toxins and bioconjugation chemistry have been utilized in the process of manipulation and structural bioengineering of peptide toxin candidates. This work has allowed for the investigation of up- and down-regulation of receptor subtypes in connection with various human pathologies and disease states. A complete list of fluorescent KCN toxins can be seen in Table 4.
Table 4. Table of fluorescently labeled peptide scorpion toxins specific for various potassium channel isoforms.

<table>
<thead>
<tr>
<th>Toxin Base Sequence</th>
<th>Mutation</th>
<th>Bioconjugate</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChTx</td>
<td>N-term R19C</td>
<td>ChTx-biotin</td>
<td>K\textsubscript{Ca}, K\textsubscript{V}</td>
<td>(129)</td>
</tr>
<tr>
<td></td>
<td>R19C</td>
<td>Rhodamine-ChTx-R19C</td>
<td>K\textsubscript{Ca}, K\textsubscript{V}</td>
<td>(179)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Cy5</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Cy3</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Alex488</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Alex546</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Alex594</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Alex660</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX\textsubscript{1}-A19C-Alex680</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HgTX(1)-A19C-Cy5</td>
<td>K\textsubscript{V}1.1/2/3/6</td>
<td>(196)</td>
</tr>
<tr>
<td>HgTx</td>
<td>A19C</td>
<td>IbTx-D19C-Alexa488</td>
<td>K\textsubscript{Ca}1.1(BK)</td>
<td>(131)</td>
</tr>
<tr>
<td></td>
<td>D19C</td>
<td>IbTx-LC-biotin</td>
<td>K\textsubscript{Ca}1.1(BK)</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td>D19K</td>
<td>IbTx-LC-FAM</td>
<td>K\textsubscript{Ca}1.1(BK)</td>
<td>(198)</td>
</tr>
</tbody>
</table>

1.7. ADVANCED METHODS IN PROBE DESIGN

1.7.1. Scorpion Toxin Chimeras

A novel approach for determining the structural components that define scorpion toxin isoform selectivity in KCN is the design, bioengineering and investigation of chimeric peptide toxins. This approach was initiated when IbTx and ChTx were targeted as chimeric candidates due to their differential selectivity for KCN isoforms, K\textsubscript{Ca}1.1 and K\textsubscript{V}1.3 respectively, and high AA homology (~68%), in the presence of a conserved disulfide framework. The major consideration in choice of peptide candidates was the demonstrated selectivity of IbTx (K\textsubscript{Ca}1.1), and the promiscuity of ChTx, which inhibits both K\textsubscript{Ca}1.1 and K\textsubscript{V}1.3. Thus combining individual block sections of AA sequences/secondary structural features was a natural development for defining their unique pharmacological properties. The resulting chimeric toxin revealed that both N- and C-terminal domains are central in governing isoform selectivity, demonstrating that terminal sequences can be modified to induce artificial interactions (69).
A metal binding chimera of ChTx was designed and synthesized to examine the extent of modification possible in scorpion toxin scaffolds (199). Nine residues, based on the Zn$^{2+}$ binding site of carbonic anhydrase B, were inserted into the native ChTx sequence. The resulting chimera successfully binds metal ions confirming the functional versatility of the scorpion toxin scaffold. This provides a template for the assembly of conformationally constrained peptide libraries with both therapeutic and industrial potential.

Acetylcholine receptors (AChR) have been identified as potential clinical targets as their binding evokes flaccid paralysis, and important characteristic in anesthetics (200). Certain snake venoms contain curaremimetic neurotoxins which bind to the AChR at the postsynaptic membranes of skeletal muscle including Toxin a, from the venom of black-necked spitting cobra (Naja nigricollis). The small disulfide-stabilized structure of the scorpion toxin ChTx was used as a basic scaffold for the active residues of loop II in Toxin a, corresponding to the central part of the curaremimetic site (201). The chimera represents a less toxic, yet equally effective immunogen for the production of toxin neutralizing antibodies, an important element in vaccine design and production. The
rationale design of synthetic vaccines could be improved significantly by utilizing this approach, resulting in increased accessibility through decreased costs.

Several Noxiustoxin-Iberiotoxin (NTX-IbTx) chimeras were developed to investigate how the length of the α-carbon backbone, and differences in the α/β turn configuration contribute to target isoform selectivity (123, 128). Results indicate that backbone elongation alters steric interaction with the channel pore, while differences in the α/β turn alter the geometry of the toxin-binding surface. By tailoring toxin size, and geometric orientation of charge distribution, peptides can be designed to discriminate between KCN isoforms based on vestibule architecture.

Another peptide toxin chimera, Tsk-MTX, has been structurally bioengineered and identified as a lead prototype scaffold for synthetic toxin-probes that could be designed de novo to produce potent blockers with unique specificity and/or affinity toward targeted KCN isoforms (202). The 38 residue peptide scaffold was derived from MTX, a 34 AA residue peptide cross linked by four disulfide bonds, active on both Kv and KCa isoforms (140), and TsKapa (Tsk; Buthidae Tityus serrulatus), a 35 AA residue peptide cross linked by 3 disulfide bonds and active only of KCa channels (203). The critical difference impacting target specificity between the two toxins is the presence of either two or three β-sheets respectively.

In its native form, CTX is active on small conductance Cl− channels and displays no affinity for K+ channels. It has been widely used as a Cl− channel blocker as well as a glioma specific marker with diagnostic and therapeutic potential (204). CTX has structural characteristics in common with α-KTx peptide toxins including Lys27 and a similar α/β scaffold. A CTX-AgTx2 chimera was constructed from the base scaffold of CTX, and three residues composing the β-sheet of Agitoxin-2 (AgTx2; Leiurus quinquestriatus) which infers Kv1 specificity (205). This work showed that structural modifications could be made successfully which induce non-native inhibitory properties, an exciting prospect for protein design and engineering.

In an effort to determine whether increasing the molecular contacts between a toxin and an ion channel impacts affinity, M’barek et al. (2005) used a gain of function approach to
evaluate this hypothesis (108). A chimeric peptide toxin was developed based on the template of MTX_{Pi1}, a disulfide variant of Maurotoxin. The N-terminal of “native” MTX_{Pi1} was replaced with that of Butantoxin (BuTX; *Tityus serrulatus*, *Tityus bahiensis*, and *Tityus stigmurus*), a 40 AA peptide constrained by 4 disulfide bridges and specificity for K_{V1.2} with an IC_{50} = 165 nM (108, 206). Transfer of 9 N-terminal residues from BuTX to MTX_{Pi1} resulted in a novel chimeric peptide with 5 disulfide bridges. The active toxin BuTX-MTX_{Pi1} displayed a 22-fold greater affinity for K_{V1.2} than “native” MTX_{Pi1}, revealing that increasing the molecular contacts between toxin and receptor, can significantly enhance affinity for KCN target isoforms, an interesting concept in terms of bioengineering.

The chimeric peptide, AgTx2-MTX, was produced synthetically consisting of a truncated AgTx2 sequence which targets K_{V}, conjugated with the complete MTX sequence which targets both K_{V} and K_{Ca} type channels (68). Interestingly, the chimeric peptide displayed uncharacteristic disulfide bridging patterns, producing a more potent peptide than either parent toxin, and found to be active of K_{V1.2}. This is yet another example of the applied use of synthetic-bioengineered peptide toxins as viable molecular probes for studying varying KCN isoforms characteristics.

### 1.7.2. Native Chemical Ligation

Native chemical ligation is a powerful synthetic strategy allowing peptide synthesis to move beyond the constraints of the standard length linear SPPS (~50–60 AAs) (207). Proven by the chemical and semi-synthesis of large functional proteins (>10 kDa), including the ion channel KcsA (208, 209), this strategy has also facilitated the incorporation of small synthetic peptide fragments into larger recombinant proteins, allowing the integration of various non-native AAs and fluorophore derivatives (102, 210).

In the past, SPPS methodology has been partially restricted by the difficulty of ensuring complete \(N^\alpha\)-deprotection and AA acylation throughout a long peptide chain, especially in syntheses over 50 residues (211). This limitation is mostly attributed to their length, hydrophobic nature and high degree of cysteine content. Alleviating these issues, native
chemical ligation employs a C-terminal thioesterified peptide that is chemically ligated to a peptide bearing a free N-terminal cysteine, this occurring sequentially using small manageable size linear peptide blocks. This process was initially performed principally using Boc-SPPS (211), however Li and co-workers described a novel approach leading to the production of a peptide thioester via Fmoc-SPPS (212). Unfortunately difficulties remained, as this method produced significant aminolysis of the primary peptide thioester species. Methodologies have been sequentially improved, demonstrated by the synthesis of the scorpion toxin II Hexapeptide (211).

In 2009, using strategies developed for native chemical ligation, Bingham et al. produced a bioengineered, two point mutation of Iberiotoxin with greatly increased yields (as much as 12-fold over standard linear synthesis). This increase can be attributed to the ease of synthesis and purification, a consequence of limiting side-reactions which functionally reduces accumulation of stepwise deletions, hydrophobic aggregation, and disulfide cross-linking (213). All of which are common occurrences encountered during extended syntheses that detrimentally affect yields. Native chemical ligation also proves useful when seeking to generate racemic mixtures of peptide toxins to ease the production of crystals for X-ray determination of overall three-dimensional toxin structure. Synthetic D-kalitoxin and L-kalitoxin enantiomers (KTX; Androctonus mauretanicus mauretanicus) were synthesized by NCL and used to produce highly ordered racemic crystals, which facilitated X-ray structural determination (102, 125). Crystallization proved difficult when solely employing the L-protein isoform.

This synthetic approach provides a straightforward method for the preparation of multiple milligram quantities of peptide toxins, required for the production of superior quality electron density maps and structural models with lower error. This information is extremely valuable in the design and bioengineering of future KCN-specific scaffolds. Furthermore, this approach is successfully being utilized in the production of cyclized toxins for the development of potential molecular therapeutics (214, 215).
1.7.3. **Peptide Backbone Cyclization**

Scorpion toxin scaffolds specific for K⁺ channels have tremendous potential in rational drug design and development (216). Unfortunately, they suffer from a number of disadvantages in vivo owing to their inherent peptidic structure including proteolytic and thermal degradation, as well as poor bioavailability. Peptide backbone cyclization, or N-to C-terminal ligation, is capable of dramatically increasing this therapeutic potential by minimizing aforementioned drawbacks (217, 218). To achieve this, several factors must be considered in regard to the toxin candidate including, (i) distance between termini; (ii) relative orientation of termini; (iii) flexibility of the termini; (iv) position of disulfide bonds and (v) understanding of key residues involved in biological activity or binding (219). This bioengineering approach has thus far proven successful exemplified by the production of in vitro fluorescent probes (189), the design of therapeutic scaffolds for epitope chimerization (220), and the re-engineering of conotoxins for the treatment of neuropathic pain (221).

Naturally occurring in plants, animals and bacteria, circular peptides known as cyclotides are hypothesized as a natural component in host defense systems. Displaying a diverse range of biopharmaceutical properties including anti-HIV, antimicrobial and insecticidal activities (137), cyclotides are inherently stable, surmounting many of the primary obstacles observed when bioengineering peptides as potential drug therapies (219). Structurally, cyclotides are comprised of a circular peptide backbone, and a disulfide framework commonly referred to as a cysteine knot motif (222, 223). Using techniques developed for native chemical ligation (224), several therapeutic, acyclic toxins have been cyclized, including the scorpion peptide CTX (218), and the χ-conotoxin Mr1A, the latter of which has subsequently entered clinical trials as a potential treatment for neuropathic pain (225, 226).

As with other peptide toxins, cyclotides can be bioconjugated to fluorescent moieties, however in this form N-terminal labeling is not possible and limitations are presented by the stericus of the parent sequence backbone. Potentially, topologically accessible lysine residues can be used, or can be inserted into functionally inert region of the parent sequence. Akcan et al. (2011) attempted to determine if fluorophore bioconjugation and
cyclization of CTX would improve the stability of CTX bioconjugates by eliminating the accessibility of terminal AAs to circulating peptidases \((189)\). Utilizing native chemical ligation, Cyclic-CTX was synthesized by Boc chemistry. A seven-residue linker (GAGAAGG) was required for cyclization due to the proximity of the termini \((11.7 \pm 1.5 \text{ Å})\) \((189)\). Glycine and alanine were chosen for the linker due to their small size, and based on the premise that side chain functionality would not perturb the native three-dimensional conformation of the parent peptide backbone. Later bioconjugation of near infrared fluorophores to create fluorescent probes increased serum stability of cyclic toxins from 70% to 90% \((\text{after a 24h incubation})\) \((189)\).

![Diagram of cyclization of Chlorotoxin (CTX)](image)

Figure 13. Cyclization of the scorpion peptide Chlorotoxin (CTX). A flexible 7 residue linker (GAGAAGG) was used to bridge the ~11.7 Å gap between the N- and C-terminals. Adapted from Akcan et al. 2011 \((189)\).

The ability to deter proteolysis represents a major bioengineering advancement when viewing peptides as molecular therapeutics. However, consideration must be given to efficacy, pharmacokinetics, pharmacodynamics and toxicity - which are influenced by a peptide’s ability to retain its structural integrity. As reported earlier, serum stability of CTX was subsequently improved by peptide bioengineering. This suggests that proteolytic cleavage can be reduced following fluorescent bioconjugation and cyclization, while \textit{in vivo} excretion and metabolism remain unaltered \((189)\). This positive attribute infers that conformational stability will facilitate oral administration, while not changing
pharmacokinetic and pharmacodynamic (PK/PD) parameters. This advances bioengineering peptide therapeutics, making them biologically safe, when compared to their non-cyclic counterparts - this likely having an impact on how we view the future progression of scorpion toxin therapeutics.

Augmenting this technology, using cyclic peptide backbone frameworks in a process known as grafting, synthetic therapeutics can be bioengineered or ‘stitched together’ with pre-determined targets, much like chimeric toxins. This has developed into what has become one of the most successful forms of rational drug design to date. This approach combines small bioactive peptide epitopes with known molecular scaffolds, stabilized by cyclic frameworks thereby increasing oral bioavailability and potency (215). This approach has been verified by grafting analogs of the plant peptide Kalata B1 (220). Cyclization and grafting techniques now present the opportunity to re-engineer medically relevant KCN scorpion toxins to produce stable, directed, molecular therapeutics with novel KCN targets.

1.8. POTASSIUM CHANNEL TOXINS IN THE CLINIC - BIOENGINEERING DIAGNOSTIC AND THERAPEUTIC PEPTIDES

1.8.1. Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS)

1.8.1.1. Charybdotoxin (ChTx)

In 2007, the CDC estimated almost 36,000 new AIDS cases in the United States alone. This number contributes to the nearly half-million people already living and dealing with this stigmatic autoimmune disease. These numbers fail to account for the 1.2 million HIV infections, a retroviral infection which has yet to develop into full-blown AIDS. HIV/AIDS cases in the United States pale in comparison to the staggering numbers documented in Sub-Saharan Africa, most notably, South Africa (227). Current treatments, although successful, report significant adverse side effects including nausea, fatigue, emotional distress and headaches (228). The development of novel therapies could enhance the quality of life for those individuals receiving treatment for HIV/AIDS infections.
A ChTx based miniprotein was used in phage epitope randomization studies to identify synthetic peptide constructs that bind gp120 and block the gp120-CD4 interaction. Inhibiting this mechanism stops infection by binding CD4 with gp120 and preventing CD4-induced conformational isomerization which initiates co-receptor binding and viral cell fusion (229). This technology may have potential therapeutic value for the treatment of HIV infection and AIDS.

TXM1, is a novel bioengineered peptide mimetic based on ChTx, where the CD4 CDR2 loop sequence, Gln-Gly-Ser-Phe (40QGSF43), was substituted for residues 24–27 in the \( \beta \)-turn of ChTx (229). Experimentally, the ChTx chimera/FLAG gene was assembled by T7 DNA polymerase extension and found to bind selectively to gp120 (ELISA competition and biosensor direct binding assays).

These results demonstrate that CD4 recognition mimetics can be bioengineered within a \( \beta \)-turn of the ChTx miniprotein scaffold, a development which illustrates how scorpion toxins can be synthesized to contain epitopes imitating the cell receptor CD4. With a diverse pool of scorpion toxin scaffolds available with similar \( \alpha/\beta \) motifs, the extension of the phage randomization approach to other scaffolds in this family could fuel the development of a number mimetic antagonists suitable for the treatment of HIV/AIDS as well as other debilitating human pathologies.

1.8.1.2. Scyllatoxin (ScyTx)

In another example of chimeric scorpion toxin-epitope assembly for the treatment of HIV/AIDS, nine residues from CD4, vital to the binding of HIV-1, were inserted into a homologous region (\( \beta \)-hairpin loop) of ScyTx in a three step process (230). This was functionally achieved by transferring the side chains of the CD4-gp120 binding interface to a structurally equivalent region of the ScyTx scaffold.

The small size of these miniproteins infers many advantages including ease of synthesis and manipulation, including the ability to incorporate fluorophores and non-native AAs. This technology is especially valuable due to the large size of the intricate CD4 binding
surface that typically renders the rational bioengineering of small mimetics that mimic these interfaces, daunting if not impossible.

1.8.2. **T-cell Mediated Autoimmune Disease**

1.8.2.1. **OSK-1[E16K, K20D]**

The treatment of T-cell mediated autoimmune disease has undergone many iterations over the years, however diseases such as diabetes and multiple sclerosis remain untreated, affecting large percentages of the human population. The two-point mutation, Bioengineered OSK-1[E16K, K20D] (IC$_{50} =$ 3 pM) has been designed as a novel treatment for disease processes regulated by $K_v 1.3$. OSK-1[E16K, K20D] is a synthetic toxin based on the template sequence of OSK-1 ($\alpha$-KTx3.7: *Orthochirus scrobiculosus*) which displays an increased affinity (~5×) for $K_v 1.3$ over the native peptide (IC$_{50} =$ 14 pM) (231). Several residues (Arg$_{12}$, Glu$_{16}$, Lys$_{20}$ and Thr$_{36}$) were explored for their impact on toxin-receptor pharmacology. Two mutations were ultimately introduced into the native sequence during Fmoc-SPPS, E16K and K20D, having the greatest impact on affinity. Unfortunately, the synthetic bioengineered OSK-1[E16K, K20D] toxin retains biological activity towards other channel isoforms ($K_{Ca3.1}$), a major drawback for a potential clinical therapeutic.

1.8.2.2. **ADWX-1**

Several T-cell mediated autoimmune diseases are mediated by $K_v 1.3$ including multiple sclerosis, Type-1 diabetes, rheumatoid arthritis, and psoriasis (232). A potent, and highly selective inhibitor of $K_v 1.3$, the molecular therapeutic ADWX-1 (IC$_{50} =$ 1.89 pM), was designed based on the structure of BmKTX (*Buthus Martensi*) (232, 233). Three non-native mutations were engineered into ADWX-1 (G11R, I28T, and D33H) which increases its affinity 100-fold over native BmKTX ($K_d =$ 0.2 nM). Several factors were considered in this modern, and poignant example of rational drug design including distribution of peptide functional residues, residue polarity, and especially the negatively charged residues known to be intimately involved in peptide docking (38).
ADWX-1 (refer to sequence in Table 5) was rationally designed in a three-step process, aided by pharmacological data obtained by alanine-scanning mutagenesis and computational modeling. Step 1: The distribution of negatively charged residues in the BmKTX peptide template were adjusted in order to increase proximity of Lys$^{26}$ to the channel mouth, augmenting the ability of ADWX-1 to occlude the pore of Kv1.3. The mutation D33H reduced strong electrostatic repulsion between the neighboring AA, Asp$^{33}$, and a conserved aspartic acid residue in the S-6 linker domain of Kv1.3. Step 2: The polar interaction between residues in the toxin and channel were strengthened. The key residue that occludes the pore of Kv1.3 is Lys$^{26}$, therefore the neighboring hydrophobic AA, Ile$^{28}$, was replaced with a polar threonine residue which resulted in a hydrogen bond between Thr$^{28}$ (ADWX-1) and Asp$^{402}$ (Kv1.3) creating a more suitable docking environment. Step 3: A positively charged Arginine residue was introduced in the beginning of the $\alpha$-helix domain (G11R) in order to create a salt bridge between four negatively charged residues in the upper most portion of Kv1.3 with the critical residue identified as Asp$^{386}$ (232).

Although challenges remain in improving selectivity and potency of potential peptide-toxin therapeutics, this work illustrates the utility of peptide-channel complex modeling in the development of diagnostic and therapeutic agents from existing toxin scaffolds.

### 1.8.2.3. Mokatoxin-1 (moka-1)

Using a scaffold-based/target-biased strategy developed by Takacs et al. (2009), a phage display was used to design and identify a synthetic peptide, Mokatoxin-1 (moka-1), that is highly specific for Kv1.3 (133). Block of Kv1.3 in T-cells counters the effects of anti-CD3/28 stimulation and suppresses effector cytokine secretion without cross-reactive gastrointestinal hyperactivity (side effects). Other scorpion toxins have been isolated which block Kv1.3 (i.e., KTX), however due to lack of channel specificity (KTX also blocks Kv1.1 and Kv1.2), undesirable side effects are produced (i.e., diarrhea).

A phage display library of 11,200 de novo proteins (as well as 20 of the original $\alpha$-KTx peptides) was produced based on the $\alpha$-KTx family scaffold (comprising 31 toxins; KTX being the primary template) and sorted using a high throughput selection strategy (133).
Three criteria were identified as critical in order to successfully utilize this technique, in addition to conservation of cysteine framework, bioengineered toxin variants must: (i) synthesize and fold correctly (regardless of phage anchorage); (ii) express topically on the phage surface so that toxins are accessible to the target (by genetic linkage to phage particle coat protein pIII); and (iii) successfully bind target channel despite phage cargo.

The peptides produced displayed three major structural domains dubbed A (AAs 1–12), B (AAs 15–26), and C (AAs 30–38). Bioengineered Mokatoxin-1 has four positive charges at neutral pH, and is effectively chimeric with its domains corresponding to A (Ce3 toxin from *Centruroides elegans*), B (is present in AgTx2 & Agitoxin-3 (AgTx3; *Buthus occitanus*)), and C (is present in both ChTx and Lq2 from the venom of *Leiurus quinquestriatus*) (see Figure 14). Once identified and structurally defined, Mokatoxin-1 was synthesized by Boc-SPPS and confirmed pharmacologically to be highly selective for *K*<sub>V</sub>1.3 (133). Selectivity was based primarily on several key residues (a combination of residues not seen in any of the parent toxins): Leu<sup>7</sup> and Pro<sup>8</sup> (Ce3), Phe<sup>22</sup> (AgTx2), and Arg<sup>31</sup> and Tyr<sup>33</sup> (ChTx), making Mokatoxin-1 an extremely successful example of rational drug design, and a template for further bioengineering of KCN scorpion toxins for the development of novel biopharmaceuticals.

![Mokatoxin-1 Bioengineering](image)

**Figure 14.** The chimeric bioengineering of Mokatoxin-1 consisting of three individual structural segments originating from the toxins Ce3, AgTx2 and CTX respectively. Adapted from Takacs *et al.* 2009 (133).
Current methods to identify and develop molecular therapeutics based on KCN scorpion toxins have been retarded because isolating crude venom, shotgun venom gland sequencing, and site-directed mutagenesis are slow, and have thus yielded little in terms of tangible results. Because of this, efforts to rationally improve toxin-receptor selectivity are being explored.

1.8.3. Malaria

Annually there are upwards of 300 million new diagnoses of Malaria worldwide, one million of which can be fatal (234). Artemisinin-based combination therapies are currently the standard treatment. Recently however, increasing resistance to existing pharmaceuticals has exacerbated the need for innovative antimalarial agents and treatment strategies.

A novel, low affinity KCN blocker named MeuTXKβ1 (Mesobuthus eupeus) has been isolated which produces unique cytolytic effects (235). This activity is diverse, including inhibition of the parasite Plasmodium berghei (a gametocyte producer strain), binding of KCNs on rat brain synaptosomes and lysing of bacterium and various eukaryotic cells (oocytes and erythrocytes). Amazingly, a truncated N-terminus analog, N(1–21), displays anti-plasmodium activity without inducing haemolysis. This unique characteristic qualifies the peptide as an ideal template for the treatment of malaria, and opens the door for the development of transgenic malaria-resistant mosquitoes. Advanced bioengineering including cyclization, could potentiate the serum stability and half-life of MeuTXKβ1-N(1–21), furthering its distinctive qualifications as a valuable molecular therapeutic. A second antimalarial scorpion toxin, meucin-24, has been identified from the genome (cDNA) of Mesobuthus eupeus (236). Although the biological target of meucin-24 has not been confirmed, it shares high sequence identity with the N-terminus of a family of long chain KCN toxins (LcKTx), and represents an additional candidate for the development of antimalarial strategies.
1.9. CONCLUSION

As illustrated, scorpion toxins specific for potassium (K\(^+\)) channels have been vital to biomedical research resulting in the identification, localization and classification of novel channel types and families, as well as the creation of structural models pertaining to gating and binding of numerous ligands. The development of radiolabeled and fluorescent diagnostic probes has enhanced our understanding of receptor pharmacology and membrane biophysics: information that has culminated in the development of pathological models involving KCN isoforms.

The exhaustive use of combinatorial libraries by the pharmaceutical industry, and the historical role of naturally derived molecules in the clinic, has amplified interest in biologics in recent years. Stimulated by our understanding of the importance of potassium channels in human physiology, improved technology and innovative techniques including cyclization and chimeric assembly, has expanded the role of peptide toxins in medical research.

As this work progresses, superior bioengineering techniques are continually being developed as evidenced by the use of phage epitope randomization and the scaffold-based/target-biased strategy in the design of novel molecules. The sophisticated application of these peptides will undoubtedly lead to a dramatic advance in the utilization of diagnostic and therapeutic KCN scorpion toxins in molecular medicine. The design, synthesis, purification and characterization of a novel KCN probe is discussed at length in Chapter 2.
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<tr>
<th>TOXIN</th>
<th>SEQUENCE</th>
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<tr>
<td>Margatoxin (MgTX)</td>
<td>TINVKCTSPQCLPCKACQFAQGSAAGAKCMNGKCKCYPH</td>
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<td>Charybdotoxin (ChTx)</td>
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<td>Maurotoxin (MTX)</td>
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<td>Vn24</td>
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<td>Iberiotoxin (IbTx)</td>
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Table 5. Sequence information for all toxins discussed in Chapter 1.
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<th>Definition</th>
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<tbody>
<tr>
<td>αα (AA)</td>
<td>amino acid</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>μM</td>
<td>micro molar</td>
</tr>
<tr>
<td>μL</td>
<td>micro liter</td>
</tr>
<tr>
<td>AAA</td>
<td>amino acid analysis</td>
</tr>
<tr>
<td>ACN (MeCN)</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AQC</td>
<td>6-aminoquinolyl-N-hydroxy-succinimidyl carbamate</td>
</tr>
<tr>
<td>BK</td>
<td>Large conductance Ca(^{2+})-activated K(^+) channel</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>CD</td>
<td>cytosolic domain</td>
</tr>
<tr>
<td>ChTx</td>
<td>Charybdotoxin</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>N-ethyl diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>EDT</td>
<td>1,2-ethanedithiol</td>
</tr>
<tr>
<td>FAM</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>FDA</td>
<td>Food &amp; Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl chloride</td>
</tr>
<tr>
<td>Fmoc-Ahx-OH</td>
<td>N(_e)-Fmoc-aminohexanoic acid</td>
</tr>
<tr>
<td>Fmoc-Arg(Pbf)-OH</td>
<td>N(_e)-Fmoc-N(_o)((2,2,4,6,7)-pentamethyl</td>
</tr>
<tr>
<td>Fmoc-Asp(tBu)-OH</td>
<td>Fmoc-L-aspartic acid 4-\textit{tert}-butyl ester</td>
</tr>
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<td>Fmoc-Cys(Trt)-OH</td>
<td>N(_o)-Fmoc-S-trityl-L-cysteine</td>
</tr>
<tr>
<td>Fmoc-Gln(Trt)-OH</td>
<td>N(_o)-Fmoc-(N(_o)-\textit{trityl-L-glutamine</td>
</tr>
<tr>
<td>Fmoc-Glu(OtBu)-OH</td>
<td>Fmoc-L-glutamic acid 5-\textit{tert}-butyl ester</td>
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<tr>
<td>Fmoc-Lys(Boc)-OH</td>
<td>N(_o)-Fmoc-N(_e)-Boc-L-lysine</td>
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<tr>
<td>Fmoc-Lys(Mtt)-OH</td>
<td>N(_o)-Fmoc-N(_e)-4-methyltrityl-L-lysine</td>
</tr>
<tr>
<td>Fmoc-Ser(tBu)-OH</td>
<td>Fmoc-\textit{O-tert}-butyl-L-serine</td>
</tr>
<tr>
<td>Fmoc-Thr(tBu)-OH</td>
<td>N-Fmoc-\textit{O-tert}-butyl-L-threonine</td>
</tr>
<tr>
<td>Fmoc-Trp(boc)-OH</td>
<td>N(<em>o)-Fmoc-(N(</em>{\text{in}})-Boc-L-tryptophan</td>
</tr>
<tr>
<td>Fmoc-Tyr(tBu)-OH</td>
<td>Fmoc-\textit{O-tert}-butyl-L-tyrosine</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[Bis-(dimethylamino)methyl]iumyl]-1H-1,2,3-triazolo[4,5-b]pyridine-3-oxide hexafluorophosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
</tbody>
</table>
HCTU 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-
tetramethylammonium hexafluorophosphate
HF Hydrogen Fluoride
HOAt 1-hydroxy-7-azabenzotriazole
HOBt 1-hydroxybenzotriazole
IbTx Iberiotoxin
Kc,1.1 Large conductance Ca\(^{2+}\)-activated K\(^{+}\) channel
KCN Potassium (K\(^{+}\)) channel
Kd dissociation constant
LC aminohexanoic acid
MaxiK Large conductance Ca\(^{2+}\)-activated K\(^{+}\) channel
MeOH methanol
meq g\(^{-1}\) milliequivalents per gram
mg milligram
mmole millimole
min minutes
Mtt 4-methyltrityl
NNAA non-native amino acid
N\(_2\) nitrogen gas
PGD pore-gated domain
PITC phenylisothiocyanate
PTM post translational modification
RP-HPLC reverse phase-high performance liquid chromatography
R\(_t\) retention time
SEC Size exclusion chromatography
SPPS solid phase peptide synthesis
HBF\(_4\) tetrafluoroboric acid
IEC Ion-exchange chromatography
TFA trifluoroacetic acid
TFMSa trimethyl sulfonic acid
TMSBr trimethylsilyl bromide
TMSOTf trimethylsilyl trifluoromethanesulfonate
VSD voltage sensing domain
CHAPTER 2

THE DESIGN AND SYNTHESIS OF A DIRECT CONJUGATE FLUORESCENT BK CHANNEL PROBE BASED ON THE IBERIOTOXIN SCAFFOLD.

2.1. ABSTRACT

One family of transmembrane receptor proteins known as ion channels are paramount in cellular excitability, signal transduction pathways and various pathophysiological conditions. Fundamental understanding of ion channels such as the Large Conductance Ca\(^{2+}\)-activated Potassium (K\(^{+}\)) Channel (BK) may have implications in elucidating and treating a multitude of disease states such as cancer, and those that effect electrical conduction. In the past, high affinity peptide toxins, with radio-labels or secondary fluorophores have been used as probes to understand the pharmacological structure, function and anatomical localization of potassium (K\(^{+}\)) channels. Recent advances in probe design, synthetic strategies and bioconjugation techniques may allow for the bioengineering of a novel, direct conjugate fluorescent probe, based on the structure of the scorpion peptide Iberiotoxin (IbTx), which is capable of targeting and binding the BK channel both \textit{In vitro} and \textit{In vivo}. Manual solid phase peptide synthesis has been employed to facilitate the production of a non-natural peptide, IbTx[D19K] (4243.1 Da.), based on the template of the scorpion toxin IbTx. In the proceeding chapter, the parent molecule, IbTx[D19K] has been used to explore various bioconjugation strategies resulting in the development and production of IbTx[D19K]-LC-FAM (4734.6 Da.), a fluorescent probe which was purified via RP-HPLC and mass confirmed by ESI-MS. The fluorescent BK channel probe was then quantified via amino acid analysis for future electrophysiological characterization, and pharmacological evaluation prior to the investigation of BK channel expression in human glioblastoma.
2.2. INTRODUCTION

2.2.1. The Large Conductance Ca\(^{2+}\)-activated Potassium Channel (BK)

2.2.1.1. BK Channel - General Background

The calcium (Ca\(^{2+}\))-activated potassium (K\(^{+}\)) channel (K\(_{Ca}\)) isoform which is the central focus of the current investigation is the Large Conductance Calcium (Ca\(^{2+}\))-activated Potassium channel, K\(_{Ca}1.1\) (MaxiK), or BK. BK is important due to its intimate involvement in a number of human pathologies including cardiovascular disease, Atherosclerosis, Hypertension, Diabetes, various neurological disorders and Cancer. Encoded by the human slo gene (slo1, KCNMA1, 10q22), and known to have at least 20 splice variants (UniProtKB), BK channels activate in response to membrane depolarization and binding of intracellular Ca\(^{2+}\) and Mg\(^{2+}\) (Figure 1) (1-5). This action subsequently results in termination of the action potential, producing fast after-hyperpolarization and ultimately resulting in the closure of Ca\(^{2+}\) channels (6). Additionally, their function is known to be stimulated by molecular interaction with secondary ligands including carbon monoxide, heme and estrogen (7, 8).

Channel opening has been described to occur via one of two autonomous mechanisms, either voltage-dependant or Ca\(^{2+}\)-dependant activation. Early cloning experiments revealed multiple, regularly spaced Arginine (Arg) residues in the S4 transmembrane segment, a known characteristic of K\(_{V}\) channels and indicative of a voltage-dependant mechanism (7). This structural characteristic confirmed early views on the intrinsic nature of voltage-dependant activation for K\(_{Ca}\). It was additionally shown that each \(\alpha\)-subunit contains two high affinity calcium (Ca\(^{2+}\)) binding sites located in the cytoplasmic domain. These two sites have differing binding affinities for Ca\(^{2+}\), \(K_d = 0.8\) and 11.0 \(\mu\)M respectively, and confer channel activating sensitivity to the docking of calcium (9, 10).

Expressed ubiquitously, the importance of the BK channel is evident by its association with a number of essential cellular functions including neuronal electrical activity, smooth muscle contractility, hormonal secretion, skeletal muscle contractility, hair cell tuning, Ca\(^{2+}\) homeostasis and cellular excitability (1, 10, 11). Due to this omnipresent nature, BK has been identified as a clinical target for the treatment of arthritis, asthma,
autism, epilepsy, hypertension, inflammation, mental retardation, paroxysmal dyskinesia, schizophrenia and even certain cancers including aggressive glioma (1, 3, 12, 13).

Figure 1. A molecular model of the large conductance Ca\(^{2+}\)-activated potassium channel (BK). (A) Calcium bound open configuration. (B) Closed configuration lacking calcium. Adapted from Yuan et al. 2011 (10).

### 2.2.1.2. BK Channel Structure

Structurally, the BK channel is a tetrameric assembly of pore-forming α-subunits (gene: slo) that co-assemble with a set of four multifunctional accessory β-subunits, where the auxiliary β-subunits often show tissue-specific distribution and are partially responsible for the variation of channel characteristics including Ca\(^{2+}\) sensitivity (6, 14-16). This concept is discussed in detail below in Section 2.2.1.3.
Table 1. List of BK channel β-subunits, which functionally associate with a tetrameric assembly of slo-1 α-subunits, and their known tissue of specificity.

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Known Tissue Expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKβ1</td>
<td>KCNB1</td>
<td>Smooth muscle</td>
<td>(3, 14, 17)</td>
</tr>
<tr>
<td>BKβ2</td>
<td>KCNB2</td>
<td>Brain, Chromaffin cells</td>
<td>(3, 6, 17)</td>
</tr>
<tr>
<td>BKβ3</td>
<td>KCNB3</td>
<td>Testes, Pancreas, Spleen, Brain</td>
<td>(3, 6, 17, 18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Splice variant</td>
<td></td>
</tr>
<tr>
<td>BKβ3a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKβ3b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKβ3c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKβ3d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKβ4</td>
<td>KCNB4</td>
<td>Brain</td>
<td>(6, 17, 19)</td>
</tr>
</tbody>
</table>

The full assembly has three distinct structural domains including the voltage sensing domain (VSD), the pore-gate domain (PGD) and the cytosolic domain (CD) (Figure 2) (7). The VSD is responsible for monitoring membrane potential while the PGD opens and closes in order to control cellular permeability to potassium (K⁺). Collectively, the VSD and the PGD are known as the membrane spanning domain, formed primarily by transmembrane segments S1-S4 and S5-S6 respectively. The CD is composed of two regulator of K⁺-conductance subdomains, located near the C-terminus, known as RCK1 & 2, each having a distinct Ca²⁺ binding site (3). The fully assembled, functional channel displays a large single channel conductance of ~100-300 pS where channel gating involves two major steps (7). First, the sensor domain recognizes a stimulus which is followed by the second phase where the channel undergoes a conformational change opening the activation gate, an overall process known as coupling (7).
2.2.1.3. BK Channel Expression

Expressed predominantly at the cell surface, trafficking of BK to the plasma membrane occurs via the cis- and trans-Golgi networks (1). Here the channel may undergo post-translational modification in the form of phosphorylation, glycosylation, myristoylation and palmitoylation (20, 21). Intracellular expression has also been documented where BK has been observed in the nucleus, mitochondria, golgi complex and endoplasmic reticulum (22-27). Intracellular BK channels are thought to function as Ca\(^{2+}\) sensors and possibly play a role in ischemic cardioprotection and possibly in an internal organelle signaling mechanism (1).

The location of BK channel expression has been documented in a diverse array of tissues ranging from nervous to non-nervous cell types including neurons, neuroendocrine cells, epithelial cells, endothelial cells, sensory cells and smooth muscle cells (1, 11). Tissue specific expression is governed by two mechanisms consisting of alternative splicing and differential association with a β-subunit. Occurring at four distinct sites in the C-terminal
region of the BK channel protein, alternative RNA splicing further contributes to channel diversity creating a unique compliment of individual isoforms, each exhibiting distinctive expression patterns (28). The directed expression of these isoforms is further controlled by differential association with a subset of β-subunits (Table 1).

2.2.1.4. BK Channel as a Pharmaceutical Target

As alluded to previously, Potassium (K+) channels have become an increasingly relevant pharmaceutical target for the treatment of diverse range of human disease states including cardiovascular disease, diabetes, neurological disorders, epilepsy and cancer (17, 29). As a pharmaceutical target, BK has come into focus specifically for its involvement in a vast, characteristic set of pathophysiological states (Table 2). This becomes magnified as aging progresses due to the altered expression and function of BK channel over time, with complete knockout ultimately being associated with multiple organ failures (8, 21).

Table 2. List of disease states associated with the BK potassium channel.

<table>
<thead>
<tr>
<th>Disease States &amp; BK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia (21)</td>
</tr>
<tr>
<td>Diabetes (12, 21, 30, 31)</td>
</tr>
<tr>
<td>Incontinence (8, 12, 32)</td>
</tr>
<tr>
<td>Neurological disease (33)</td>
</tr>
<tr>
<td>-Mental retardation (3, 34)</td>
</tr>
<tr>
<td>-Schizophrenia (3, 35)</td>
</tr>
<tr>
<td>-Autism (3, 34)</td>
</tr>
<tr>
<td>Cancer</td>
</tr>
<tr>
<td>-Osteosarcoma (29, 38)</td>
</tr>
<tr>
<td>-Glioblastoma (1, 29, 39, 40)</td>
</tr>
<tr>
<td>-Ovarian (29, 41)</td>
</tr>
<tr>
<td>-Breast (29, 42)</td>
</tr>
<tr>
<td>-Prostate (29, 43)</td>
</tr>
<tr>
<td>Paroxysmal dyskinesia (3, 14)</td>
</tr>
<tr>
<td>Deafness (6)</td>
</tr>
<tr>
<td>Epilepsy (3, 14, 18, 19)</td>
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<tr>
<td>Subarachnoid hemorrhage (21)</td>
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<tr>
<td>Cardiovascular diseases</td>
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<td>-Hypertension (8, 12, 36)</td>
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<tr>
<td>-Atherosclerosis (12, 21, 37)</td>
</tr>
<tr>
<td>Hypercholesterolemia (21)</td>
</tr>
<tr>
<td>Metabolic disease (33)</td>
</tr>
<tr>
<td>Erectile dysfunction (8, 12, 44)</td>
</tr>
<tr>
<td>Autoimmune disease (33)</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
</tr>
</tbody>
</table>

Additionally, there are several FDA approved compounds that have known secondary effects which include interaction with the BK channel. These compounds are used to treat ailments including sickle cell anemia and depression, further highlighting the need to consider BK when devising patient treatment options for a diverse range of physiological processes and disease states (Table 3).
Table 3. List of pharmaceutical compounds with documented BK cross-reactivity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>Mechanism of Action</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupivacaine</td>
<td>local anesthetic</td>
<td>blocks Na+ channels</td>
<td>(45)</td>
</tr>
<tr>
<td>Vinpocetine</td>
<td>vasodilator</td>
<td>inhibits cyclic GMP phosphodiesterase</td>
<td>(11, 46, 47)</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>sickle cell anemia</td>
<td>imidazole P-450 inhibitor</td>
<td>(11)</td>
</tr>
<tr>
<td>Riluzole</td>
<td>amyotrophic lateral sclerosis</td>
<td>blocks TTX-sensitive sodium channels</td>
<td>(11, 48)</td>
</tr>
<tr>
<td>2-Methoxyestradiol (2ME2)</td>
<td>anticancer agent</td>
<td>inhibits angiogenesis</td>
<td>(11, 49)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>calorie restriction mimic/antiaging/antidiabetogenic</td>
<td>inhibits cAMP-degrading phosphodiesterases</td>
<td>(11, 50)</td>
</tr>
<tr>
<td>Fenamates (mefenamic acid, tolfenamic acid, flufenamic acid, meclofenamic acid)</td>
<td>NSAIDs</td>
<td>gap junction blockers</td>
<td>(11, 51)</td>
</tr>
<tr>
<td>Cilostazol</td>
<td>intermittent claudication</td>
<td>inhibits of type 3 phosphodiesterase</td>
<td>(52, 53)</td>
</tr>
<tr>
<td>Chlorzoxazone</td>
<td>muscle relaxant</td>
<td>Activates KCa3.1 in endothelial cells</td>
<td>(54, 55)</td>
</tr>
<tr>
<td>Hexachlorocyclohexane (delta-HCH)</td>
<td>lipophilic neurodepressant</td>
<td>inhibits neurotransmitter release</td>
<td>(56)</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>Neuroleptic/schizophrenia</td>
<td>K+ channel opener</td>
<td>(35)</td>
</tr>
</tbody>
</table>

2.2.2. The Role of BK Channel in Human Disease

2.2.2.1. Cardiovascular Disease

As discussed antecedently, cardiovascular disease has a direct, intrinsic link to the expression and function of the BK channel due to its regulatory role in vascular tone (12). Mitochondrial BK expression in cardiomyocytes has also been proposed as a mechanism for anti-ischemic effects and hypoxic cardioprotection (1, 57, 58). Due to these novel physiological characteristics, BK channels could potentially have implications in transplant medicine, and are promising targets in the treatment of Atherosclerosis and Hypertension (12, 59). This emerging area of medical research could have profound effects on quality of life in older populations in terms of physical activity levels, however additional studies in fully characterized animal models, primary human tissue and suitable patient populations, need to be conducted.
2.2.2.2. Atherosclerosis

Classified as hardening of the arteries by the Animated Dissection of Anatomy for Medicine (A.D.A.M.) Medical Encyclopedia, atherosclerosis is a disorder caused when excessive cholesterol and fat aggregate as plaques on the internal arterial walls. The progression of atherosclerosis in the human aorta is characterized by the up-regulation of BK channel. Some have hypothesized that the appearance of BK in response to the buildup of arterial plaques, implies a link to the mechanisms involved in the manifestation of atherosclerosis (12). However this may be a hypoxic response, or a buildup of channel infrastructure in order to create a cardioprotective barrier against the ischemic conditions created in the immediate environment of the plaque. In either case, these questions pose relevance in the study and understanding for the role of BK in atherosclerosis.

2.2.2.3. Hypertension

Diagnosed by high blood pressure, hypertension is becoming increasingly prevalent in human populations affecting nearly 25% of the adult U.S. population (60). Interestingly, abnormal function of BK channels has been associated with hypertension, however the degree to which these changes are reflected in abnormal channel function varies, and is dependent on the underlying cause of the hypertension itself (12). Mechanisms have also been proposed for the role of BK channel in the pathogenesis of diabetic hypertension. It is thought that the onset of hypertension in diabetic patients stems from mutations to the molecular composition of the α/β subunit assembly (30). Pharmaceutical targeting of BK channel in the arterial cell wall may well represent a new avenue for the treatment of hypertension based on the fact that channel activation physiologically stimulates arteriolar vasodilatation by inducing relaxation in the smooth muscle (12).

2.2.2.4. Diabetes

According to the Centers for Disease Control, diabetes affects more than 25 million people a year and is associated with the pathogenesis of heart disease, hypertension, blindness, kidney disease, problems with the nervous system, dental disease and
sometimes results in limb amputation (61). In diabetes, oxidative stress in the vascular wall causes functional impairment of the BK channel (62). Additionally, and due to its ubiquitous nature, BK has been further linked to abnormal regulation of retinal hemodynamics resulting in the onset of diabetic retinopathy (16). As shown in Thirunavukkarasu et al. 2007, the activation of BK may reduce blood glucose levels thereby offering an supplementary approach to the treatment of diabetes and its associated pathologies (31).

2.2.2.5. Neurological Disorders

2.2.2.5-A. Schizophrenia, Autism and Mental Retardation

Neurological disorders are considered a substantial threat to public health and can have a considerable impact on quality of life for not only the patient but also family members tasked with caring for their sick relatives. Symptoms often include limitations to physical mobility and function, cognitive impairment, problems with verbal communication as well as behavioral and psychosocial issues (63). Three neurological disorders, autism, schizophrenia and mental retardation have documented links to altered regulation and or expression of the BK channel (3, 34, 35). In schizophrenia, the abnormal translation of mRNA has been intrinsically linked to the etiology of the disease state. In these patients, channel expression is found to be significantly lower in the prefrontal cortex when compared to those in a control group (35). A mechanism has been proposed recently which may contribute to the progression of symptoms for both autism and mental retardation where h-slo haploinsufficiency leads directly to the pathogenesis of the disease state owing to the decreased expression of BK channel (34). The role BK in the regulation of neuronal excitability and the release of neurotransmitters, has led to the emergence of BK as a novel pharmaceutical target for the treatment of certain neurological disorders. Evidence for this theory is supported by studies that show the commonly used antipsychotic drug diazoxide, a neuroleptic, enhances K⁺ conductance in central neurons by increasing the frequency of channel opening physiologically augmenting fast action hyperpolarization (35).
2.2.2.5-B. Epilepsy

According to the Epilepsy foundation of America, epilepsy is a chronic age related neurological disorder characterized by the manifestation of recurrent seizures in the absence of brain lesions or metabolic abnormalities, which affects a variety of psychological and physical functions (18). It is estimated that more than 2 million Americans are afflicted with epilepsy, with 150,000 new cases diagnosed annually (64). Interestingly, a large number of mutations associated with epilepsy are linked to genes which encode either voltage or ligand gated potassium channels including but not limited to KCNQ2, KCNQ3 and KCNA1 (18, 65-70).

Two mutations in BK channel genes have been linked to the etiology of epilepsy. The first gene that has been linked to epilepsy is KCNMB3 (3q26), which codes for the β3 accessory subunit of the BK channel and is expressed at high densities in synaptic termini (9, 14, 18, 71, 72). This mutation manifests phenotypically as an early truncation of channel protein, and translates physiologically as idiopathic generalized epilepsy (18).

The second mutation is associated with the Slo gene and has been linked to generalized epilepsy characterized by underlying paroxysmal dyskinesia (3, 73). Affecting the Ca\(^{2+}\) binding site in RCK1, this mutation interferes with the mechanism which couples the binding of Ca\(^{2+}\) to channel opening functionally altering Ca\(^{2+}\) dependant activation (74).

2.2.2.6. Cancer

By definition, cancer is a vast and diverse group of diseases primarily characterized by uncontrolled growth and the spread of abnormal cells, this according to the American Cancer Society. Originating from both internal (i.e. inherited mutations) and external factors (i.e. radiation), the progression of cancer, if left untreated, can and often does ultimately culminate in fatal patient outcomes. Statistically, cancer is the second leading cause of death in the United States with an estimated 1.6 million newly diagnosed cases, and over a half million deaths in 2013 alone (75). Currently, the most widely accepted approach to the treatment cancer involves either a single, or combination of methods including but not limited to surgery, chemotherapy, radiation, hormone therapy, biological therapy or targeted therapy (29, 75). Unfortunately, these treatment options
are often associated with severe side effects and poor clinical outcomes reflected by low survival rates. In order to mitigate these harsh side effects, increase survivability and improve clinical outcomes, it is essential that the identification of novel therapeutic targets and the development of innovative new treatment strategies occurs.

In light of this need, the classic paradigm focused on the cytotoxic treatment of cancer in vivo (cytotoxic era) is rapidly being replaced by the genomic era, as the acceleration in genetics research has facilitated the conceptualization of mechanism based therapies and treatment options. Two primary mechanisms harnessing ion channels have been proposed as innovative new treatment strategies. Due to their role in the onset, growth, proliferation, migration and invasion of various cancers, the selective inhibition and/or modulation of these ion channels has been shown to disrupt the proliferation of cancer cells in vitro, and further, to counteract their progression in vivo (29, 76). Additionally, transcription profiling has revealed tissue specific expression and distribution of various ion channels making them ideal targets for the selective and targeted delivery of traditional and experimental chemotherapeutics (29, 77).

Of the many types of ion channels investigated, potassium (K+) channels in particular were identified in many of the physiological process vital to the pathogenesis of disease including the proliferation, progression and apoptosis of cells representing a vast number of cancer and tumor cell types (29, 78). The phenotypic manifestation of KCN in these cancer types results in either the up- or down regulation of channel expression. A qualitative list defining the KCN family groups and their expression within a diverse set of cancer and tumor cells has been compiled in Table 4.
Table 4. KCN groups and their specific relationship (expression) to human cancer.

<table>
<thead>
<tr>
<th>KCN Group</th>
<th>Cancer and Tumor Cell Types</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir</td>
<td>glioblastoma, uterine leiomyoma (fibroid tumor), breast carcinoma, astrocytic tumors</td>
<td>(29, 40, 79-83)</td>
</tr>
<tr>
<td>KCa</td>
<td>Osteosarcoma, glioma, ovarian cancer, breast cancer, prostate cancer, colon cancer</td>
<td>(38, 39, 41-43, 84, 85)</td>
</tr>
<tr>
<td>Kv</td>
<td>breast cancer, uterine cancer, leukemia, gastric cancer</td>
<td>(42, 79, 86-89)</td>
</tr>
<tr>
<td>K2P</td>
<td>breast cancer, prostate cancer, glioma</td>
<td>(29, 90-92)</td>
</tr>
</tbody>
</table>

BK channels represent a well-studied isoform from the KCa channel grouping, and one central to this investigation due to its omnipresent expression in a wide array of cancer types, outlined below in Table 5. BK channel up-regulation has been linked to malignancy grade, metastasis and invasion, while channel activation has been linked to tumor cell proliferation, altered growth characteristics and transendothelial migration (41-43, 93, 94). There have however been conflicting reports regarding the role of BK in tumor cell growth. In at least one case, BK was documented to exhibit antitumoral effects which was demonstrated by an observed increase in tumor growth following the knockdown of hSlo, the gene responsible for encoding the α-subunit of the BK complex (38). Paradoxically, in another study, the inhibition of BK channel by a highly specific ligand was shown suppress tumor growth (94). Regardless of the discrepancy, a clear relationship has been established between the presence and activity of BK, and pathophysiology of cancer including the channels ability to modulate the microenvironment surrounding a tumor in vivo.
Table 5. Three BK variants and their documented expression in a wide variety of cancer and tumor cell types.

<table>
<thead>
<tr>
<th>BK Variant</th>
<th>Cancer or Tumor Type</th>
<th>Anatomical locus</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK (native)</td>
<td>Osteosarcoma</td>
<td>Bone, Lung</td>
<td>(29, 38)</td>
</tr>
<tr>
<td></td>
<td>Glioma</td>
<td>Brain, Spine</td>
<td>(13, 29, 43, 95, 96)</td>
</tr>
<tr>
<td></td>
<td>Glioblastoma</td>
<td>Brain</td>
<td>(94, 97, 98)</td>
</tr>
<tr>
<td></td>
<td>Astrocytoma (Glioblastoma multiform)</td>
<td>Brain, Spine</td>
<td>(43, 94, 99)</td>
</tr>
<tr>
<td></td>
<td>Breast Cancer</td>
<td>Mammary gland, Brain, Lung</td>
<td>(29, 42, 79, 81, 100)</td>
</tr>
<tr>
<td></td>
<td>Ovarian Cancer</td>
<td>Ovary</td>
<td>(29, 41, 101)</td>
</tr>
<tr>
<td></td>
<td>Prostate Cancer</td>
<td>Prostate, Bone</td>
<td>(93)</td>
</tr>
<tr>
<td></td>
<td>Cervical Cancer</td>
<td>Cervix, Abdomen, Lung</td>
<td>(101)</td>
</tr>
<tr>
<td></td>
<td>Pituitary tumor</td>
<td>Pituitary gland</td>
<td>(102)</td>
</tr>
<tr>
<td>gBK</td>
<td>Gastric Cancer</td>
<td>Stomach</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Lung Cancer (adenocarcinoma)</td>
<td>Lung</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Lung Cancer (small cell carcinoma)</td>
<td>Lung, Cervix, Prostate, GI Tact</td>
<td>(103-105)</td>
</tr>
<tr>
<td></td>
<td>Breast Cancer</td>
<td>Mammary gland, Brain, Lung</td>
<td>(42, 103)</td>
</tr>
<tr>
<td></td>
<td>Glioma</td>
<td>Brain, Spine</td>
<td>(95, 103, 106)</td>
</tr>
<tr>
<td></td>
<td>Colon Cancer</td>
<td></td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Duodental Cancer</td>
<td></td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Liver Cancer (Hepatocellular carcinoma)</td>
<td>Liver</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>Pancreatic Cancer</td>
<td>Pancreas</td>
<td>(103)</td>
</tr>
<tr>
<td>BK₁</td>
<td>Prostate Cancer</td>
<td>Prostate, Bone</td>
<td>(29, 43)</td>
</tr>
<tr>
<td></td>
<td>Breast Cancer</td>
<td>Mammary gland, Brain, Lung</td>
<td>(29, 42, 43)</td>
</tr>
</tbody>
</table>

2.2.3. Design of a Fluorescent BK Channel Probe: IbTx[D19K]-LC-FAM

2.2.3.1. Design Overview

With the extensive contribution of the BK channel to human physiology and its broad impact on a wide spectrum of disease processes, a direct conjugate fluorescent probe, capable of diagnostically targeting BK both in vitro and in vivo could potentially result in an increased understanding, expedited diagnoses and advanced treatment options. In 2006, Bingham et al. created a biotin derivative of Iberiotoxin (IbTx-LC-biotin), which, when coupled with a fluorescent conjugate of streptavidin (Streptavidin-Alexa Fluor 488;
Molecular Probes), produced *in vitro* visualization of the BK channel in a transformed cell line (107).

![Figure 3. The design and assembly of the probe IbTx-LC-biotin. (A) The amino acid sequence of IbTx showing the point mutation "X" where mutation occurred. (B) Structure of the aminocaproic linker conjugated to the biotin molecule. (C) A molecular model of the conjugated peptide highlighting (green) the residues vital to the bi-molecular interaction. Adapted from Bingham et al. 2006 (107).](image)

The experimental staining method for IbTx-biotin mimics the immunocytochemistry protocols utilized with primary and secondary antibody systems where the primary is epitope/antigen specific and the secondary selectively targets the primary and carries the fluorescent moiety. Although effective as an *in vitro* diagnostic agent, this system has inherent drawbacks and experimental limitations. The most significant of these limitations are the extended incubation time-frames which accumulate between two and three hours of additional protocol time, due to the necessity for pre-incubation blocking steps which neutralize endogenous cell-surface biotin. Blocking effectively prohibits non-specific binding of StrAv-Alexa Fluor 488, significantly reducing background signal. The two-stage incubation functionally prevents any *in vivo* application of the fluorescent probe, effectively eliminating the possibility of use as an internal visualization agent for the identification and intraoperative resection of tumoral masses.

The design and production of a successful fluorescent probe takes careful consideration and technical planning and includes several overriding factors that must be considered. The initial consideration is the identification of an appropriate target. In this case, the Ca$^{2+}$-activated Potassium channel, BK, has been identified and selected due to its
intimate involvement in the disease processes outlined previously (Section 2.2.2), and most importantly, its unique involvement in human cancer and pronounced over-expression in glioblastoma. Following target selection, a peptide toxin with the appropriate natural characteristics, must be selected. These characteristics include specificity, selectivity and dissociation kinetics ($K_d$). Additionally, there must be a comprehensive understanding of the three dimensional molecular structure of the active toxin with respect to binding surface and electrostatic interactions. These factors must be well understood in order to begin molecular modifications and sequence manipulation (i.e. bioconjugations) without effecting biological activity.

### 2.2.3.2. Structure Activity Relationship (SAR)

An early case study which provided the template for the design of advanced peptide probes came from the investigation of the bimolecular interaction between Charybdotoxin (ChTx), a scorpion toxin with high identity to that of Iberiotoxin (IbTx), and the bacterial potassium channel KcsA, which is analogous to the mammalian isoform, BK ($^{108}$). In 2005, Yu et al. performed an Alanine scan of ChTx in order to determine the amino acid residues located within the critical bi-molecular binding interface. A residue impact report has been summarized in Figure 4. Omitting the cysteine residues which infer three dimensional conformation, mutation of which would render the toxin inert, it was determined that residue K27 was of critical importance due to its role in toxin orientation during the binding process, and insertion into the channel pore, functionally blocking the flow of $K^+$ ions. Residues F2, and N30 were substantially involved in the binding interface and mutation at either of these positions, depending on the inserted residue, can potentially nullify binding. Secondary residues, which moderately effect binding but do not eliminate totally the binding characteristics were identified as S37, R25, W14, Y36 and M29. This interaction has been modeled using computer simulations in order to better visualize the binding interaction, Figure 4.
Figure 4. A computer generated model of the known bi-molecular interaction between Charybdotoxin (ChTx) and the bacterial K⁺ channel homolog KcsA. The amino acids highlighted in purple represent the bioactive interface between the toxin and receptor. The binding of ChTx has been linked primarily to involvement of residues F2, K27 and N30 while secondary involvement has been linked to S37, R25, W14, Y36 and M29. Adapted from Yu et al. 2005 (166).

Possessing sequence identity approaching 70%, ChTx and IbTx are extremely similar in terms of molecular structure, electrostatic interaction, binding kinetics and target, Figure 5. There is one major difference however, which centers on the target specificity of the
molecule. ChTx tends to be quite promiscuous, having several different K⁺ channel targets, while IbTx is specific for only the BK channel. This characteristic significantly advances IbTx as a potential probe candidate.

Using the knowledge gained from the study of the sequence and computer generated model, it was determined that sequence modification could be made by manipulating D19 in IbTx, a residue 180° removed from critical K27, without affecting biological activity. The substitution of a non-native amino acid, with specialized chemistry, in the 19 positions enables the bioconjugation of external moieties such as biotin or a fluorophore.

### 2.2.3.3. Design of IbTx[D19K]

Producing a novel fluorescent BK channel-probe based on the molecular structure of Iberiotoxin, first requires the design, synthesis and characterization of a non-native peptide isoform with a point mutation allowing for post-synthetic chemical conjugation.
IbTx[D19K] was designed based on the bi-molecular template revealed by investigating the binding interaction between ChTx and KcsA. Knowing that Lysine-27 (L27) is critical in binding due to its physical insertion directly into the channel pore, modifications can be made 180° from the known binding interface (green residues - Figure 6) without detrimental impacting biological activity. Interface reversal correlates to the Aspartic acid at position 19 (D19), which can be functionally replaced during the synthetic process (Section 2.2.4) with the non-native amino acid, Lysine-MTT, a mutation which has a minimal negative impact on pharmacological activity (Chapter 3).

![Molecular model of IbTx[D19K]](IIbbTTxx)

**Figure 6.** Molecular model of IbTx[D10K]. (Top) Computer model highlighting in green the functional residues and the binding interface. (Bottom) Sequence comparison between native IbTx and the mutated IbTx[D19K].

<table>
<thead>
<tr>
<th>IbTx</th>
<th>ZFTDVDcSVskEcWSvCkDlfGvdRgKCMGKKcrCYYq</th>
</tr>
</thead>
<tbody>
<tr>
<td>IbTx[D19K]</td>
<td>ZFTDVDcSVskEcWSvCk (K-MTT) LFGvDRgKCMGKKcrCYq</td>
</tr>
</tbody>
</table>

Lysine-MTT (K-MTT) is a functionalized amino acid, synthesized with a specialized protectional group, 4-methyltrityl, blocking its ε-amino function. This protection of the ε-amino function can be selectively deprotected and hence, the bioconjugation of various prosthetic moieties can proceed (discussed in Section 2.2.4.7). With the completion of the design stage of the novel peptide, the synthetic process must be planned carefully prior to undertaking peptide assembly. Detailed thought must be infused into the choice of the orthogonal protection scheme, solid support and method of bioconjugation (if
necessary). In the current study, a strategically planned SPPS has been carried out on the carefully designed IbTx isoform, IbTx[D19K], as described in Section 2.3.2.3.1.

2.2.4. **Solid Phase Peptide Synthesis**

2.2.4.1. **Overview**

Synthetic peptide-toxins are gaining momentum in the biopharmaceutical industry as novel diagnostic and therapeutic tools, and are now a staple of many industrial research programs. These molecules have had a tremendous impact on the fields of receptor pharmacology, electrophysiology, biochemistry and medicine. As discussed in Chapter 1, traditionally, peptide-toxins have been produced in one of three ways: (i) isolation from a natural source (animal, plant, bacteria etc.), (ii) solution phase chemistry, or (iii) by means of recombinant technology. In the modern realm of bio-sustainable research, over-aggressive harvesting of source species for isolation is discouraged while bioethics promotes a search for "green" alternatives. Additionally, the solution phase synthesis of peptides, unlike small organic molecules, requires extensive purification steps following each stage of assembly \( (109) \). Unfortunately, the cost and time-inefficiency of this process limits its utility and application. Recombinant technology has advanced significantly in terms of peptide production by beginning to address production issues related to yield, and the inclusion of post-translational modifications (PTMs) and non-native amino acid (NNAA) substitutions \( (110) \).

Conceptualized in the early 1950's by Nobel laureate Dr. Bruce Merrifield, Solid-Phase Peptide Synthesis (SPPS), officially introduced in a 1963 publication, has remained the standard for the production of synthetic peptides for use in research and medicine \( (111) \). Solid phase peptide synthesis has four major advantages over alternative approaches to peptide assembly, (i) it expedites the synthetic process by simplifying the chemical approach and minimizing the practical application, (ii) it minimizes the losses accumulated during disproportionate purification steps required for multi-step solution synthesis, (iii) there is a high degree of solubility inherent to the solvent platform which functionally reduces aggregation, and (iv) it typically results in higher yields when compared to alternative options \( (112) \). SPPS allows for relatively swift production of
high purity peptides at high yield, while retaining the flexibility to incorporate PTMs, NNAA's as well as additional designer ligands.

Figure 7. Generalized reaction scheme representing Solid Phase Peptide Synthesis. Adapted from Kates and Albericio 2000 (113).

Solid phase peptide synthesis is based on the concept of cyclical, repetitious addition of a single amino acid (AA) residue, joined to a continuously growing peptide chain (Figure 7). The growing peptide is covalently anchored to a solid support that facilitates the synthetic process. Principal to controlling the sequence of the growing peptide chain, the reaction between the α-amino (α-NH$_3^+$) function of the anchored amino acid, and the carboxylic (COO$^-$) function of the incoming amino acid must be strategically managed. Additionally, side chain functionalities must be blocked in order to maintain chemoselectivity and minimize the formation of byproducts.
2.2.4.1. Solid Support

The utilization of solid support imparts many favorable characteristics on the synthetic process including the use of excessive reagents which drive the reaction to completion, a simplified method for the removal of excess reagents, and a reduction in peptide aggregation and precipitation (113). Though a number of solid supports were explored initially, such as cellulose, polyvinyl alcohol, polymethacrylate and sulfonated polystyrene, the first insoluble polymer with suitable functionality (amino groups) to create a covalent bond to the first amino acid was identified as a chloromethylated copolymer of styrene and divinylbenzene (111). Since this development, additional materials have been tested for potential application in SPPS including polysaccharides, polyamides, modified polyolefin particles, polyacrylamides, polyethylene glycol, polystyrene, controlled-pore silica and phenolic resins (112, 114).

The term solid support is somewhat of a misnomer, as resins are actually highly dynamic structures that display unique solvation entropy when swelled in a broad range of solvents to reveal functionality. The gel matrix with suitable characteristics (i.e. uniform bead size, thermo-stable) provides large loading values (mmol/g resin) facilitating high yield syntheses (115). When choosing the appropriate resin for a specific synthesis, one must consider the desired C-terminal chemistry, solvent compatibility, swelling ratio as well as the length of the sequence.

In some cases, a readily reversible linker, or handle, is required to protect the C-terminal $\alpha$-carboxyl group (116). Upon liberation from the handle and therefore resin, linkers

![Diagram of handle insertion in SPPS](image-url)

**Figure 8. Example of the handle insertion in SPPS. Adapted from Songster et al. 1997 (116).**
allow for the creation of many C-terminal functionalities such as acids, amides, active esters (i.e. thioesters), and secondary amides (117). Linkers come in a plethora of configurations for a variety of applications.

### 2.2.4.2. α-Amino (N\(^{\alpha}\)) Protection

Known as temporary protection due to reversibility under specific conditions, N\(^{\alpha}\)-protection is accomplished primarily using one of two schemes. The first, developed in 1957, known as tert-butyloxycarbonyl (Boc) chemistry (Figure 9), was the forerunner of solid phase peptide synthesis, proceeds through acylation by \(t\)-butyl azidoformate (118). This form of α-elimination (where the proton and leaving group are co-localized on the same atom) is effective due to reversibility using mild acids. It does have distinct drawbacks however, mainly the required use of Hydrogen Fluoride (HF), a highly caustic strong acid, during the removal of orthogonal (side chain) protection (Section 2.2.4.3) during the cleavage process (discussed in Section 2.2.4.5). These drawbacks served as the impetus for the development of cleaner protection schemes.

![Figure 9. The schematic diagrams a synthesis using t-Boc groups protecting the N\(^{\alpha}\)-amino function. Adapted from Barany et al. 2004 (119).](image)

A replacement for Boc chemistry was introduced in 1970 which utilized β-elimination and made irrelevant the need for HF, allowing for substitution with the safer, moderate acid, trifluoroacetic acid (TFA). Known by its chemical name 9-Fluorenlymethoxycarbonyl, or Fmoc for short, this protection scheme is compatible with base-labile orthogonal side-chain protection, and can be removed using ammonia or...
pyridine (120). Although reactions times often surpass those observed in Boc chemistry, Fmoc synthesis has become the preferred method due to its cleanliness and ease of use. A representation of Fmoc $\text{N}^\alpha$-protection is seen below (Figure 10).

![A representation of Fmoc $\text{N}^\alpha$-protection](image)

*Figure 10. The basic Fmoc approach to Solid Phase Peptide Synthesis. Adapted from Barany et al. 2004 (119).*

### 2.2.4.3. Orthogonal Protection

Until 1977, peptide purity and yield suffered due to uncontrolled reactions with the side chain functionality of certain amino acids (i.e. Cys, Lys). This resulted in the formation of byproducts, prolonging reaction times and increasing the use of primary reagents. Orthogonal protection provided a mechanism for the comprehensive removal of side chain protection during the liberation of the peptide from the solid support (cleavage). Orthogonal protections schemes have been developed which are either acid or base labile, and compatible with Fmoc and Boc $\text{N}^\alpha$-protection respectively.
Table 6. Representative examples of orthogonal protection utilized in Boc and Fmoc chemistry. Adapted from Barany et al. 2004 (119).

| Examples of Orthogonal Protection for Cys & Lys in Boc and Fmoc Chemistry. |
|---|---|---|---|
| **Boc Approach** | Cysteine 4-Methylbenzyl | Cys(Mcb) | TFA Piperidine |
| Lysine 2-Chlorobenzoxy carbonyl | Lys(2-CIZ) | TFA Piperidine | Pd(0) Hydrazine |
| **Fmoc Approach** | Cysteine Triphenylmethyl | Cys(Trt) | Piperidine Pd(0) Hydrazine |
| Lysine n-Butoxy carbonyl | Lys(Boc) | Piperidine Pd(0) Hydrazine |

2.2.4.4. Activation

There are three primary classes of in-situ reagents used for the activation of incoming amino acids for N-terminal attachment to the elongating sequence in the sequential synthetic process. Initially carbodiimide-mediated activation methods were employed in Boc chemistry based on the parent molecule, \( N,N' \)-dicyclohexylcarbodiimide (DCC). Additional DCC coupling reagents were developed including 1-hydroxy-7-azabenzotriazole (HOAt) and 1-hydroxybenzotriazole (HOBr) which diversified the chemistry (121).
Figure 11. Example reaction scheme illustrating *in-situ* activation using DCC based reagents. Adapted from Albericio *et al.* 1997 (122).

Activation using a carbodiimide mediated system proceeds through the formation of an amide bond between the labile *O*-acylisourea and an amino leaving group (Figure 11). Although acylation capabilities are excellent (Figure 11), problems are experienced periodically including the production of symmetrical anhydrides in the presence of excess carboxylic acid, and the production of active esters which can disrupt *in-situ* activation in the presence of HOBt (122).

Another class of *in-situ* activation agents are the Phosphonium reagents which exhibit oxophilicity in the presence of a tertiary base which produces phosphonium and aminium salts that produce oxybenzotriazole or OBt esters. Under normal conditions, AOP and PyAOP are more efficient than BOP and PyBOP (122).
The final class of *in-situ* activation agents, the Uronium salts HATU, HBTU, HCTU, HAPyU, HAMDU and HDTU, see extensive utility in Fmoc chemistry. Disproved by X-ray crystallography, Uronium salts were once believed to be tetramethyluronium salts, but are now known to be guanidinium N-oxide isomers (121). Certain Uronium agents require a tertiary amine such as N-ethyl diisopropylamine (DIEA) to reach their full potential and produce efficient coupling rates (Figure 12).

**2.2.4.5. Cleavage**

The synthetic process culminates in the cleavage of the linear peptide from its solid support, which liberates the molecule and systemically removes orthogonal protection restoring native functionality. Boc and Fmoc chemistries require considerably different cleavage conditions that principally account for the popularity of the Fmoc approach. Global deprotection of side chain protection groups during cleavage generates highly reactive cationic species which can lead to the chemical modification of amino acids with electron dense side chains including those of Tyrosine, Tryptophan, Methionine and
Cysteine \((117)\). The generation of these reactive species must be carefully controlled by the addition of chemicals known as scavengers, which neutralize the harmful effects of the free radicals.

Peptides assembled using Boc chemistry require the strong acid, Hydrogen Fluoride (HF), for global deprotection of orthogonal groups and liberation from the solid support. The scavengers anisole, dimethyl sulfide and \(p\)-cresol are added to neutralize carbonium ions, and the addition of thiocresol is protects the sulfhydryl groups of Cysteine \((123)\). A stringently controlled low-high system of HF allows for controlled regulation over the cleavage process and results in high purity peptides. HF is highly corrosive reacting with numerous materials including oxides and glass. Alternate strong acids including Tetrafluoroboric acid (HBF\(_4\)), trimethylsilyl bromide (TMSBr), trimethylsilyl trifluoromethanesulphonate (TMSOTf) and trimethyl sulfonic acid (TFMSa) can also be used for cleavage, however results are comprised \((121)\). Specialized equipment and detailed handling requirements make the use of HF difficult, dangerous and expensive. This hurdle is the major contributing factor in the popularity of the Fmoc approach.

Cleavage of peptides synthesized under Fmoc conditions is a much simpler process with a reduced potential for serious accidents and injury, and requiring little specialized equipment. Fmoc scavengers are solvated in Trifluoroacetic acid (TFA) (Table 7). Water moderately scavenges \(t\)-butyl cations as well as cleavage products from arylsulphonyl protecting groups while 1,2-Ethanedithiol (EDT), a noxious thiol, is used for the removal of \(t\)-butyl cations and trityl groups from Cystiene. EDT also protects aromatic amino acids such as Tryptophan from sulfonation. Thioanisole suppresses Methionine oxidation, and removes protectional moieties from Arginine. Phenol is utilized for its protective effects on Tyrosine and Tryptophan \((7)\).

<table>
<thead>
<tr>
<th>NAME</th>
<th>SCAVENGERS</th>
<th>RATIO (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent K</td>
<td>TFA/Thioanisole/water/phenol/EDT</td>
<td>82.5:5:5:5:2:5</td>
</tr>
<tr>
<td>Reagent R</td>
<td>TFA/Thioanisole/anisole/EDT</td>
<td>90:5:3:2</td>
</tr>
</tbody>
</table>

Table 7. Two popular reagents used in the cleavage of peptides produced using Fmoc chemistry \((117)\).
The final step in the cleavage process is the precipitation of peptic material using cold tert-butyl ether (117). Centrifugation results in a pellet that is resuspended several times in acetic acid to remove trace scavengers before lyophilization for stable storage. Repeated lyophilization steps have anecdotally improved the physical properties of the peptide product enabling simplified handling, purification, and storage.

### 2.2.4.6. Purification and Characterization

Following the design, synthesis, cleavage, precipitation and re-folding, synthetic peptides must finally be purified. There are several chromatographic methods for the isolation and purification of synthetic peptides including Size-Exclusion Chromatography (SEC), Ion-Exchange Chromatography (IEC), and lastly Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (124).

High Performance Liquid Chromatography (HPLC) was first utilized for the purification of synthetic peptides in 1976 leading to expedited production and increased purity (125). An organic solvent (Solvent - B) is run against an aqueous solvent (Solvent - A) from 5 to 65% reversing phase over the course of 60 min, creating a 1%/min gradient (Table 8). The stationary phase consists of a cylindrical column containing alkyl chains of varying length (4, 8 or 16 carbons) which provide varying degrees of hydrophobic resolution (124). The elution profile run over the stationary phase facilitates the separation of peptides based on hydrophobicity. A process that can be monitored via ultraviolet/visual (UV/Vis.) spectroscopy at 214 nm. The amide bonds present in the peptide backbone are detected at this wavelength (λ = 214 nm) allowing for purification of synthetic compounds to nearly 99.9%.
Table 8. Examples of mobile phase solvents used in RP-HPLC (113).

<table>
<thead>
<tr>
<th>Example</th>
<th>Solvent A (Aqueous Buffer)</th>
<th>Solvent B (Organic Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example 1</td>
<td>0.1% TFA in Water</td>
<td>0.1% TFA in 90/10 MeCN/water (v/v)</td>
</tr>
<tr>
<td>Example 2</td>
<td>0.01 M Ammonium acetate in Water</td>
<td>0.01 M Ammonium acetate in 90/10 MeCN/water (v/v)</td>
</tr>
<tr>
<td>Example 3</td>
<td>0.05 M Triethylammonium phosphate (pH 2.5) in water</td>
<td>0.05 M Triethylammonium phosphate (pH 2.5)/MeCN; 2:3 (v/v)</td>
</tr>
</tbody>
</table>

The purification of the target material can be confirmed by mass spectrometry which has been utilized extensively in the characterization of novel peptide toxins (126). Electrospray Infusion-Mass Spectrometry (ESI-MS) uses high temperature, high pressure and electrical energy to ionize a peptide creating a charged molecule which can be detected and analyzed (127). After traveling the length of a pressurized vacuum tube, the ionized peptides impact a specialized detector that measures mass accurately down to ±1 Dalton (Da). This process confirms that the correct sequence of the target molecule has been synthesized and purified correctly and accurately. Following this process, the peptides can be quantified by amino acid analysis for use (Section 2.2.4.8).

2.2.4.7. Bioconjugation

Bioconjugation, and techniques utilized in the design of KCN probes have been discussed at length in Chapter 1. Radio-labels and fluorophores have been used extensively to produce novel bioactive molecular calipers to study various physiological aspects of KCN expression and activity. Here we aim to design a direct conjugate fluorescent peptide probe based on the Iberiotoxin (IbTx) template (Figure 13). An ideally suited fluorescein isoform will be covalently linked the IbTx[D19K] mutant on resin. The newly functionalized peptide will be cleaved, oxidized, purified and quantified for use in various in vitro expression systems.
2.2.4.8. Quantification & Amino Acid Analysis

First introduced in the 1950's, amino acid analysis (AAA) has been the standard quantification method for peptides and proteins for some 60 years. There are three major phases to quantification via AAA. Peptides are first hydrolyzed in strong acid (i.e. HCl) to separate individual amino acids followed by derivatization for detection. Finally the individual amino acids are separated and quantified against known standards (128).

Initially amino acids were derivatized with a chromophore following separation on Ion-exchange columns using sulfonated divinyl benzene/polystyrene resins, a method known as post-column derivatization. Advances in chromophore/fluorophore and separation technology has resulted in the development of pre-column derivatization, a more sensitive approach.

In pre-column derivatization (Figure 14), a high purity peptide sample is hydrolyzed in 6N Hydrochloric acid for 18-24 hours at 110°C, in the absence of oxygen. The liberated amino acids are then derivatized with a fluorescent compound such as ninhydrin, phenylisothiocynate (PITC), or 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC, or Accq*Fluor), and then be separated via RP-HPLC (129). The individual amino acid profiles are compared to known standards for quantification.
2.3. EXPERIMENTAL PROCEDURES

2.3.1. Manual Solid Phase Peptide Synthesis: IbTx[D19K-MTT]

Iberiotoxin[D19K-MTT] was manually synthesized (0.5 mMole scale) on an Fmoc-Gln(Trt)-CLEAR-Acid Resin (0.38 meq g⁻¹; Peptides International, Louisville, KY) providing the necessary C-terminal amide functionality. In-situ neutralization with Fmoc-amino acid (2 mMole) for 30-45 min (@ room temp.) per αα was repeated until a coupling efficiency of 99.5% was achieved. Amino acids were activated and coupled using HCTU (0.5 mMole in DMF) and DIEA (0.025 mMole). Coupling efficiency was monitored following each cycle by measuring residual free amine via quantitative Ninhydrin assay (130). Removal of Fmoc group was achieved by washing (2x 1 min) with 50% Piperidine (v/v in DMF). Side chain protecting groups were as follows: Cys(Trt), Asp(tBu), Arg(Pbf), Gln(Trt), Thr(tBu), Ser(tBu), Lys(boc), Glu(OtBu), Trp(boc), Tyr(tBu) and Lys(Mtt) (Peptides International, Louisville, KY. The Aspartic acid residue at position 19 was substituted with the non-native AA (NNAA) Fmoc-Lys(Mtt)-OH. On completion of the synthesis, the resin was washed with DMF followed
by DCM/MeOH (50% v/v), dried under N₂ and stored for later use (-20°C). The synthetic process was adapted from Schnölzer et al. (131).

2.3.2. Bioconjugation

2.3.2.1. Removal of MTT

The fully assembled peptide (Section 2.3.1), on resin, was swelled first in DCM (30 min) followed by DMF (overnight). The specialized deprotection of Fmoc-Lys(Mtt)-OH was achieved under mild acidic conditions over 30 minutes using 1% TFA (v/v in DCM; 1 min x 30 washes). Finally, the resin was washed thoroughly with DMF to eliminate any trace TFA.

2.3.2.2. Fmoc-Ahx-OH

In certain conditions, an aliphatic carbon linker was used to alleviate steric hinderance and improve coupling. Following removal of the MTT protectional group from Lysine19, Fmoc-Ahx-OH (Novabiochem, Darmstadt, Germany) was activated and coupled to the free amine using HCTU (0.5 mMole in DMF) and DIEA (0.025 mMole) as described previously (Section 2.3.1).

2.3.2.3. Fluorescein isothiocyanate (FITC)

The fluorophore, fluorescein isothiocyanate (FITC: AnaSpec Inc., Fremont, CA) was solvated (2x molar excess) in pyridine/DMF/DCM (12:7:5 v/v) and incubated with the resin overnight. Following overnight incubation, the resin was washed thoroughly (DMF) to remove latent fluorophore. This method was adapted from Anaspec; Peptide Tips. Coupling efficiency was monitored via Ninhydrin as described previously (Section 2.3.1).

2.3.2.4. Carboxyfluorescein-NHS (FAM-NHS)

The fluorophore, carboxyfluorescein (FAM: ChemPep Inc., Wellington, FL) was activated and coupled using HCTU (0.5 mMole in DMF) and DIEA (0.025 mMole) as described above (Section 2.3.1). Following overnight incubation, excess fluorophore was carefully removed as described previously (Section 2.3.2.3).
2.3.3. **Cleavage**

Fully assembled peptides (on resin - 500 mg) were subjected to cleavage in 40 mL Reagent K (82.5% TFA, 5% thioanisole, 5% Phenol, 5% H₂O, 2.5% EDT) for 2 hours at 24°C. The resulting cleaved peptide material was recovered by filtration and precipitation (cold t-butyl ether). Resulting crude peptide was lyophilized and stored at -20°C prior to oxidation and purification.

2.3.4. **Air Oxidation**

Peptide folding (formation of disulfide bridging) was carried out in oxidation buffer (2 M Urea, 0.1 M Glycine, 0.1 M NaCl) for 18 hours at room temperature (typically 15 mg peptide in 50 mL buffer). Oxidation mixture was then acidified (Acetic acid to pH ~3), centrifuged (3500 rpm for 5 min @ room temp) and filtered (0.45 μm Nylon) following the addition of the ion-pairing agent TFA (~20 μL), in order to prepare the sample for purification.

2.3.5. **Purification**

Synthetic peptides were separated using: (i) Preparative Scale (Vydac; C₁₈, 10 μm, 300 Å, 22 x 250 mm, flow 8 mL min⁻¹) – used to desalt peptides from oxidation buffer. (ii) Analytical Scale (Vydac; C₁₈, 5 μm, 300 Å, 2.1 x 250 mm, flow 1 mL min⁻¹) used for bulk purification of desalted oxidized peptides. (iii) Capillary Scale (Phenomenex; C₁₈, 5 μm, 300 Å, 1.0 x 250 mm, flow 100 μL min⁻¹) – used for comparative RP-HPLC profiling, quality control of peptide purity, and peptide quantification experiments. System (i) used a 625 Waters HPLC pump and controller interfaced with a 996 Waters Photo Diode Array Detector. Both gradient control and data acquisition was facilitated by the use of the Waters Millennium³² software. Systems (ii) and (iii) used a Waters 2695 Alliance RP-HPLC System interfaced with a 996 Waters Photo Diode Array Detector for automated sample analysis and detection. Data was acquired and analyzed using Waters Millennium³² (v3.2) software. Samples were eluted using a linear 1% min⁻¹ gradient of organic (90/10% v/v CH₃CN/0.08% v/v aq. TFA; Solvent B) against 0.1% v/v TFA aq. (Solvent A). Elutants were monitor from 200–300 nm and extracted typically at 214 or 223 nm.
2.3.6. Mass Confirmation

PE-Sciex API 3000 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) was used in this investigation. Samples were directly delivered to the atmospheric pressure ionization (API) source of the mass spectrometer via a 30 cm, 50 μm i.d. fused silica capillary (Polymicro Technology Inc., Phoenix, AZ, USA) interfaced with Rheodyne 8125 Injector (20 μL external loop; Rheodyne, Cotati, CA, USA). Carrier solvent (50/50% v/v of a 0.09% v/v aq. Formic acid and 90/10% v/v CH₃CN/0.09% v/v aq. Formic acid) was provided by either an ABI 140B Dual Syringe Pump or by a micro-syringe infusion pump (Harvard Apparatus, South Natick, Mass., USA). Both systems allowed for varying flow rates (5–200 μL min⁻¹). Full-scan single MS experiments were typically obtained by scanning quadrupole-3 (Q-3) from m/z 400–2200 in 3–5 sec with a scan step size of 0.2–0.5 Da. Data was acquired using Analyst Software (v1.4.1) (Applied Biosystems/MDS Sciex, Thornhill, Ontario, Canada). Typically samples were RP-HPLC purified fractions, acidified with 5% v/v Formic acid in carrier solvent.

2.3.7. Quantification: Amino Acid Analysis

Synthetic peptide probes were quantified by comparison to a known Iberiotoxin standard using the capillary RP-HPLC methods described above (Section 2.3.2.3.5). All runs were performed in multiples (n=3) for quality assurance.

2.4. RESULTS

2.4.1. Manual Solid Phase Peptide Synthesis: IbTx[D19K-MTT]

A bioengineered version of IbTx was manually synthesized with a key modification, the non-native amino acid Lysine-MTT (Peptides International Inc., Louisville, KY) was incorporated in the 19 position (D19K-MTT) to allow for direct fluorophore bioconjugation in specified versions of the IbTx probe. The Lys-MTT approach was feasible due to the positioning the N-terminal pyroglutamic acid (Z) in the native sequence. The orthogonal protection strategy, and selective deprotection of Lys-MTT was simplified by the natural blockage of the α-amino function. Had a residue with a free amino been present at the N-terminal, alternate orthogonal protection schemes (i.e. Fmoc-
Lys-Boc) would have been required for selective deprotection and fluorophore bioconjugation. The synthesis proceeded smoothly requiring only three double couplings as illustrated by the blue peaks visible on the hydropathy index (Figure 15). Four isoforms were created in total and are summarized in Table 9 below.

![Figure 15. Hydropathy index for the manual solid phase peptide synthesis of IbTx[D19K-Mtt].](image)

### 2.4.2. Bioconjugation

Bioconjugation occurred under conditions described above (Section 2.3.2.3.2). Four derivatives of IbTx[D19K] were prepared. Only IbTx[D19K]-FITC and IbTx[D19K]-LC-FAM were purified to completion based on conjugation yields. IbTx[D19K]-LC-FITC and IbTx[D19K]-FAM were used in optimization experiments (Chapter 4).
Table 9. Fluorescently bioconjugated peptide probe isoforms created from IbTx[D19K]. The synthetic processes were evaluated for yield and efficiency.

<table>
<thead>
<tr>
<th>ISOFORM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IbTx[D19K]-FITC</td>
</tr>
<tr>
<td>IbTx[D19K]-LC-FITC</td>
</tr>
<tr>
<td>IbTx[D19K]-FAM</td>
</tr>
<tr>
<td>IbTx[D19K]-LC-FAM</td>
</tr>
</tbody>
</table>

2.4.3. Cleavage

Peptides were cleaved with Reagent K and precipitated using the cold ether system. A total of 3.75 g of crude peptide was recovered.

2.4.4. Air Oxidation

Air oxidation was conducted as described above (Section 2.3.2.3.4). The appropriate mass shift of 6 Da from reduced to oxidized peptide was observed by ESI-MS indicating the loss of 6 protons and the formation of three disulfide bonds (Table 10). Synthetic peptides were centrifuged and filtered for RP-HPLC purification.

Table 10. Mass shift of oxidized peptides according to ESI-MS analysis.

<table>
<thead>
<tr>
<th>Reduced Mass (Da)</th>
<th>Oxidized Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IbTx[D19K]</td>
<td>MH⁺ 4249.1</td>
</tr>
<tr>
<td>IbTx[D19K]</td>
<td>MH⁺ 4243.1</td>
</tr>
<tr>
<td>IbTx[D19K]</td>
<td>MH⁺ 4740.6</td>
</tr>
<tr>
<td>IbTx[D19K]</td>
<td>MH⁺ 4734.6</td>
</tr>
</tbody>
</table>

2.4.5. Purification

IbTx[D19K] was purified via C₁₈ Reverse Phase-High Performance Liquid Chromatography. IbTx[D19K] was observed at Rᵣ ~27 min (Figure 16) and IbTx[D19K]-FITC was observed at Rᵣ ~35 min (Figure 17). IbTx[D19K]-LC-FAM was
observed at \( R_t \sim 35 \) min (Figure 18) and the fluorescence chromatograph, monitored simultaneously, correlated to the elution profile at 214 nm (Figure 19).

Figure 16. RP-HPLC UV/Vis chromatogram of synthetic IbTx[D19K]. Probe was observed at \( R_t \sim 27 \) min.

Figure 17. RP-HPLC UV/Vis chromatogram of synthetic IbTx[D19K]-FITC. Probe was observed at \( R_t \sim 35 \) min.
2.4.6. Mass Confirmation

Direct infusion mass spectrometry was undertaken for target mass confirmation. IbTx[D19] was observed at $[\text{M+5H}]^{5+}$ 850.0 Da. (Figure 20). IbTx[D19K]-FITC was observed at $[\text{M+5H}]^{5+}$ 928.2 Da. (Figure 21). IbTx[D19K]-LC-FAM was observed at $[\text{M+6H}]^{6+}$ 790.1 Da. (Figure 22).
Table 11. Calculated Mass charge states of the three synthetic peptide probes bioengineered in the course of this investigation.

<table>
<thead>
<tr>
<th></th>
<th>IbTx[D19K]</th>
<th>IbTx[D19K]-FITC</th>
<th>IbTx[D19K]-LC-FAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH⁺</td>
<td>4243.1 Da.</td>
<td>4638.5 Da.</td>
<td>4734.6 Da.</td>
</tr>
<tr>
<td>[M+2H]²⁺</td>
<td>2122.6 Da.</td>
<td>2320.3 Da.</td>
<td>2368.3 Da.</td>
</tr>
<tr>
<td>[M+3H]³⁺</td>
<td>1415.4 Da.</td>
<td>1547.2 Da.</td>
<td>1579.2 Da.</td>
</tr>
<tr>
<td>[M+4H]⁴⁺</td>
<td>1061.8 Da.</td>
<td>1160.6 Da.</td>
<td>1185.65 Da.</td>
</tr>
<tr>
<td>[M+5H]⁵⁺</td>
<td>849.6 Da.</td>
<td>928.7 Da.</td>
<td>947.92 Da.</td>
</tr>
<tr>
<td>[M+6H]⁶⁺</td>
<td>708.2 Da.</td>
<td>774.1 Da.</td>
<td>790.1 Da.</td>
</tr>
<tr>
<td>[M+7H]⁷⁺</td>
<td>607.2 Da.</td>
<td>663.6 Da.</td>
<td>677.4 Da.</td>
</tr>
</tbody>
</table>

Figure 20. Direct infusion mass confirmation of IbTx[D19K].
Figure 21. Direct infusion mass confirmation of IbTx[D19K]-FITC.

Figure 22. Direct infusion mass confirmation of IbTx[D19K]-LC-FAM.
2.4.7. **Quantification: Amino Acid Analysis**

Peptides were quantified by comparative RP-HPLC analysis using an Iberotoxin standard. A predetermined number of moles from the IbTx standard were run by RP-HPLC (n=3) and the area under the curve (AUC) for the three replicates was averaged. This was then compared to AUC\textsubscript{avg.} (n=3) for IbTx[D19K], IbTx[D19K]-FITC and IbTx[D19K]-LC-FAM to determine the number of moles injected for each sample. This number was then back calculated to account for dilution volume and injection volume to determine the total amount of pure sample in moles. The molecular weight of each peptide was then used to determine total mass of the pure material and the total yield was calculated by dividing the overall mass of pure peptide by the amount of starting material (crude peptide).

Table 12. Yields from peptide toxin probes created in the course of this investigation.

<table>
<thead>
<tr>
<th>YIELDS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IbTx[D19K]</td>
<td>9.0%</td>
</tr>
<tr>
<td>IbTx[D19K]-FITC</td>
<td>1.7%</td>
</tr>
<tr>
<td>IbTx[D19K]-LC-FAM</td>
<td>&gt;9.0%</td>
</tr>
</tbody>
</table>

2.5. **DISCUSSION**

Manual solid phase peptide synthesis of IbTx[D19K] proceeded smoothly as can be seen in Figure 15. Difficulties were encountered as expected with Fmoc-Lys(Mtt)-OH, and recoupling was necessary. Post cleavage product revealed one major peak resolved at 214 nm by RP-HPLC.

The presence of pyroglutamic acid (Z) at the N-terminal functionally blocked the $\alpha$-amino function, limiting labeling to the $\varepsilon$-amino function of Lys19. This strategy allowed IbTx[D19K]-FITC to be prepared and purified. In the absence of Z at the N-terminal, alternative orthogonal protection strategies could be employed in order to direct
fluorophore labeling to specific residues within the primary sequence. The yield of IbTx[D19K]-FITC was extremely poor. This was attributed to pitiable efficiency in the formation of the thiol-urea bond between the ε–amino function of Lys19 and the fluorophore Fluorescein isothiocyanate (FITC). Quality assurance by Ninhydrin reaction (described above) revealed maximum coupling efficiency of about 75%, nearly 24.5% lower than acceptable. One possible explanation for this occurrence could be that FITC is sterically hindered from reacting by the side chain R-groups of the adjoining amino acids. To alleviate this, a spacer, Ahx-OH, N-ε-Fmoc-aminohexanoic acid, was first conjugated to the ε–amino function of Lys19 with great success (99.5%). Next, FITC was bioconjugated to the newly accessible spacer. Surprisingly, coupling efficiency remained low. This indicated that there was a problem with the formation of the thiol-urea bond. The fluorophore Carboxyfluorescein succinimidyl ester (FAM) was then bioconjugated in order to investigate the role of the attachment chemistry in coupling efficiency. Great success was achieved with FAM as coupling efficiencies reached 99.5%. The formation of an amide bond between Carboxyfluorescein succinimidyl ester and the ε–amino function of Lys19 is the most efficient, and best suited for this application. Maximizing potential yields is paramount in the design of potential probes in order to minimize loss and keep production costs low.

Cleavage was carried out in reagent K, under high acid conditions. One important design consideration for the creation of fluorescent probes is the stability of the fluorophore in this extreme environment. Some fluorophores decompose and will not retain fluorescent characteristics through the cleavage process. Certain compounds (i.e. FITC/FAM) are well suited for this process, while others (i.e. Alexa Fluors, Invitrogen/Molecular Probes) would be better applied in post cleavage ligation processes.

Air oxidation was achieved in amphoteric buffer containing urea and salt. Some precipitation of the target material occurred during oxidation, but this effect could be minimized with the addition of small amounts of propanol. Caution was taken while filtering and purifying peptides from the buffer as viscosity caused by the presence of Urea increased backpressure substantially and can potentially cause problems if not
monitored closely. Dilution of the sample with equal volumes of solvent A limited issues with backpressure during sample loading.

Purification of IbTx[D19K] proceeded smoothly due to the cleanliness of the synthetic process. A single dominant peak was isolated at Rt ~27 minutes as can be seen in the RP-HPLC chromatogram above (Figure 16). Purification of IbTx[D19K]-FITC (Rt ~25 min) proved incredibly difficult, as the low coupling efficiency effectively buried the peak of interest within a mass of byproducts (Figure 17). This likens the purification process to essentially finding the proverbial needle in a haystack, and significantly reduced yield. By improving chemistry, coupling efficiency of fluorophore bioconjugation and therefore yield, the purification of fluorescent probes can be simplified, as was the case with IbTx[D19K]-LC-FAM (Rt ~25 min) (Figure 18). Monitoring the peptide elution simultaneously with a fluorescence detector (ex. 492 nm, em. 519 nm) confirmed the covalent attachment of the fluorophore (Figure 19).

The calculated mass of bioengineered IbTx[D19K] (4243.1 Da) corresponds to the residue mutation, a 17 Da. difference from native Iberiotoxin (Figure 20). Mass charge states for this toxin are summarized in the Table 11, with the dominant state observed being [M+5H]^{5+} at 850.0 Da. The fluorescent probe IbTx[D19K]-FITC has a calculated mass of 4638.5 Da., and was observed in a dominant mass state [M+5H]^{5+} at 928.2 Da (Figure 21). Last, the toxin IbTx[D19K]-FAM has a calculated mass of 4735.8 Da. and was observed in at in the dominant mass form [M+6H]^{6+} 790.1 Da. (Figure 22).

Quantification of probes was achieved by RP-HPLC for use in Chapters 3 and 4. The area under the curve was for each synthetic peptide probe was compared to a known Iberiotoxin standard. Percent yield was calculated by determining the final number of moles and dividing that by the mass of peptide pre-oxidation (typically 100 mg in this investigation). The yield of IbTx[D19K] (9.0%) reflects the ease of purification resulting from optimal synthesis. The decreased, and poor yield of IbTx[D19K]-FITC was first thought to be a result of steric hinderance. However, upon further investigation it was found that the efficiency of the bioconjugation strategy was responsible for the poor yield. After switching bioconjugation chemistries, yield was maximized to equal, if not better than the non-fluorescent version of the probe (>9.0%). Although it should not be
possible to have a yield higher than the original product, we can attribute this increase to the ease in purification provided by a product that is fluorescent yellow. You can quite literally, see it coming off the column.

2.6. CONCLUSION

In summary a bioengineered isoform of the peptide toxin probe IbTx[D19K] was successfully synthesized at high yield using manual Fmoc solid phase techniques. The chemistry required for efficient bioconjugation of fluorophores in probe production was optimized and employed, and several fluorescent probes, based on the framework of Iberiotoxin, were produced for the \textit{In vitro} study of BK channel in Electrophysiological (Chapter 3) and Pharmacological (Chapter 4) studies.

Fluorophore efficiency could be improved as FITC and FAM are susceptible to photobleaching and instability when compared to the Alex Fluors or Cy dyes. As discussed in Chapter 4, the need for a near infrared version of the IbTx[D19K] probe becomes quite apparent for application in future, \textit{In vitro} investigations. IbTx[D19K]-LC-Cy5, could potentially fulfill this need. Additionally, post-resin derivatization could be explored using functionalized linkers and amine reactive Texas red fluorophores.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micro molar</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BK</td>
<td>Large conductance Ca^{2+}-activated K^+ channel</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FLIPR</td>
<td>Fluorometric Imaging Plate Reader</td>
</tr>
<tr>
<td>FMP</td>
<td>FLIPR membrane potential</td>
</tr>
<tr>
<td>GΩ</td>
<td>gigaohm</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell 293</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screen</td>
</tr>
<tr>
<td>IbTx</td>
<td>Iberiotoxin</td>
</tr>
<tr>
<td>IbTx-LC-FAM</td>
<td>Iberiotoxin-aminohexanoic acid-carboxyfluorescein</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo Hertz</td>
</tr>
<tr>
<td>mg</td>
<td>milli gram</td>
</tr>
<tr>
<td>mL</td>
<td>milli Liter</td>
</tr>
<tr>
<td>mM</td>
<td>milli Molar</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MTA</td>
<td>material transfer agreement</td>
</tr>
<tr>
<td>mV</td>
<td>milli volts</td>
</tr>
<tr>
<td>nM</td>
<td>nano Molar</td>
</tr>
<tr>
<td>NTX</td>
<td>Noxiustoxin</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
</tbody>
</table>
CHAPTER 3

PHARMACOLOGICAL CHARACTERIZATION OF IbTx[D19K] AND IbTx[D19K]-LC-FAM: A COMPARISON OF ELECTROPHYSIOLOGICAL TECHNIQUES.

3.1. ABSTRACT

Electrophysiological techniques have been used extensively to describe the pharmacological characteristics of peptide toxins including their molecular targets and kinetic parameters \( K_d \). Early experiments utilized various voltage-clamp approaches including the axial-wire method, the gap method and the microelectrode method before evolving into more modern patch-clamp techniques including cell-attached, whole-cell, excised and perforated patch configurations. Recently however, new technologies have prompted a review of a new classification method has been developed to describe the pharmacological parameters of peptide toxins. A voltage sensitive dye known as the Fluorometric Imaging Plate Reader (FLIPR) Membrane Polarization (FMP) dye has been developed and introduced by Molecular Devices. Based on the detection of changes in membrane polarization, the FMP dye can be used in standard fluorescent plate readers to investigate agonists and antagonists of G-protein coupled receptors, voltage-gated and ligand-gated ion channels. Whole cell patch clamp recordings from stably transfected HEK293/BK cells revealed a \( K_d \)'s of 48.35 nM (± 9.11 nM) and 161.29 nM (± 36.5 nM) from IbTx[D19K] and IbTx-LC-FAM respectively. The FLIPR (FMP) assays using the same cell line produced inconclusive data for IbTx[D19K] and IbTx-LC-FAM. This data highlights some of the inherent limitations associated with the proprietary FMP-dye including propensity for false positives, limited fluorescent signal intensity and the reliance large changes in membrane polarization for detection. The accurate, reproducible data obtained from whole cell patch clamp experiments, in conjunction with the limitations experienced, and inconclusive data obtained from the FLIPR (FMP) assay reconfirmed the use of patch clamp analysis as the primary method for the classification of synthetic peptide toxins for the advanced bioengineering of novel fluorescent probes.
3.2. INTRODUCTION

3.2.1. A General Introduction to Electrophysiology

Electrophysiology is the science of studying the electrical signatures of various cells and tissues from throughout the body. Generated by the flow of ions through specialized receptors known as ion-channels (discussed at length in Chapter 1), electrical currents are responsible for the manifestation and propagation of fundamental signals such as nerve impulses and action potentials. The measurement and study of these electrical signals can be used to classify different families and sub-types of ion-channels as well as the pharmacological ligands that inhibit them. Unfortunately, these measurements can be difficult to obtain for three main reasons, (i) the flow of ions through channel proteins is a dynamic process due to changing driving forces, (ii) voltage dependant gating mechanisms respond to changes in membrane potential, and (iii) certain intracellular (cytosolic) factors (i.e. Mg$^{2+}$, K$^+$) can alter gating a restrict the flow of ions ($I$). These limitations restricted the efforts of neuroscientists until advances in technology facilitated the invention of an apparatus which overcame the aforementioned difficulties and led to the establishment of a new scientific field of study, electrophysiology.

The primary method for measuring the electrical properties of ion channels is an approach known classically as the voltage-clamp technique, and later, the patch-clamp technique. With the early development of the voltage clamp in 1949, Kenneth S. Cole successfully measured the transverse impedance of a giant squid axon (Loligo vulgaris) by holding membrane potential static with a feedback circuit created by inserting two electrodes directly into the axon fiber (2). This work was followed shortly by the defining experiments of Hodgkin and Huxley for which they shared the 1963 Nobel Prize in Medicine. By eliminating differences in membrane potential, Hodgkin and Huxley were successful in defining the mechanisms responsible for the current-voltage relationship that physiologically manifests the propagation of the action potential (3-6).

Voltage-clamp techniques use intracellular microelectrodes to study large areas of membrane or whole tissues preparations, which contain several hundred ion channels and produce current on the macroscopic scale. Alternatively, the development of extracellular recording methods such as the patch-clamp approach in the 1970's,
facilitated the study of single channel, microscopic scale conductance's and helped to revolutionize the growing field (7). This feat was achieved formally in 1976 by Erwin Neher and Bert Sakmann when they published work which outlined an approach to measure single channel conductance from a biological preparation (8).

Both voltage- and patch-clamp techniques have been adapted and utilized extensively for the pharmacological classification of ion channel ligands including small organic molecules, peptides and proteins. Using patch-clamp analysis, the highly specific relationship between Noxiustoxin (NTX: Centruroides noxius) and its Potassium (K+) channel target was described in 1982 by Carbone et al. (9, 10). This revelation ushered in a new era in the identification of medically significant peptides, and in recent years, advanced patch-clamp methods have been used to classify astounding numbers medically significant toxins from various animal species including sea anemones, cone snails, spiders, and snakes (11-14).

Voltage-sensitive dyes have emerged recently as a possible alternative to the voltage- and patch-clamp approaches for describing the pharmacological characteristics of novel toxins. The advanced dyes offer unique and specific advantages over the established technology in that they require minimal specialized training, minimize the need for technologically advanced, costly equipment, and reduce the amount of time required to classify novel peptide toxins (15). This technology is still in its infancy however, requiring extended viability studies, comprehensive validation and unequivocal acceptance from the scientific community. Electrophysiological classification has been principal to the current investigation for understanding if sequence bioengineering and fluorophore bioconjugation has impacted, or hopefully, not impacted the pharmacological relationship between Iberiotoxin[D19K], IbTx-LC-FAM and the specific target, the BK potassium channel.

3.2.2. The Voltage-clamp Method

The forefather of electrophysiological classification, the voltage-clamp technique for intracellular recording has been utilized extensively since its inception in 1949, and continues today. This approach allows for the study of transmembrane electrical
potential, produced by the charge differential across a physiological membrane (Figure 1A). By viewing this system as an electrical circuit, the storage or flow of ions across this membrane can be treated as either potential \( (E) \) or current \( (I) \) respectively (Figure 1B). Using a feedback amplifier to hold transmembrane voltage at a static, known value, the difference between the measured electrical potential and a potential which has been set artificially by the experimenter can be quantified \( (16) \).

\[
I = \frac{E}{R} \quad \text{or} \quad E = IR \quad (3.1)
\]

This relationship can be described mathematically by Ohms Law (equation 3.1), where current \( (I) \) through a resistor is proportional to the potential \( (E) \), and inversely proportional to resistance \( (R) \).

A major advantage of the voltage-clamp technique is that it facilitates the differentiation between membrane ionic current and capacitive current \((17)\). In order to understand the technical theory of electrophysiology, a brief review of biophysics is required including the relationship between the experimental parameters and their expressed units (Table 1).
Table 1. A brief overview of the electrical parameters used in basic electrophysiology. Adapted from Molleman et al. 2003 (1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Unit of Measurement</th>
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<tbody>
<tr>
<td>Capacitance</td>
<td>C</td>
<td>farad (F)</td>
</tr>
<tr>
<td>Charge</td>
<td>Q</td>
<td>Coulomb (C)</td>
</tr>
<tr>
<td>Conductance</td>
<td>g</td>
<td>siemens (S)</td>
</tr>
<tr>
<td>Current</td>
<td>I</td>
<td>ampere (A)</td>
</tr>
<tr>
<td>Frequency</td>
<td>ν</td>
<td>Hertz (Hz)</td>
</tr>
<tr>
<td>Potential</td>
<td>E</td>
<td>volt (V)</td>
</tr>
<tr>
<td>Resistance</td>
<td>R</td>
<td>ohm (Ω)</td>
</tr>
</tbody>
</table>

Several adaptations of the voltage-clamp technique (Figure 2) have been developed and can be employed based on the size and/or shape of the cell preparation under investigation. Techniques include the axial wire method which works well with large cylindrical tissues, the gap method which facilitates the investigation of myelinated fibers such as neurons, the microelectrode clamp which can be applied in either single, double, or triple electrode configurations, and the suction pipette method works well on isolated cells.

Figure 2. A simplified schematic of the voltage-clamp circuit. Adapted from Moore et al. 1971 (18).
3.2.2.1. Axial Wire Method

The axial wire method is best suited for preparations of long cylindrical cells, and has been used with preparations of barnacle muscle fibers, giant squid axons, Myxicola axons and crayfish axons (17, 19-21). In order to apply this technique, the diameter of the cells in preparation must be large enough to allow penetration of the axial wire without anatomical damage. The axial wire, a low-resistance current electrode typically made of platinum, is inserted longitudinally down the center of the cut end of the fiber. The electrode functionally serves dual purpose by passing current and providing clamp conditions (22). One major advantage of the axial wire method is the low resistance access which provides for rapid measurements under clamp conditions (17).

3.2.2.2. Gap Method

Initially developed to investigate naturally isolated patches from excitable membranes located in the node of Ranvier, the gap method is used typically to measure the intracellular potential distribution or external series resistance in a small patch of membrane from nerve axons or muscle fibers (22). A gap is created by running the preparation through a number of pools (usually consisting of intracellular solution) separated by air, sucrose or vaseline (17). These separations create artificial nodes of high electrical resistance, similar to those found naturally in myelinated fibers. This method is suited for elongated preparations, the diameter of which does not need to be as large as that required for the axial wire method. The gap method has been employed for investigations to define signature characteristics such as tail or gating currents from myelinated axons and vertebrate muscle fibers (23, 24).

Additionally, the gap method can be applied to preparations of large spherical cells such as molluscan neurons and Xenopus oocytes (25, 26). In this arrangement, cells are held in a funnel shaped partition with a hole separating two solution filled chambers. The cell is seated against the funnel walls to form a tight electrical seal while the cell membrane is permeabilized to permit access to the inside of the cell. The cell is then clamped between the two pools, one contacting the cytoplasmic solution and the other, the cellular exterior.
Importantly, this approach provides a rapid clamp permitting the study of gating currents with low noise (17).

### 3.2.2.3. Microelectrode Clamp

Depending on the physical characteristics of the expression system under investigation, the voltage-clamp method can be configured using either a single, double or triple microelectrode to penetrate the biological preparation. The single electrode configuration is most often used on small cells which may be too fragile to handle impalement with multiple electrodes (Figure 1A), while a double electrode arrangement is suited for channels expressed in large and robust cells such as *Xenopus oocytes* (Figure 1B) (27, 28). Quickly switching between measuring voltage and injecting current when using the single microelectrode set-up, is accomplished using specialized equipment. Conversely, the double electrode set up utilizes one electrode for recording the voltage, and the other to inject current (1). Until the development of time-sharing systems, the single electrode variation was notoriously difficult to use because the speed in which switching occurs (2-20 kHz) can quickly lead to uncontrolled electrical oscillations within the preparation (29).

![Figure 3](image1.png)

**Figure 3.** Simplified schematics of the single (A) and double (B) microelectrode voltage clamp circuits. Adapted from Molleman *et al.* 2003 (1). A description of the various technical functions can be found in the appendix, Figure A1.

A third configuration, used only for special cases, makes use of three microelectrodes and is aptly known as the three electrode clamp. This method was developed initially for use
on long, cylindrical cells such as muscle fibers, and works well for the measurement of delayed currents (30). Functionally, one microelectrode is used to deliver current into the preparation while the other two are used for measurement purposes (Figure 4).

![Figure 4. Simplified schematic of the three microelectrode voltage clamp circuit. Adapted from Adrian et al. 1970 (30).](image)

### 3.2.3. The Gold Standard: Patch-clamp

The development and use of the voltage-clamp approach was followed closely by the establishment of new techniques by Bert Sakmann an Erwin Neher which facilitated high fidelity measurement of minute (picoampere) ionic currents from single channels and ushered in a new era in electrophysiology (31). The new techniques overcame two fundamental limitations of early voltage-clamp experiments, mainly (i) the space-clamp problem of spatial non-uniform control over voltage, and (ii) the lack of dynamic control over the composition of intracellular ionic species. Overcoming these obstacles made possible the characterization of ion channel biophysical properties including conductance, voltage dependence, selectivity, open probability, as well as the identification and kinetic classification of pharmacological ligands (i.e. peptide toxins) (8).

By all accounts, most patch-clamp experiments are, at their core, voltage-clamp experiments. Initially, a glass pipette with a clean, smooth, fire polished tip is pressed against the membrane of a single, isolated cell. The pipette is connected to a voltage-clamp circuit allowing measurement of a very few, or even a single channel (Figure 5) (29). Sequential iterations of the technique involved the use of suction to form a Gigaohm (GΩ) seal between the glass pipette and cell membrane. This set-up produced the microenvironment allowing rupture of the patched membrane thus providing dynamic
control over intracellular composition, and thus ionic forces which drive cellular electrophysiology.

The development and evolution of the equipment used for patch-clamping has paralleled the evolution of the field itself. Fundamentally the experimental setup would consist of a Faraday cage containing a microscope to visualize the cells and an air-table to isolate the microscope from vibrations, and a patch-clamp amplifier and a pulse generator are located on a rack nearby in order to voltage clamp the cells (8). Finally, a patch pipette/amplifier probe is held in the arm of micromanipulator which is attached to an oscilloscope, computer and chart recorder in order to record and document the data and results (8). Improvements in this equipment led to the evolution of the patch clamp technique and expanded the applicability of the approach to virtually all biological preparations including both animal and plant cells as well as isolated tissue, prokaryotic cells and cell organelles (1, 16). Primary variations of the patch-clamp technique such as the cell-attached, whole-cell, excised and perforated patch approaches have helped to facilitate this flexibility while secondary variations including the double-patch, loose-patch, giant-patch, detector-patch and the tip-dip bilayer method have further expanded the field and contributed in unique and complimentary ways.
3.2.3.1. Cell-attached Patch

The most straightforward, and simplest of all patch configurations is the cell-attached patch approach. In this mode, the pipette is pressed against the cell membrane creating a tight seal. This high resistance connection, typically in excess of one GΩ, allows for single channel measurement of channel proteins captured within the tip of the pipette (Figure 6) (8). Several key conditions must be met in order to achieve a seal amenable to patch clamp experiments. First, the cell surface (membrane) must be clean and free of extracellular debris. Second, experimental buffers must be filtered to remove macromolecules and dust that can disrupt the seal. Third, the pipette tip should be fire polished to smooth and clean the contact surface, and last, positive pressure must be applied through the pipette just prior to seal formation to generate outflow keeping the pipette tip clean and free of debris (32).

![Figure 6. Representative circuit of the cell-attached patch clamp configuration. Adapted from Molleman et al. 2003 (1).](image)

Because the biological membrane remains intact following formation of the seal, this approach is the easiest to achieve and provides the closest to a native, physiological environment for the measurement of single channels. This same advantage also imparts the major drawback to the cell-attached approach in that it hinders measurement of
membrane potential and limits access to, and control of the intracellular environment \( (I) \). The cell attached approach is the starting point for two advanced forms of patch known as the perforated patch, and the excised, inside-out patch-clamp. These approaches will be discussed further in Sections 3.2.3.4 and 3.2.3.3.B respectively.

### 3.2.3.2. Whole-cell Patch

The whole-cell approach begins similar to the cell attached mode. The primary difference being the portion of the biological membrane within the pipette is ruptured by applying slight suction, thus creating direct electrical contact between the electrode and cytoplasm and providing low-resistance (mega-ohm scale) access to the intracellular environment (Figure 7) \( (8) \). The tightness of the seal (\( \Omega \)) prevents current leakage between the two electrodes and facilitates measurement of whole cell (macro) current while manipulating, or perfusing both the intra- and extracellular fluid (environment) \( (22) \).

![Figure 7. Representative circuit of the whole-cell patch clamp configuration. Adapted from Molleman et al. 2003 \( (1) \).](image)

This approach is useful for determining cellular populations of ion channels. Under these conditions however, washout of cytosolic factors can occur essentially destroying the native physiological make-up of the cell \( (I) \). In order to overcome some of the main
limitations of the whole-patch method, a new approach was developed known as the perforated patch which allows for direct cytosolic contact through artificial pores while retaining intracellular fluid by maintaining the majority of the lipid bilayer. Much like the cell-attached patch configuration is the basis for one form of excised patch, so too is the whole-cell patch mode. By withdrawing the pipette from the cell following attachment and rupture of the biological membrane, the bilayer reseals to form an outside-out excised patch from the whole-cell setup (22). This technique will be discussed subsequently in Section 3.2.3.3.A.

3.2.3.3. Excised Patch

Isolating a small portion of the cell membrane can facilitate single channel recording under carefully controlled conditions. Known as the excised-patch approach, isolated membranes can be set up in either the outside-out, or inside-out configuration based on the initial patch mode, either the whole-cell or cell-attached technique respectively. Under these conditions, "inside" and "outside" refer to the intracellular and extracellular side of the membrane while "out" refers to the bath solution. Although technically demanding, the excised-patch approach provides unequivocal control over both the electrical and chemical microenvironments surrounding the cell, offering a unique setting to investigate single-channel response to compounds (i.e. peptide toxins) applied to the exposed cellular matrix (1).

3.2.3.3.A. Outside-out Patch

Outside-out patches are created in a five-step process starting from the cell-attached configuration. The cell-attached patch is quickly converted to whole-cell mode using standard suction processes. Once in whole-cell mode, the excised patch is formed by pushing the microelectrode further into the cell and then pulling the pipette away from the cell body until separation occurs. Small portions of the cell membrane are excised from the surrounding lipid bilayer and remain attached to the pipette tip. Following withdrawal of the pipette, the remaining portions of the bilayer, still attached to the pipette tip, fold back onto themselves due to the polar nature of phospholipid
constituents, effectively creating a parabolic patch where the extracellular side of the cell membrane faces the bath solution (Figure 8) (16).

Figure 8. Creation of an outside-out patch configuration in five-steps. Adapted from Kornreich et al. 2007 (16).

This approach is more difficult to obtain than the inside-out excised patch configuration, however the planar orientation and size of the outside-out patch creates conditions that permit direct manipulation of the extracellular side of membrane making it extremely useful for studying the pharmacological properties of receptor-operated channels such as those activated by ion channel agonists including neurotransmitters, hormones and peptide toxins (8). Unfortunately, an increased level of background noise is often encountered while recording under these conditions. This noise can likely be attributed to the lowered resistance of the pipette seal caused by excising a membrane patch with a relatively large surface area. Additionally, channel kinetics may not adhere to those observed under "native" conditions due to the artificial microenvironment created by the bath solvents (32).

3.2.3.3.B. Inside-out Patch

The inside-out excised patch configuration, much like the outside-out, is again initiated from the cell-attached patch mode, however unlike the outside-out patch, there is no need to progress to the whole-cell set-up. Following the formation of the GΩ seal, the pipette is pulled away from the cell membrane, breaking free a small section of lipid bilayer. Once excised, the phospholipids briefly form a vesicle before the outer face is broken in one of three ways. The intracellular components of the vesicle (membrane) are exposed either through immersion in a concentrated Ca^{2+} solution, exposure to the
interface between the bath solution and the open air environment, or through brief contact with a paraffin droplet (Figure 9) (32).

![Figure 9](image)

Figure 9. Creation of an inside-out patch configuration in five steps. Adapted from Kornreich et al. 2007 (16).

Since most channels are modulated in some form by intracellular processes, this configuration allows for the study of channel protein activation or modulation through exposure to high levels of Ca$^{2+}$, ATP or the catalytic subunit of protein kinase (33-35). Additionally, the inside-out patch facilitates the study of cytosolic signaling molecules and enzymatic activity on the behavior of second-messenger-activated channels (8).

Certain drawbacks to the inside-out patch configuration exist including a loss of vital cytosolic factors which function to control channel behavior, and the occurrence of trapped vesicles within the pipette tip which prevent the formation of planar patches (32).

### 3.2.3.4. Perforated Patch

One of the primary problems with the whole-cell patch approach is the loss of cytosolic factors during the perfusion and dialysis of micropipette fluids over the course of long-lasting experiments (36). This effect can result in changes in the properties of ionic currents and secondary messenger responses (32). Introduced in 1988 by Horn and Marty, the perforated patch configuration was developed to eliminate this washout effect by utilizing selective perforation in order to retain vital cytosolic constituents such as divalent ions and proteins while maintaining the free-flow of small molecules such as Na$^+$, K$^+$ and Ca$^{2+}$ ions (37). The perforated patch is achieved first by initiating the cell-attached configuration. Once the attachment is made, pore forming compounds such as
nystatin, α-toxin, gramicidin and amphotericin B are introduced to induce artificial channels or pores in the portion of the membrane patched within the electrode (8, 16, 32). As with the whole-cell approach, the perforated patch method is useful for studying global ion channel activity within a cell.

### 3.2.3.5. Secondary (niche) Patch Applications

Several secondary patch clamp approaches have been developed to overcome the limitations described with the primary patch methods. The techniques are used in specialized cases and in unique circumstances where experiments cannot be performed readily with standard set ups. The double-patch approach is basically a whole cell measurement of two attached cells which allows for the study of gap junctions or junction channels (38). Loose-patch techniques are considered an improved version of the cell-attached approach which uses a large-diameter pipette tip (5-120 μm) with a low resistance seal of only a few megaohms (39). Here, special electronics are used to compensate for leak currents in conjunction with specialize pipettes which facilitate the measurement of large currents. This approach was developed to map ion-channel distribution and current densities observed in large cells (40). By utilizing specialized hydrocarbon mixtures in the giant-patch approach, large-tipped pipettes (10-40 μm), dusted with the hydrocarbons, can form GΩ seals with giants patches of cell membrane (41). Under normal conditions, GΩ seals become increasingly more difficult to achieve the larger the membrane patch becomes. This configuration was developed to study macroscopic currents produced by ion-channels and transporters (8). The detector-patch was first used to study ion channels that are specifically sensitive to neurotransmitters and second messengers. Using this approach, it is possible to take an inside-out patch containing specialized ion-channels, and inserting it into a larger cell such as an oocyte to study intracellular changes in ion concentration (42). Additionally outside-out patches containing channels capable of detecting neurotransmitters can be used to study synaptic activity (43-45). Finally, the tip-dip bilayer technique is fundamentally a modified approach to study reconstituted ion channels in a small patch configuration known as the lipid bilayer technique. In this case, a synthetic lipid bilayer is produced on the pipette tip through repetitive dipping into a monolayer lipid film (46, 47). This method
facilitates the study of reconstituted ion channels while minimizing the capacitative artifacts indicative of standard bilayer experiments (8).

3.2.4. The FLIPR (Fluorometric Imaging Plate Reader) Assay

There are several technologies currently used to investigate ion-channels including radioligand binding, radioactive flux assays, cell viability assay in both mammalian and yeast cell lines, bioluminescence and fluorescence detection (48). However the gold-standard for monitoring ion-channel activity has historically been the various patch-clamp recording techniques, as previously discussed. The versatility of the patch-clamp technique is unfortunately hampered by the cell-by-cell approach, effectively limiting rate, and functionally prohibiting its applicability to high throughput screening (HTS) processes (49).

![FLIPR mechanism](image)

Figure 10. FLIPR mechanism. Fluorescence intensity changes with either an increase or decrease in cell membrane potential. Membrane depolarization causes dye to follow (+) ions into the cell causing an increase in fluorescence. Hyperpolarization causes the dye to follow (+) ions out of the cell causing a decrease in fluorescence. Adapted from www.moleculardevices.com (50).

To overcome the rate limiting effects of the patch-clamp approach in the search for, and classification of, novel modulators of channel activity, fluorescence-based HTS assays have been developed in recent years to replace the conventional technologies (51). In the mid-1990's, fluorescent, voltage-sensitive dyes were developed which are capable of monitoring changes in membrane potential by responding to changes in cellular stimuli including hyperpolarization and depolarization with dynamic, quantified fluorescence (52). Known as Fluorometric Imaging Plate Reader (FLIPR) Membrane Potential (FMP)
dyes (FMP-Blue and FMP-Red), the partitioning of the dye across intra- and extracellular membrane compartments occurs as a response to changes in membrane potential and results in changes in fluorescence intensity (48). Generally, depolarization of the cell membrane induces movement of the FMP dye into the cell and causes binding to intracellular hydrophobic sites, functionally producing an increase in the fluorescent signal (Figure 10). Alternately, hyperpolarization of the cellular membrane causes extrusion of the dye from the intracellular environment producing a decrease in fluorescence (53). Changes in membrane potential, monitored via FMP assay, are not a linear function of the number of channels opened by the agonist, rather as the number of open channels increases, membrane potential approaches the equilibrium potential asymptotically, as predicted by the Goldman equation (Equation 3.2) (54). Therefore the relationship between the concentration of the agonist, and the cellular response can be distorted when using membrane potential to monitor response (51).

$$E_m = \frac{RT}{F} \ln \left( \frac{\sum_i^n P_{M_i^+} [M_i^+]_{out} + \sum_j^M P_{A_j^+} [A_j^+]_{in}}{\sum_i^n P_{M_i^+} [M_i^+]_{in} + \sum_j^M P_{A_j^+} [A_j^+]_{out}} \right) \quad (3.2)$$

FMP assays can be used to investigate the pharmacological characteristics of selective inhibitors of voltage-gated ion channels, ligand-gated ion channels and G-protein coupled receptors (51, 55). FMP dyes are advantageous due to the compatibility with FLIPR plate based technology, and their suitability for conventional fluorescence plate readers, compatibility which has increased the number of compounds that can be classified in a given time by as much as 20-fold (48, 56). Some drawbacks to the use of FMP assays have emerged including difficulties in assay optimization, signal intensity and potential artifacts. There are also a number of factors that can result in false positives in the data including fluorescent compounds (ligands), various ionophores (i.e. Ca^{2+}, K^+) and compounds that cause membrane permeabilization or receptor internalization (desensitization) (57).
3.3. EXPERIMENTAL PROCEDURES

3.3.1. Outsourced Approach: Patch Clamp Analysis

Whole cell patch clamp experiments were performed in collaboration with Dr. Youshan Yang in the laboratory of Dr. Frederick Sigworth at the Yale University School of Medicine in New Haven, Connecticut. Analysis was undertaken using the synthetic peptides IbTx[D19K] and IbTx-LC-FAM. Recordings were made from stably transfected HEK293/BK cells maintained at physiological conditions (37°C, 5 % CO₂) as described in Section 3.3.2.1. Ramp voltage was staged from -100 to +100 mV delivered in 2.2 sec intervals while currents were measured at +80 mV. Cells were perfused with HEPES buffer (10 mM) containing KCl (160 mM), EGTA (1 mM), free calcium (1 μM) and varying concentrations (10 - 2000 nM) of either IbTx[D19K] or IbTx-LC-FAM maintained at physiological pH (pH = 7.4). The pipette buffer was identical with exception of the experimental peptide. A minimum of six (n ≥ 6) measurements were taken for IbTx[D19K] while at least four (n ≥ 4) measurements were made using IbTx-LC-FAM, both from 2 μM stocks diluted in DH₂O and spiked with BSA (1 mg/mL). Data was analyzed using Igor Pro 6.32A and a fixed Hill coefficient of 1 was used for curve fitting and standard deviation.

3.3.2. In-house Approach: 96-well Plate Based FLIPR Assay

3.3.2.1. Cell Culture & MTT-Cell Growth Analysis

Cell Culture: A documented vehicle for the expression of exogenous proteins, HEK293 cells are an ideal platform for the expression of BK channel (58). Native human embryonic kidney cells (HEK293) were stably tansfected using a pcDNA3 vector encoding a His6-Flag-epitope-tagged human Slo gene (gi: 507922) and resistance to the antibiotic Geneticin (G418), described elsewhere by Wallner et al., 1995 (59). Transfected HEK293/BK cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Cellgro, Mediatech Inc., Manassas, VA) supplemented with 10.0% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, Ontario, Canada), 10,000 U/mL penicillin/streptomycin (Cellgro, Mediatech Inc., Manassas, VA), 1% L-glutamine (Lonza...
Inc., Allendale, NJ) and 0.3 mg/ml Geneticin (G418; Gibco, Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO₂.

**MTT-assay:** After allowing 2 weeks for selection (at 0.6 mg/mL G418), cells (maintained as described previously, Section 3.3.2.1) were plated in, culture treated, flat-bottom, 96-well plates (Greiner, CELLSTAR; Sigma Aldrich) at a density of 1x10⁴ cells/well and left to attach overnight in a humidified incubator (37°C, 5% CO₂). At times of 0, 12, 24, 36, 48, 60 and 72 h, media was aspirated from n=3 wells and cells were washed with warm, sterile PBS (200 μL). Fresh, sterile complete media (100 μL) containing MTT reagent (20 μL) to a final concentration of 5 mg/mL was added to each well and the plates were returned to the incubator. Following 4 h incubation, the MTT-media was aspirated and neat DMSO (200 μL) was added to each well overnight. The following day (12 h), optical density (OD) was measured at 595 nm in a Spectramax Gemini plate reader (Molecular Devices) running SoftMax Pro microplate data acquisition and analysis software (Molecular Devices). The experiment was repeated three times to reduce variability and to infuse confidence into the analyzed data. Data was analyzed using GraphPad Prism v4.

**3.3.2.2. FLIPR**

As described previously (Section 3.3.2.1), cells were maintained under selective pressure for 2 weeks (0.6 mg/mL G418) prior to investigation. Cells were plated in flat-bottom, culture treated, 96-well plates (Greiner, CELLSTAR; Sigma Aldrich) at a density of 1x10⁴ (or 1 x 10⁵) cells/well and left to attach overnight in a humidified incubator (37°C, 5% CO₂). Following overnight attachment, cells were incubated for 30 min at physiological conditions (37°C, 5% CO₂) with 100 μL of the proprietary FMP-Dye (FMP-Red - Molecular Devices; Sunnyvale, California) prepared as per the manufacturer's instructions. Toxins were diluted in PBS (1 mg/mL BSA), brought to a Tᵥ of 50 μL and added at varying concentrations (0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 10, 50, 100, 500 and 1000 nM) to respective wells (n=3). After addition of the toxin, cells were again incubated for 20 min in a humidified environment (37°C, 5% CO₂) to achieve maximum block. Finally, plates were analyzed using a multipoint read in a Gemini
SPECTRAmax fluorescent plate reader (ex: 530 nm, em: 565 nm) at high sensitivity with no wavelength cutoff. Data was analyzed using GraphPad Prism v4.

3.4. RESULTS

3.4.1. Outsourced Approach: Patch Clamp Analysis

Following whole-cell patch clamp analysis, the $K_d$ of IbTx[D19K] was found to be 48.35 nM ($\pm$ 9.11 nM) while IbTx-LC-FAM was found to have a $K_d$ equal to 161.29 nM ($\pm$ 36.5 nM) (Figure 11). Conductance was measured at toxin concentrations of 10, 50, 100, 200, 500 and 2000 nM. The plots indicate that signal was effected at a minimum concentration of ~10-20 nM, with full block observed nearing ~1μM (Figure 11). Blocking showed stability and selectivity for BK as washout slowly returned signal to initial values.

![Figure 11. Whole cell patch analysis of IbTx[D19K] and IbTx-LC-FAM recorded from stably transfected HEK293/BK cells.](image-url)
3.4.2.  In-house Approach: 96-well Plate Based FLIPR Assay

3.4.2.1.  Cell Culture & Cell Growth Analysis (MTT-assay)

The growth characteristic native HEK293 cells and stably transfected HEK293/BK cells were evaluated using the MTT cell viability assay (measured in optical density at 595 nm). The first analysis was carried out over 5 days (120 h), however it was discovered that a three day (72 h) experiment sufficiently provided the data necessary to describe the growth characteristics of the cell lines (Figure 12). For the final 2 trials, the experiment was concluded after 72 h. Both the native HEK293 and transformed HEK293/BK cells had a lag phase of 12 h following plating prior to initiation of the growth phase. There was a significant difference in the maximum cell density achieved by the two cell lines with native HEK293 cells growing almost 1.5x more dense than HEK293/BK cells after 60 h. The growth of both cell lines plateaued simultaneously at ~60 h, indicating the cells had reached maximum density.

![Growth Curve (geometric mean)](image)

Figure 12. Measurement of growth characteristics of native (HEK293) and transformed (HEK293/BK) cell lines via MTT-cell viability assay.

3.4.2.2.  FLIPR

The FLIPR (FMP)-assay was performed using HEK293/BK cells (Figure 13) while control experiments were conducted in parallel using wild type HEK293 cells (Figure 14). Experimental data indicated a $K_d$ value of ~1 nM for IbTx[D19K] and about ~2 nM
for IbTx-LC-FAM. The data is unreliable as the standard deviations are large and varied. Additionally, false positives were observed in the experiments conducted in the non-expressing, control cell line, HEK293. The false positives negate any information obtained from the experiments in the expressing HEK293/BK cell line. The data was analyzed using GraphPad Prism v4.

Figure 13. FLIPR (FMP) assay of IbTx[D19K] recorded using FMP-Red dye (Molecular Devices). (A) Experimental data from HEK293/BK cells. (B) Control data from HEK293 wild type cells.

Figure 14. FLIPR (FMP) assay of IbTx-LC-FAM recorded using the FMP-Red dye (Molecular Devices). (A) Experimental data from HEK293/BK cells. (B) Control data from HEK293 wild type cells.
3.5. DISCUSSION

Traditionally, the whole cell patch clamp technique has been used by the Bingham laboratory to determine experimentally, the pharmacological properties of synthetic peptides for further use in in-vitro receptor expression and visualization experiments (60, 61). This work has been outsourced due to the necessary expertise and specialized equipment required to perform the analyses. First, an electrophysiology laboratory must be identified which possesses the appropriate channel isoforms, in this case, the Ca\(^{2+}\)-activated K\(^+\) channel (BK). Next collaborative partnerships must be established which requires the generation and signing of material transfer (MTA) and Non-disclosure agreements, legally binding documents required by research institutions. Once the initial requirements are fulfilled, the samples can then be prepared accordingly (lyophilized) and shipped to the contracted laboratory for investigation. Whole cell patch clamp requires large amounts of experimental samples for classification that are both expensive to produce, and time consuming to prepare. In order to reduce some of the constraints of whole cell patch characterization, including the length of time, amount of materials and the reliance on 3rd party investigators, the FLIPR (FMP) assay has been identified as a potential in-house alternative to the outsourced process. Here, the accuracy of the in house assay has been compared to traditional patch analysis for two synthetic peptides, IbTx[D19K] and IbTx-LC-FAM in an attempt to validate the FLIPR (FMP)-assay as a valid replacement of current approaches.

Whole cell patch analysis revealed $K_d$'s of 48.35 nM (± 9.11 nM) and 161.29 nM (± 36.5 nM) for IbTx[D19K] and IbTx-LC-FAM respectively (Figure 11). From the analysis of the generated data, it appears that the point mutation at position D19, and substitution with a specialized Lysine(MTT) residue, impacted slightly the binding kinetics of the synthetic peptide when compared to that of the native toxin, whose $K_d$ is 1.7 nM (± 0.5 nM) (62). Comparatively, previous single point synthetic mutants of the Iberiotoxin peptide had extremely varied kinetic properties, thus, the current results were not entirely unexpected (Table 2). The isoforms listed in Table 2 reveal that point mutations and side chain additions to the AA residue at position 19 in the native IbTx sequence sequentially increase the binding characteristics ($K_d$) of the toxin based on size, charge and polarity.
A previous experiment in 2006 by Bingham et al. investigating the biotinylation of IbTx, revealed a 15-fold increase in the dissociation constant. With this under consideration, IbTx[D19K] (K_d = 48.4 nM) retained sufficiently strong binding kinetics, and most importantly, specificity for the BK channel. The favorable pharmacological properties revealed by whole-cell patch clamp permitted progression to the bioconjugation step of the current investigation allowing the addition of the carbon linker (LC: aminohexanoic acid) and the fluorophore (FAM: 5'-carboxyfluorescein) to proceed (Chapter 2). Had there been issues with the binding kinetics of the IbTx[D19] peptide, reevaluation of the synthetic strategy and fluorophore placement would have been necessary. Although further reduced following the final bioconjugation steps, the IbTx-LC-FAM peptide (K_d = 161.3 nM) was found to retain sufficient biological activity and the entirety of its channel specificity (Figure 11). This data provided sufficient evidence to proceed with in vitro experiments using the IbTx-LC-FAM probe to investigate BK in engineered an native expression systems (Chapter 4).

Table 2. Examples of Iberiotoxin mutants and how the mutations effect the pharmacological properties of the peptide. *The experimental K_d of IbTx-Alexa488 was not determined in the study by Hafidi et al. 2005, however the paper states the peptide was active at 50 nM.

<table>
<thead>
<tr>
<th>IbTx Mutant</th>
<th>K_d (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IbTx</td>
<td>1.7 (± 0.5)</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx K27R</td>
<td>6.8 (± 0.9)</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx K27N</td>
<td>32 (± 15)</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx K27Q</td>
<td>1380 (± 159)</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx R34N</td>
<td>600 (± 130)</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx R34D</td>
<td>&gt;&gt;17000</td>
<td>(62)</td>
</tr>
<tr>
<td>IbTx D19K</td>
<td>48.4 (± 9.1)</td>
<td>Current work</td>
</tr>
<tr>
<td>IbTx[D19K]-LC-biotin</td>
<td>26.0 (± N/A)</td>
<td>(61)</td>
</tr>
<tr>
<td>IbTx[D19K]-LC-FAM</td>
<td>161.3 (± 36.5)</td>
<td>Current work</td>
</tr>
<tr>
<td>IbTx[D19C]-Alexa488</td>
<td>active @ 50 nM (K_d = N/A)*</td>
<td>(63)</td>
</tr>
</tbody>
</table>

The experimental values determined by whole-cell patch clamp analysis were then compared to results obtained from the FLIPR (FMP)-assay to explore the replacement...
potential of the new approach. First, both expressing and control cell lines were analyzed using the MTT-cell viability assay in order to understand how the transfection process and expression of a non-native ion channel (BK) affected the growth characteristics of the HEK293/BK cell line. It was determined from the analysis of the data the experimentation must be offset by approximately 18 h, in order to have a level and consistent platform for investigation (Figure 15). The diminished growth rate of the HEK293/BK cell line could potentially be linked to one of two attributes of the experimental conditions. The metabolic load created by the over-expression of the BK channel could be slowing the growth rate of the cells. Alternatively, the presence of an additional antibiotic, G418, in the growth media could be impacting the rate of growth in the HEK293/BK cell line. Most likely, a combination of the two effects are impacting the cell line and causing a difference in the rate and intensity of the HEK293/BK growth.

![Growth Curve](image.png)

**Figure 15.** Analysis of the differential growth rate between the wild type HEK293 and transformed HEL293/BK cell lines.

There were two significant issues which were exposed while working with the FLIPR (FMP) assay, which limits it's applicability under current conditions. The data obtained from the plate-based experiments indicated that there was a minimal change in fluorescence upon addition of the synthetic toxins, even at high concentration. This observation has been reported elsewhere from previous investigations of $K^+$-regulated
mechanisms (64). The lack of signal intensity observed from the FMP-dye hindered the large variations in signal required to produce significant, reliable data. In an attempt to increase base signal intensity, cell density was increased from 10,000 cells/well to 100,000 cells/well in the plating process. The assumption being that an increased number of cells would allow for retention of more FMP-dye, resulting in increased baseline fluorescence. Unfortunately, a significant increase in fluorescence intensity was not detected following this adjustment, indicating a physical limitation with the proprietary FMP-dye. Issues with detection limits have been reported elsewhere and Molecular Devices is currently attempting to reformulate the dye to counteract this limitation (65). The primary problem experienced while employing the FLIPR (FMP)-assay was the occurrence of false positives in the wild type, HEK293 control experiments (Figure 13B and 14B). The false positives effectively negate any data obtained from the principal experiments conducted in the expressing, HEK293/BK cell line (Figure 13A and 14A). Some investigators have experimented with the addition of high concentration K\(^+\), which could potentially counteract the occurrence of false positives (64). Unfortunately the high cost of the FLIPR reagents and value of the synthetic toxins prohibited further attempts to optimize the assay conditions. In all, the observed inefficiencies experienced while employing the FLIPR (FMP)-assay provided additional evidence for the continued use of whole-cell patch clamp analysis in the evaluation and characterization of boiengineered toxins and fluorescent probes.

3.6. CONCLUSION

The synthesis of IbTx[D19K] resulted in a peptide with reduced binding capabilities, however the specificity of the molecule remained consistent, and selectivity for the Ca\(^{2+}\)-activated K\(^+\) channel (BK) was retained. The direct bioconjugation of a fluorophore functionally disrupted the binding interface of the IbTx peptide. Initially, it was believed that this conformation caused steric hindrance, physically blocking the K27 residue from insertion in the channel pore, however it is plausible that the proximity of the fluorophore to the peptide backbone caused pinching of the molecule effectively distorting three-dimensional conformation and inhibiting the native biological activity. In a step-wise fashion, the addition of a six-carbon aminohexanoic acid linker provided clearance of the
fluorophore from the peptide backbone, alleviating the conformational distortion, and restoring the biological activity of the probe. Despite reducing the binding properties of the IbTx[D19K] peptide by a factor of three, whole-cell patch clamp analysis confirmed that IbTx-LC-FAM was a viable probe to investigate in vitro expression models of the BK channel (Chapter 4). Pharmacological characterization further validated the bioengineering strategy of fluorescent probes described in this work. This design template could potentially be translated to the production of a diverse toolbox of molecular calipers facilitating the investigation of a vast set of clinically relevant ion channels.

There were several prominent factors that confirmed the use of whole-cell patch clamp as the primary method for the analysis and pharmacological characterization of synthetic peptides. By comparison, the well established accuracy of the patch clamp technique trumps that of FLIPR (FMP) assay in terms of reproducibility and accuracy, and although the FLIPR (FMP) approach could potentially be modified to generate more reliable, accurate and reproducible data, the prohibitive cost of the supplies and reagents limited attempts to optimize protocols. Molecular Devices, the maker of the FMP-dyes, is currently working to reformulate the assay components in an attempt to increase signal intensity, and sensitivity for monitoring changes in membrane potential for both its potassium K\(^+\)- and Ca\(^{2+}\)-based platforms. Although an in-house validation method would expedite and simplify the synthetic processes, until the reliability and accuracy of a potential replacement can be validated, patch clamp will remain the "gold standard" of pharmacological characterization. Once introduced however, the next generation FLIPR (FMP)-assays should be reevaluated for their potential to replace current patch clamp techniques for the analysis and pharmacological characterization of synthetic peptides over the course of the bioengineering process.
**APPENDIX:**

<table>
<thead>
<tr>
<th></th>
<th>Amplifier</th>
<th>Capacitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differential amplifier</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Earth (usually both electrode)</td>
<td></td>
<td>Voltage source</td>
</tr>
<tr>
<td>Input or output</td>
<td></td>
<td>Switch</td>
</tr>
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Figure A1. Symbols used to represent various functions in schematic diagrams.
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(50) (2013), Molecular Devis LLC, Sunnyvale, CA.


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<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>μM</td>
<td>micro molar</td>
</tr>
<tr>
<td>μL</td>
<td>micro liter</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>5'-FAM (FAM)</td>
<td>5'-Carboxyfluorescein</td>
</tr>
<tr>
<td>AgTx</td>
<td>Agitoxin</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>BK</td>
<td>Calcium (Ca(^{2+}))-activated Potassium (K(^{+})) Channel - (K(_{Ca1.1}))</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CTX</td>
<td>Chlorotoxin</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>Cyanine5.5</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>EMEM</td>
<td>Eagles Minimum Essential Medium</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>Fmoc-Ahx-OH</td>
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<tr>
<td>FSG</td>
<td>Fish Skin Gelatin</td>
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<tr>
<td>G418</td>
<td>Geneticin</td>
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<tr>
<td>g</td>
<td>grams</td>
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<tr>
<td>gBK</td>
<td>Glioblastoma BK channel</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GBM (MG)</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>GluRs</td>
<td>ionotropic glutamate receptor</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>HEK293</td>
<td>Humen Embryonic Kidney Cells</td>
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<td>hours</td>
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<tr>
<td>IbTx</td>
<td>Iberiotoxin</td>
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<tr>
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<tr>
<td>kDa</td>
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<tr>
<td>KCN</td>
<td>Potassium (K(^{+})) channel</td>
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<tr>
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<tr>
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<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NaChs</td>
<td>Sodium Channels</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NaP(_{i})</td>
<td>Inorganic Phosphate</td>
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<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>NCaChs</td>
<td>Calcium Channel</td>
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<tr>
<td>NHS</td>
<td>N-Hydrocysuccinimide</td>
</tr>
<tr>
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<td>nanometer</td>
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<tr>
<td>NP-40</td>
<td>Nondet P-40</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline with Tween-20</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TrisCl</td>
<td>Tris Chloride</td>
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<tr>
<td>U-251 MG</td>
<td>Human Glioblastoma cell line</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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CHAPTER 4

VALIDATION OF THE NOVEL MOLECULAR PROBE, IbTx-LC-FAM, FOR IN VITRO TARGETING OF HIGHLY AGGRESSIVE GLIOBLASTOMA MULTIFORME.

4.1. ABSTRACT

Glioblastoma multiforme (GBM) is a highly aggressive primary brain tumor originating from astrocytic glial cells in human populations. Responsible for nearly 14,000 deaths annually, GBM respond poorly to conventional therapy and our ability to treat these tumors surgically remains ineffective due to extensive migration through otherwise healthy regions of the brain. The lack of robust intraoperative tools capable of identifying and localizing metastatic tissue in vivo further magnifies this therapeutic ineffectiveness. Scorpion toxins have recently shown tremendous potential to serve as molecular scaffolds for the design of diagnostic fluorescent probes capable of targeting characteristic tumor-specific ion channels in vivo. The BK, Calcium (Ca$^{2+}$)-activated Potassium (K$^+$) Channel (K$_{Ca}1.1$), a known cancerogenic biomarker significantly upregulated in GBM, has been identified as a promising target for in vivo molecular targeting. The scorpion peptide, Iberiotoxin (IbTx - Buthus tamulus), a highly selective BK inhibitor has been used as a template to bioengineer the experimental fluorescent probe, IbTx-LC-FAM. The selective targeting of BK in vitro has been validated using both engineered (HEK293/BK) and native (U-251 MG) expression systems. The biologic interaction between IbTx-LC-FAM and BK has been further confirmed by demonstrating the decreased proliferation of native glioblastoma cells following exposure to the experimental fluorescent probe. This data highlights the potential for bioengineered IbTx to be functionalized as a tumor-specific vehicle for the targeted delivery of chemotherapeutic drugs, a novel treatment option for human glioblastoma.
4.2. INTRODUCTION

4.2.1. Primary Brain Tumors: Glioma and BK

4.2.1.1. Introduction to Human Glioma

The human brain broadly consists of two types of cells. Neurons, which are excitable cells capable of transmitting information throughout the body via electrical and chemical signals, and glial cells, which primarily function as structural support but which also play a secondary role in guiding development and neuronal insulation (1). Glial cells are typically considered less important in terms of human physiology however about 30% of all brain tumors, and the most aggressive types, classically manifest from glial cells (2, 3). There are three types of glial cells including Astrocytes which maintain homeostatic concentrations of ions and neurotransmitters in the extracellular environment, Oligodendrocytes which co-assemble as myelin sheets to insulate neurons, and Ependymal cells which form the epithelial lining of cavities in the brain (1). A fourth type, Microglia, exists but are not truly glial cells and function primarily in immune response. From these three glial cell types, a broad class of brain tumors known as Glioma arise, and include ependymoma, oligodendroglioma and astrocytoma (2). This family of primary brain malignancies are characterized by extensive cellular migration which in turn produces widely dispersed infiltration of healthy brain tissue, ultimately rendering surgical resection nearly impossible.

Ependymoma represent only 2% of brain tumors in human populations and are usually the most treatable form of brain cancer (3). In most cases, these tumors do not infiltrate healthy brain tissue, a unique feature which makes possible full resection of ependymoma through surgical intervention, leading to a relatively high success rate in terms of treatment outcomes (2). Oligodendroglioma, unlike ependymomas, spread throughout healthy brain tissue making surgical resection nearly impossible. Although usually restricted to the brain, these tumors can on rare occasions spread to the spinal cord rendering them difficult to treat (2). Finally, astrocytoma account for about 20% of all brain tumors and are usually the most aggressive form (3). These tumors can be classified as low, medium or high grade based on of the speed of their proliferation, and migrate extensively throughout healthy tissue making them difficult to treat surgically.
One of the most severe and deadly forms of brain cancer, and the focus of this study, are high grade astrocytoma, also known as glioblastoma or glioblastoma multiforme (GBM) (2).

Treatment options are limited once glioblastoma is diagnosed, requiring surgery as a primary intervention and often combined with frequent and debilitating rounds ionizing radiation (IR) and chemotherapy (4). Surgical resection physically minimizes the space occupying effect of the tumor, and provides histological samples for clinical classification. Unfortunately, as with most glioma, complete removal of the tumor is near impossible due to extended migration and widespread net-like infiltration of parenchyma tissue resulting in poorly defined tumor margins and leading to practitioner fears of damaging healthy brain tissue, potentially resulting in mental retardation and psychological deficiencies (5-7). Additionally, sublethal IR has a contradictory side effect, stimulating specific ion channels such as BK, in turn leading to enhanced migration of glioblastoma (4). Due to this, the need for new tools to visualize tumor foci and reduce intraoperative margins could increase therapeutic value and benefit patient outcomes, as current post-surgical survival rates hover at a paltry 15 months (8). Ion channels have been proposed as a possible target for these new diagnostic and therapeutic tools, as their tissue specific expression has been noted extensively, see Chapter 2. This approach has been validated preliminarily with the targeting of Chloride channels using a bioengineered version of the scorpion peptide Chlorotoxin (CTX), and the creation of tumor paint, see Figure 1 (9).
4.2.1.2. The General Role of Ion Channels in Glioma

As previously stated, glioma cells are known to anatomically express a plethora of ion channel groups including ionotropic glutamate (GluRs), nucleotide and GABA receptors, glycine receptors, ligand-gated ion channels (neurotransmitter receptors), anion channels, as well as a diverse array of Sodium (NaChs), Calcium (CaChs) and Potassium (KCNs) channels (10). Recent investigations, focused on whole genome analysis of human glioma, have revealed mutations in the genes responsible for the expression of ion channels in nearly 90% of the histological samples encountered (11). Physiologically, ion channels establish and maintain resting membrane potential, and therefore facilitate cellular proliferation by assisting in the progression through cell cycle check points (12). Due to this relationship, the uninhibited activity of ion channels can ultimately result in oncogenesis. One of the most abundant, and therefore well studied of these groups are the potassium channels (KCN) which are known to be involved in pathogenesis and directly correlate to malignancy grade of glioma type primary brain tumors. This group of channels has recently been recognized as an exciting target for molecular therapies aimed at the development of novel treatment options.

As with many other ion channel groups, altered expression of a multitude of KCN families and subtypes has been reported in human glioma including passive potassium channels, inward rectifying, voltage-gated, outward rectifying and Calcium (Ca\(^{2+}\))
activated potassium channels \((1, 10, 12)\). Passive potassium channels, or leak channels, are expressed in astrocytes and are, as their name implies, always open facilitating continuous permeability of \(K^+\) ions, independent of membrane potential or voltage. In glioma cells, a direct link has been established between passive \(K^+\) currents, cell survival and tumor health \((13)\). Inwardly rectifying channels are localized to the plasma membrane in healthy astrocytes, and function to buffer extracellular \(K^+\) and establish a negative resting potential (-80 to -90mV) across the plasma membrane \((12, 14)\).

In glioblastoma however, their expression is altered, localizing channels to the nuclear membrane and functionally resulting in unrestrained tumor growth or proliferation \((1, 15)\). Voltage-gated potassium channels are known to have divergent functions depending on the type of glioma, but are generally thought to play a role in cell growth and the progression through standard cell cycles \((16)\). In certain cases the expression of \(K_V\) has been directly linked to the severity of the tumor, or malignancy grade, and blockage of these same channels can physiologically result in decreased proliferation rates \((17, 18)\). Outward rectifying potassium channels play a well-known role in the repolarization of action potential in excitable cell types, making their expression in human glioma paradoxical. Preliminary evidence exists indicating that outward rectifying potassium channels play a pivotal role in astrocytic proliferation \((10, 19)\). The most well known, and thoroughly studied family of KCN in human glioma are the Calcium (\(Ca^{2+}\)) activated potassium channels, of which BK is a predominant physiological target of study and a popular object of therapeutic intention, as several studies have established that BK is the predominantly expressed KCN isoform \((20-22)\). In glioma cells, \(K_{Ca}\) respond to fluxuations in \(Ca^{2+}\) concentration and contribute to outwardly rectifying potassium currents \((12)\).

### 4.2.1.3. The Role of BK in Glioma

The BK channel is arguably the highest profile potassium channel target in the study of carcinogenesis and glial-derived cancers, largely due to its existential role in tumor cell growth, survival, and migration \((23)\). Unlike most other cancers of the body, the BK channel is significantly over expressed in human glioma and its up-regulation correlates
directly with the malignancy grade of primary brain tumors and therefore clinical patient outcome \((12, 24)\). Specifically, BK channel has been associated with meningioma and glioblastoma including involvement in tumor invasion and ultimately, the formation of brain metastasis \((25, 26)\). BK channels are expressed topically, segregated in the plasma membrane, but additionally, are also found in intracellular organelles including the mitochondria, golgi and the endoplasmic reticulum \((27, 28)\). Two BK channel isoforms have been documented in human glioma, the first being the native BK channel deriving from the \(hSlo\) gene, and the second, gBK, a splice variant with 97% identity found only in glioma cells and differentiated by the addition of a 34 amino acid (AA) insert which is localized to the intracellular region of the native channel sequence near the C-terminal tail and close to the \(Ca^{2+}\) sensor, see Figure 8 \((1, 27, 29)\). Interestingly, the gBK splice variant exists only in the brain of patients with human glioma, and is absent from patients with healthy, normal brains. This anatomical anomaly allows for the classification of gBK as a novel tumor associated biomarker and a prospective target for molecular probes to study the role of \(K_{Ca}\) in tumor physiology and the potential delivery of novel chemotherapeutic treatments.

![Figure 2](image_url)

**Figure 2.** Human glioblastoma cells (U251-MG) differentially stained for both gBK (green) and BK (red) using primary antibodies. A gBK specific antibody was designed which targets the 34 aa insert allowing differentiation between the two isoforms. Adapted from Ge et al. 2012 \((27)\).

In glioma cell physiology, BK channels are active at a characteristic resting potential of about -90 mV and an internal \(Ca^{2+}\) concentrations near 1 mM \((20)\). When active, BK
channels mechanistically translate changes in intracellular Ca\(^{2+}\) to changes in K\(^{+}\) conductance, a process which, in various glial cells, is known to accompany cellular proliferation and alter neuronal migration (21, 30). The intracellular changes in Ca\(^{2+}\) can be attributed to the preferential localization of BK channels near lipid raft microdomains where the expression of inositol 1,4,5-triphosphate receptors (IP3R) also occurs. IP3Rs release intracellular Ca\(^{2+}\), and hence function as the proximal Ca\(^{2+}\) source for BK channel activation (5). Expressed minimally in healthy glial cells, BK channels are known to be up-regulated concurrently with neoplastic transformation, thereby altering native cellular biophysics, and contributing to malignant cell behavior (4, 20).

Cell motility is an integral aspect of malignancy, and migrating glioma cells must undergo localized cell volume changes including shrinking and flattening in order to squeeze through the extracellular spaces in the brain (12). Net salt flux, partially regulated by the efflux of K\(^{+}\) through BK channels, contributes to the regulation of osmotic forces acting upon the cell and ultimately, regulates the volume control that permits invasive migration in the brain. Several studies investigating the administration of BK specific inhibitors including paxilline, tetraethylammonium (TEA), NS-1619 and Iberiotoxin (IbTx) revealed that channel block functionally inhibited cellular migration, reduced velocity and limited metastasis by up to 40\% (12, 20, 30, 31). Interestingly, a contrasting study in 2000 claims that BK activation inhibits migration completely (32). It is evident from the previously discussed physiology, that BK channels play a significant and important role in the biology of glial-based cancers. Clarification of contradictory data could however lead to a deeper understanding of BK channels in cancer biology and facilitate the progression toward advanced treatments that would benefit patient outcomes and improve quality of life.

4.2.2. Scorpion Toxins as Tools to Study Potassium (K\(^{+}\)) Channels

Scorpion toxins are disulfide rich peptides that have been recognized recently as effective tools to target, visualize and study the multifaceted role of potassium channels in human physiology and disease including glioma. Recently, there have been two examples of arachnid toxins, CTX and AgTx, bioengineered as effective channel selective fluorescent probes for the study of tumors in physiological systems. CTX:Cy5.5, which targets Cl\(^{-}\)
channels, has been successfully used for the intraoperative visualization of malignant glioma, medulloblastoma, prostate cancer, intestinal cancer and sarcoma, see Figure 1 (9). This probe functionally improved surgical capabilities and minimized resection margins thereby minimizing the need for adjuvant therapies. Similarly, AgTx was conjugated to a near-infrared fluorescent dye and used in non-invasive in vivo imaging to localize tumors in murine models, see Figure 9 (33). A 2003 study elaborated further on this approach when a $K_{Ca}$ specific agonist, NS-1619, was used to target channels in the endothelium of murine brain tumor vessels, selectively delivering a chemotherapeutic drug in a sustained fashion while completely avoiding toxicity to the surrounding healthy tissue (34). Similarly, a BK specific peptide such as Iberiotoxin (IbTx) could be bioengineered, and used as a probe to study the involvement of BK in glial-based carcinogenesis, as an intraoperative imaging tool to target glioma, and to specifically and selectively deliver novel chemotherapeutics directly to aggressive brain tumors \textit{in vivo}.

![Figure 3. Non-invasive \textit{in vivo} imaging of a tumorigenic murine model injected with the NIR bioconjugated imaging probe AgTs. Adapted from Moore \textit{et al.} 2013 (33).](image)

A thorough review of this topic has been discussed extensively in Chapter 1, "Scorpion Toxins Specific for Potassium (K\(^{+}\)) Channels: A Historical Overview of Peptide Bioengineering". One thing however has lacked from each of the previous studies, which is the validation of \textit{in vitro} expression models and the establishment of target specific binding characteristics, an action that would subsequently eliminate questions and concerns surrounding non-specific binding. Here we aim to use classic molecular biology to establish \textit{in vitro} expression models and to validate the binding of an
experimental fluorescent probe, IbTx-LC-FAM, which specifically targets BK channels *in vitro* and ultimately *in vivo*.

4.2.3. **Engineering Iberiotoxin (IbTx) to Study BK Channel**

A fluorescent probe, IbTx-LC-FAM, based on the molecular scaffold of the scorpion peptide Iberiotoxin (IbTx), has been bioengineered to study the role of the large conductance Ca$^{2+}$-activated K$^+$ channel (BK) in human glioblastoma, and further, to investigate the multifaceted potential of IbTx as an intraoperative diagnostic probe and a novel delivery vehicle for advanced chemotherapeutics. This topic has been discussed in its entirety in Chapter 2.

4.3. **EXPERIMENTAL PROCEDURES**

4.3.1. **SDS-PAGE Gel Electrophoresis and Western Blot Confirmation of BK Protein Expression Levels in HEK293/BK - Validation of the in vitro Model**

The expression of BK channel in a model cell line was assessed by Western blot analysis using the protocol as described by Collier et al., 2002 (35). Native human embryonic kidney cells (HEK293) were stably transfected using a pcDNA3 vector encoding a His6-Flag epitope-tagged human *Slo* gene (gi: 507922) and resistance to the antibiotic Geneticin (G418), described elsewhere by Wallner et al., 1995 (36). Transfected HEK293/BK cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Cellgro, Mediatech Inc., Manassas, VA) supplemented with 10.0% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, Ontario, Canada), 10,000 U/mL penicillin/streptomycin (Cellgro, Mediatech Inc., Manassas, VA), 1% L-glutamine (Lonza Inc., Allendale, NJ) and 0.3 mg/mL Geneticin (G418; Gibco, Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO$_2$. After allowing 2 weeks for selection (at 0.6 mg/mL G418), cells were washed once with ice cold PBS and then lysed with RIPA buffer (50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% Nondet P-40 (NP-40), 0.5% sodium deoxycholate, 1% SDS) supplemented with protease and phosphatase inhibitor cocktail for 30 min. Cell lysate was collected and centrifuged at 12,000g for 5 min at 4°C. The resulting supernatant was quantified using a BCA protein determination assay and
standardized at a concentration of 1 mg/mL. Standardized lysates (30 μg) were resolved on a 7.5% SDS-polyacrylamide electrophoresis gel under reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes with semidry transfer (Bio-Rad Laboratories, Hercules, CA), and blocked overnight at 4°C in blocking buffer (5% nonfat dried milk, 2% bovine serum albumin, 2% normal goat serum in TBS plus 0.1% Tween 20). Membranes were subsequently washed (3x, 10 min in TBST) and incubated with primary antibody (rabbit anti-BK, 1:200 in blocking buffer) for 2 hrs at room temperature. The affinity-purified rabbit polyclonal primary antibody, purchased from Alomone Labs (Jerusalem, Israel; APC-151), was raised against an extracellular peptide domain (epitope 199-213) of rat origin. Following incubation, membranes were again washed (3x, 10 min in TBST) and incubated with a solution containing secondary antibody (horseradish peroxidase conjugated donkey anti-rabbit, 1:2000, in blocking buffer) for 1 h at room temperature. Membranes were again washed (3x, 10 min in TBST) prior to detection with enhanced chemiluminescence horseradish peroxidase (HRP) substrate (Western Lightening Plus ECL; PerkinElmer Inc., Walthem, MA) for 2 min in the dark. Bands were visualized by exposing membranes to film (2 min) and developed using a Konica Medical Film Processor (SRX-101A). Confirmation of even protein loading was established by staining SDS-polyacrylamide gels with Coomassie Blue. Band intensities were calculated using ImageJ software (National Institutes of Health, Bethesda, MD). Relative expression levels of BK channel in postive controls, experimental samples and negative controls were also quantified via ImageJ.

4.3.2. Immunocytochemistry: HEK293/BK - Visual Confirmation of the in vitro Model

HEK293/BK and fluorescent tagged HEK293/BK-YFP cells were grown as previously describe in Section 4.3.1 and seeded on glass coverslips (22x22 mm, 1 oz; Fisher Scientific, Walthem, MA) pretreated with acetone and Poly-l-lysine (1 mg/mL; Sigma-Aldrich, St. Louis, MO) for 24-48 h in 6-well dishes prior to use. Cells grown on coverslips were washed once with warm (37°C) phosphate buffered saline (PBS; 125 mM NaCl, 25 mM NaPi, pH 7.4) and fixed/permeabilized in ice-cold MeOH (-10°C) for 10 min. Cells were washed thoroughly in neat PBS and blocked with 0.5% fish skin
gelatin (FSG) in PBS for 1 h at room temperature. An affinity purified rabbit-polyclonal primary antibody (Anti-Maxi Potassium channel alpha antibody; ab3586) purchased from Abcam, was raised against a synthetic peptide corresponding to amino acid residue 945-961 of the human BK alpha subunit. The antibody was biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) following the manufacturer's protocol and prepared (1:500) in blocking solution (1:10 in PBS). Coverslips were incubated at room temperature for 2 h and washed (3x, PBS). Following the wash, cells were incubated with either the secondary detection reagent, Alexa Fluor 488 Streptavidin (2.0 μg/mL in PBS), or Alexa Fluor 568 Goat Anti-Rabbit IgG (co-localization experiment) for 2 h at room temperature. Cells were subsequently washed (1x) in room temperature PBS containing Hoescht 33342 (Invitrogen, Life Technologies, Grand Island, NY), washed in neat PBS (2x), and finally dipped in DH2O prior to mounting on glass slides using Fluoromount-G (Southern Biotechnology, Birmingham, AL). Slides were allowed to set up overnight before sealing with clear nitrocellulose lacquer (nail polish). Labeled cells were observed using a Zeiss Axioskop 2 plus microscope and digital images were captured using a Zeiss Axiocam MRe (R3.0) and Axiovision software (Release 4.7.2). HEK293/BK cells were treated equally (minus primary antibody) as a negative control of non-specific binding.

4.3.3. **IbTx-LC-FAM: Probe Validation in HEK293/BK**

HEK293/BK cells grown on coverslips as previously described, Section 4.3.2, were washed once with warm (37°C) phosphate buffered saline (PBS; 125 mM NaCl, 25 mM NaPi, pH 7.4) and incubated with 60 μL of IbTx-LC-FAM (200 nM) in PBS (1 mg/mL BSA) for 30 min at 4°C. Cells were then placed directly into ice-cold paraformaldehyde (2% PFA in PBS) for 20 min at 4°C to allow for fixation. Cells were washed and prepared for imaging as described previously in Section 4.3.2. Native HEK293 wild type cells (HEK293/WT) were treated concurrently as a negative control.
4.3.4. SDS-PAGE Gel Electrophoresis and Western Blot Confirmation of BK Protein Expression Levels in a Human Glioblastoma (U-251 MG) - Validation of the Native Expression Model

The native human glioblastoma cell line U-251 MG, was maintained in Eagles Minimum Essential Medium (EMEM; Cellgro, Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Etobicoke, Ontario, Canada) and 1% L-glutamine (Lonza Inc., Allendale, NJ) at 37°C in a humidified atmosphere of 5% CO2. Cells were treated and prepared for western blot analysis as described previously in Section 4.3.1.

4.3.5. IbTx-LC-FAM: Fluorescent Visualization of BK Channels in Native Human Glioblastoma (U-251 MG)

The native human glioblastoma cell line, U-251 MG, was maintained as described previously in Section 4.3.4. IbTx-LC-FAM was used according to the previously described protocol, Section 4.3.3, to validate the probes effectiveness in a native expression system. The U-251 MG cells were incubated for 20 min with 60 μL of non-labeled Iberiotoxin[D19K] (1.0 μM in PBS with 1 mg/mL BSA) prior to standard incubation with IbTx-LC-FAM to eliminate questions regarding non-specific binding (negative control).

4.3.6. Plate Based IbTx-LC-FAM Dosage Experiment: U-251 MG.

The native human glioblastoma cell line, U-251 MG, was maintained as described previously in Section 4.3.4. Cells were plated in culture treated, flat-bottom, 96-well plates (Greiner, CELLSTAR; Sigma Aldrich) at a density of 1x10⁴ cells/well and left to attach overnight in a humidified incubator (37°C, 5 % CO2). IbTx-LC-FAM (500 nM) was diluted in serum free media (EMEM, 1% L-glutamine) and sterile filtered. Following the initial 24 h attachment period, regular media was replaced with serum free media spiked with IbTx-LC-FAM (n=3) and replaced fresh every 24 h for 5 consecutive days as described previously (21). A control set (n=3) was treated with serum free media (as before) containing no IbTx-LC-FAM. On the fifth day, cells density was measured by MTT-cell viability assay. Media was first aspirated and cells were washed with warm, sterile PBS (200 μL). Fresh, sterile complete media (100 μL) containing MTT reagent
(20 μL) to a final concentration of 5 mg/mL was added to each well and the plates were returned to the incubator. Following 4 h incubation, the MTT-media was aspirated and neat DMSO (200 μL) was added to each well overnight. The following day, optical density (OD) was measured at 595 nm in a Spectramax Gemini plate reader (Molecular Devices) running SoftMax Pro microplate data acquisition and analysis software (Molecular Devices).

4.4. RESULTS

4.4.1. SDS-PAGE Gel Electrophoresis and Western Blot Confirmation of BK Protein Expression Levels in HEK293/BK - Validation of the in vitro Model

The qualitative analysis of protein expression was undertaken to confirm the presence of BK channel in the stable model cell line HEK293/BK. A significant difference was detected between the overall expression of BK in the transfected cell line, HEK293/BK (positive control), and the non-expressing wild type cell line, HEK293/WT (negative control). The HEK293/BK cell line expresses the target channel at high levels while the wild-type, HEK293/WT cell line lacks the target protein. This evidence confirms the uptake of the pcDNA3 vector encoding a His6-Flag-epitope-tagged human Slo gene (gi: 507922) by the HEK/293 progenitor cells.
4.4.2. Immunocytochemistry: HEK293/BK - Visual Confirmation of the in vitro Model

A visual depiction of BK channel expression in an HEK293 cell line was produced using a stable transfected HEK293/BK-YFP cell line expressing an isoform of the BK channel conjugated to a Yellow Fluorescent Protein moiety (YFP: ex. 514 nm, em. 527 nm). Visually, fluorescent tagged channels appear in concentric areas of patched and punctate patterns radiating from the nucleus which was counter-stained blue using Hoescht 33342 (Figure 5). A heterogeneous mixture of expressing and non-expressing cells was observed despite the selective pressure applied using the antibiotic G418. This effect may be related to vector uptake and the retention of gene copy number by individual cells in the original culture. The non expressing, wild type cell line HEK293/WT was void of signal in the 527 nm range, thus establishing effective conditions for microscopy.
Figure 5. Fluorescent Imaging of HEK293/BK-YFP. A symbolic model of BK expression in HEK293. Hoescht 33342 (Blue) was used as a nuclear stain, and yellow fluorescent protein (yellow) is indicative of the alpha subunit of the BK channel. Images were taken using a 20x objective. (A) 527 nm channel: 550ms, Hoescht 33342: 11ms. (B) 527 nm channel: 550ms, Hoescht 33342: 11ms.

The pink coloration (signal) observed in Figure 6-A, signifies colocalization of ab3586 and the BK-YFP channel, thus confirming the target specificity of the primary antibody for the protein of interest. This feature further substantiates preliminary epitope binding data (Figure 7), and effectively confirms the expression of the BK channel in the HEK293/BK cell line. A negative control sample, also using the HEK293/BK-YFP cell line was treated concurrently, less the addition of ab3586 (Figure 6-B). This treatment method visually lacked colocalized signal (i.e. pink coloration), effectively verifying the reactivity of the secondary antibody (Alexa Fluor 568 Goat Anti-Rabbit IgG) for the ab3586 epitope, eliminating the possibility of non-specific binding or cross reactivity and corroborating the data observed in Figure 7. Taken together, the authenticated colocalization formally validates the expression of the BK channel in the HEK293/BK cell line and confirms the cell model as a viable *in vitro* expression system for investigating the experimental fluorescent probe, IbTx-LC-FAM.
Immunocytochemical investigation of BK channel expression in HEK293/BK showed strong correlation with the western blot data seen in Figure 4, and visually confirmed the presence of the target protein in the HEK293/BK model cell line (Figure 7). Patched and punctate expression patterns align closely with those observed in the HEK293/BK-YFP example cell line, leading to preliminary evidence for the legitimacy of the HEK293/BK cell line as a valid in vitro model to investigate the binding characteristics of the experimental fluorescent probe, IbTx-LC-FAM. A negative control using the HEK293/WT cell line was treated concurrently and lacked any evidence of observable antibody binding, thus eliminating questions regarding non-specific binding and cross reactivity of the ab3586 primary antibody in the HEK293 test system.
4.4.3. *IbTx-LC-FAM: Probe Validation in HEK293*

The validated BK channel *in vitro* expression system, HEK293/BK, was incubated with the experimental fluorescent probe, IbTx-LC-FAM, according to the protocol described previously, Section 1824.3.3. Fluorescent microscopy revealed a prominent green signal at 517 nm, corresponding the emission spectra of the bioconjugated fluorophore, 5′-Carboxyfluorescein (5′-FAM: exc. 492 nm, em. 517 nm) (Figure 8-A). This data is indicative of functional binding of the IbTx-LC-FAM probe to BK channels expressed in the model system. Negative control HEK293/WT cells (Figure 8-B) were treated identically, displaying no observable signal at 517 nm, and thus confirming the highly selective nature of the experimental fluorescent probe, IbTx-LC-FAM, for the BK channel.
Figure 8. Fluorescent visualization of BK channels in HEK293 using the experimental fluorescent probe IbTx-LC-FAM (yellow). Hoescht 33342 (blue) was used as a nuclear stain for contrast. Images were taken using a 20x objective. (A) 488nm channel: 1298ms, Hoescht 33342: 5ms. (B) 488nm channel: 1327ms, Hoescht 33342: 3ms.

4.4.4. SDS-PAGE Gel Electrophoresis and Western Blot Confirmation of BK Protein Expression Levels in Human Glioblastoma (U-251 MG) - Validation of the Native Expression Model

The expression of BK channels in primary human glioblastoma cells (U-251 MG), was confirmed by qualitative western blot analysis (Figure 9). The relative expression was not as pronounced as the positive control (isolated human heart mitochondrial lysate), but was significantly higher than the negative control (human red blood cell lysate). Measurable expression levels of BK channel in the human glioblastoma cell line (U-251 MG) authenticate its use as a platform to validate the biological activity and binding of IbTx-LC-FAM in a native (non-engineered) expression model.
4.4.5. *IbTx-LC-FAM: Fluorescent Visualization of BK Channel in Native Human Glioblastoma (U-251 MG)*

The validated human glioblastoma *in vitro* expression system, U-251 MG, was incubated with the experimental fluorescent probe, *IbTx-LC-FAM*, according to the previously described protocol, Section 1824.3.3. Fluorescence microscopy revealed a strong green signal at 517 nm, corresponding the emission spectra of the bioconjugated fluorophore, 5'°-Carboxyfluorescein (5°-FAM: exc. 492 nm, em. 517 nm) (Figure 16-A). This data is indicative of functional binding between the *IbTx-LC-FAM* probe and BK channels expressed in the native brain tumor model. A negative control experiment utilizing the U-251 MG cell line was conducted simultaneously (Figure 10-B). The cells were first treated with native Iberiotoxin (*IbTx*) prior to incubation with the fluorescent probe, *IbTx-LC-FAM*. Pre-treatment with the non-labeled *IbTx* eliminated the fluorescent signal at 517 nm, thus confirming the highly selective nature of the experimental fluorescent probe, *IbTx-LC-FAM*. 

Figure 9. Qualitative confirmation of BK expression in U-251 MG. The relative density of BK channel protein in a native human glioblastoma model as determined via western blot analysis (**P = 0.014) (n=3). An alpha level of .05 was used for analysis.
4.4.6. Plate Based IbTx-LC-FAM Dosage Experiment: U-251 MG.

The density, or number of U-251 MG cells was measured by MTT-assay following a 5-day incubation with an elevated concentration of IbTx-LC-FAM (Figure 11). The experiment was replicated three times for a total of 9-wells/concentration. Data was analyzed (GraphPad Prism, v4) and a significant difference was observed between the experimental (IbTx-LC-FAM) and control wells by a two-tailed, unpaired t test (P = 0.0139).
4.5. DISCUSSION

For the first time, experiments have been designed and conducted which provide evidence for highly specific binding of an experimental fluorescent probe, based on the molecular framework of a scorpion toxin specific for the BK channel, in an extensively validated in vitro expression system. These data have significant implications in advances in molecular medicine including the extension of our ability to visualize primary human brain tumors in vivo, thereby increasing the intraoperative potential for successful resection likely extending survival outcomes and quality of life. Additional therapeutic potential further extends the applicability of the IbTx-LC-FAM from a diagnostic tool, to a chemotherapeutic delivery system by exploring the conjugation of the peptide to a carefully selected library of small molecule chemotherapeutics compatible with amide conjugation chemistry.

While HEK293 has been used extensively in electrophysiological experiments (i.e. patch-clamp analysis) involving potassium channels (37), the cell line has yet to be validated as an in vitro expression system used to verify the selective binding of a diagnostic fluorescent probe, such as IbTx-LC-FAM. Data from western blot analysis of BK expression levels (Figure 4) clearly demonstrate a 10-fold increase in the surface-
expression of the BK channel in the HEK293/BK cell line when compared to the wild type HEK293 cell line ($P = 0.0014$). Incidental background expression of BK channel was observed, however this data did not correlate to the immunocytochemistry investigation of BK surface expression leading to speculation of low-level intracellular BK expression in HEK293. Recent investigations have revealed increasing levels of evidence for mitochondrial expression of the BK channel in various human cell types including cardiac myocytes (38) and more interestingly, kidney epithelial cells (39). Circumstantial evidence is indicative of BK expression in the mitochondria of wild type HEK293 cells, an observation that should be subsequently investigated in follow-up studies as this phenomenon has yet to be documented.

Green and Yellow fluorescent labeled potassium channels such as BK and hERG have been used successfully as FRET reporters to investigate gating the characteristics of ion channels (40), and as in vitro fluorescent biomarkers to visualize channel architecture (41). Unfortunately however, they are inherently ineffective as in vivo bioimaging agents due to the need for the transfection of fusion constructs and the sheer size of the fluorescent fusion protein, YFP (~26.9 kDa), which substantially affects trafficking. This same construct, HEK293/BK-YFP, can be used however to provide a template of "native" topical expression patterns of BK in HEK293/BK (Figure 5).

Immunocytochemistry (ICC) experiments in the HEK293/BK-YFP cell line revealed colocalization of a primary polyclonal anti-BK antibody, ab3586 (Figure 6), and the fluorescent tagged BK-YFP channel. The colocalized signal confirms the specificity of the antibody for BK channels in the engineered cell model. The anti-BK antibody can then be applied to the non-fluorescent HEK293/BK expression model to confirm surface expression of BK channel in the stably transfected HEK293 cell line (Figure 7). The HEK293/BK cell line, validated using both western blot analysis, and immunocytochemical visualization can be used with to address the binding specificity of IbTx-LC-FAM for the BK channel in an engineered in vitro expression model.

The BK channel is a well known biomarker that is significantly upregulated in native human glioblastoma and whose expression level has been correlated directly to the severity of tumor grade (23). The primary human glioblastoma cell line, U-251 MG, has
been used extensively as an *in vitro* model for human brain tumor (42).

Immunocytochemical studies of ion channel expression in the U-251 MG cell line have confirmed the upregulated expression of native BK channel and have further revealed the expression of a novel BK splice variant, known as glioblastoma BK or gBK (Figure 2) (27). Similar to the approach used to validate the engineered expression system, western blot analysis of BK channel expression in U-251 MG (Figure 9) confirmed a significant increase (P = 0.0014) in channel expression when compared to the negative control. Although expression levels are significantly higher in the over-expression model HEK293/BK compared to the native expression model, U-251 MG (P = 0.0017), the difference merely represents a 4-fold increase, a disparity not unexpected of an engineered expression system compared to that of a native expression model. The limited fluorescent signal observed when staining the U-251 MG cell line with IbTx-LC-FAM, correlates directly with the difference in BK expression levels. Controlled experiments using a non-labeled version of the synthetic probe, IbTx[D19K], confirmed the highly specific binding of the diagnostic probe, IbTx-LC-FAM, in the native human glioblastoma system, U-251 MG. These experiments successfully validated both an engineered and a native expression model of BK expression and further confirmed the utility of IbTx-LC-FAM, which can now be used to investigate BK channels in *in vitro* expression systems representing human glioblastoma.

Following the validation process, the effects of the IbTx-LC-FAM probe on the growth characteristics of the U-251 MG cell line were explored based on experiments described previously (21, 24). Dosing U-251 MG cells with high concentration of IbTx-LC-FAM resulted in a significant decrease in cellular proliferation (P=0.0139). This data further confirmed the highly specific biologic interaction between the bioengineered toxin and the BK channel expressed in the U-251 MG glioblastoma cell line. Importantly, this interaction demonstrates the potential for replacing the fluorophore with a host of different chemotherapeutic molecules to evaluate the ability of IbTx[D19K] to function as a tumor-specific vehicle for the targeted delivery of chemotherapeutics. This technology has merit for tumor reduction or potential elimination chemotherapies.
Table 1. Outline of experimental program designed to formulate a bioactive peptide toxin carrier system for selective delivery of experimental chemotherapeutics to tumoral masses *in vivo*. *NIR stands for Near-Infrared Fluorophore.*

<table>
<thead>
<tr>
<th>Phase</th>
<th>Step</th>
<th>Base Toxin</th>
<th>Conjugate</th>
<th>Test Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>IbTx[D19K]</td>
<td>FAM</td>
<td><em>In vivo</em> - Mouse</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>IbTx[D19K]</td>
<td>NIR fluorophore</td>
<td>Whole-cell patch</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>IbTx[D19K]</td>
<td>NIR fluorophore</td>
<td><em>In vitro</em> - U-251 MG</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>IbTx[D19K]</td>
<td>NIR fluorophore</td>
<td><em>In vivo</em> - Mouse</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>IbTx[D19K]</td>
<td>Chemotherapeutic 1</td>
<td>Whole-cell patch</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>IbTx[D19K]</td>
<td>Chemotherapeutic 1</td>
<td><em>In vitro</em> - U-251 MG</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>IbTx[D19K]</td>
<td>Chemotherapeutic 1</td>
<td><em>In vivo</em> - Mouse</td>
</tr>
<tr>
<td>IV</td>
<td>2-4</td>
<td>IbTx[D19K]</td>
<td>Chemotherapeutic Library</td>
<td>All Steps (2-4)</td>
</tr>
</tbody>
</table>

Once confirmation of the IbTx-LC-FAM binding properties have been successfully translated from *in vitro* to *in vivo*, a library of chemotherapeutics can be identified and conjugated in an attempt to formulate fully active biotherapeutic compounds capable of attacking tumors *in vivo*. Chemotherapeutics with a free carboxylic acid function could potentially be covalently ligated to the toxin, similar in fashion to the carboxyfluorescein fluorophore. *In vitro, in vivo* and electrophysiological studies would be necessary to ensure the biologic activity of both the toxin and chemotherapeutic are retained following chemical conjugation and to further evaluate efficacy. A program should be initiated to investigate further this developmental area of molecular medicine as there is the potential here to revolutionize the current approaches to chemotherapy and radiation treatment. A grant program has been described briefly in Table 1.

### 4.6. CONCLUSION

We have confirmed the specific binding of IbTx-LC-FAM in both engineered and native expression systems. Future, *in vivo* biophotonic studies should be undertaken to assess the potential for IbTx-LC-FAM to be used in glioblastoma mouse models to identify, visualize and localize tumoral masses *in vivo*. Under these conditions weak signal intensity may be observed due to the physical characteristics (limitations) of the carboxyfluorescein fluorophore, which could potentially be overcome by switching to a near infrared fluorophore such as Cy5.5 NHS Ester (Lumiprobe: cat# 57020). Upon
confirmation of \textit{in vivo} target specificity, advanced studies investigating the selective delivery of covalently linked chemotherapeutics could subsequently confirm IbTx[D19K] as a novel, highly targeted therapeutic delivery platform in addition to its utility as an \textit{in vivo} diagnostic tool.

Additional peptide prospecting studies utilizing domestic venom resources could potentially yield a large biocache of peptide toxins fundamentally active toward a myriad of ion channels and receptor proteins. These novel toxins could hypothetically be carefully selected for targets of interest and bioengineered using the design and validation templates laid out in the previous chapters. This technology may well lead to a vast toolbox of highly selective nano-tools that could revolutionize therapeutic treatment options thus advancing the concept of targeted molecular medicine. Peptide prospecting will be discussed at length in Chapter 5.
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<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>API</td>
<td>atmospheric pressure ionization</td>
</tr>
<tr>
<td>DHB</td>
<td>dihydroxybenzoic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>ESMS</td>
<td>electro spray mass spectrometry</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fmoc, 9-Fluorenylmethylxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-Asn(Trt)-OH</td>
<td>N-alpha-9-Fluorenlymethoxycarbonyl-N-beta-Trityl-L-Asparagine</td>
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<td>9-Fluorenlymethoxycarbonyl-L-Aspartic Acid-beta-t-Butyl Ester</td>
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<td>9-Fluorenlymethoxycarbonyl-S-Trityl-L-Cysteine</td>
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<td>Fmoc-His(Trt)-OH</td>
<td>N-alpha-9-Fluorenlymethoxycarbonyl-Nim-Trityl-L-Histidine</td>
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<td>9-Fluorenlymethoxycarbonyl-O-t-Butyl-L-Hydroxyproline</td>
</tr>
<tr>
<td>GBR</td>
<td>Great Barrier Reef</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-gamma-carboxyglutamic acid</td>
</tr>
<tr>
<td>HCTU</td>
<td>1H-Benzotriazolium-1-[bis(Dimethylamino)Methylene]-5-Chloro Hexafluorophosphate-(1-),3-Oxide</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational new drug</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>matrix assisted desorption/ionization mass spectrometry</td>
</tr>
<tr>
<td>MALDI-TOF-(TOF)-MS</td>
<td>MALDI-Time of light-(Time of flight)-Mass spectrometry</td>
</tr>
<tr>
<td>M$_r$</td>
<td>average molecular mass</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MV</td>
<td>milked venom</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<tr>
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<td>polymerase chain reaction</td>
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<tr>
<td>PSD</td>
<td>post-source decay</td>
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<tr>
<td>PTM</td>
<td>post-translational modification</td>
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<tr>
<td>RE</td>
<td>radula extract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RV</td>
<td>radula venom</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase - high performance liquid chromatography</td>
</tr>
<tr>
<td>Rt</td>
<td>retention time</td>
</tr>
<tr>
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<td>scanning electron micrograph</td>
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<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
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<td>trifluoroacetic acid</td>
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<td>UV</td>
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CHAPTER 5

A BRIEF PERSPECTIVE ON PEPTIDE PROSPECTING - A 'CONOVENOMIC' APPROACH TO THE IDENTIFICATION OF NOVEL TOXINS FROM THE HAWAIIAN CONE SNAIL, *CONUS TEXTILE*.


5.1. ABSTRACT

Cone snail venoms provide a largely untapped source of novel peptide toxins. To advance approaches in peptide prospecting, a detailed comparative proteomic analysis was undertaken on the milked venom of the domestic Hawaiian cone snail species, *Conus textile*. Both chromatographic and mass spectral analysis were utilized, and the dissection of the venom revealed extreme levels of intraspecies variation in the venom profiles. This effect is thought to be connected to the tailoring of venom for localized prey species. Analysis of this diversity revealed a geographically conserved, previously uncharacterized peptide from *C. textile*, Tx2081. Solid phase peptide synthesis was undertaken to produce, biosustainably, amounts of the Tx2081 peptide sufficient for pharmacological characterization. Voltage-clamp analysis and bioassay in prey species of the synthetic peptide revealed selectivity for the $\alpha 3\beta 2$ isoform of the nicotinic acetylcholine receptor with maximal inhibition of 96% and an observed IC$_{50}$ of 5.4 ± 0.5 $\mu$M, and high paralytic potency in invertebrates, PD$_{50}$ = 34.2 nMol Kg$^{-1}$. A fluorescent probe bioengineered using the Tx2081 template could potentially allow for the study of mechanisms responsible for the production and propagation of pain signals in mammals, and help to produce more effective therapeutics to replace current narcotic based treatments for chronic neuropathic pain.
5.2. INTRODUCTION

5.2.1. General Background

In the proceeding chapters we have discussed advanced methods for bioengineering both diagnostic and therapeutic peptide toxins using template molecules, natural peptide toxins isolated from the venom of various scorpion species. This model is not limited to peptides found in scorpion venom however, and several other animal species are known to produce venom containing peptide toxins amenable to the outlined bioengineering model. Assembling a molecular toolbox comprised of various diagnostic and therapeutic peptides, requires the identification and characterization of novel toxins from these venoms which are capable of targeting clinically relevant ion channels such as the voltage-gated K$^+$ channel $K_V$1.3, which has been associated with HIV/AIDS and Multiple Sclerosis (MS), and the nicotinic acetylcholine receptor (nAChR) which has been associated with chronic neuropathic pain, to name a few (1). Hawaii’s unique tropical environment conceals a vast, domestic source of venomous species that produce peptide toxins capable of targeting a wide array of receptor proteins. Here we will review an innovative, biosustainable approach to peptide prospecting from the genus Conus which facilitates the isolation and characterization of novel peptide toxins, with potential value as advanced diagnostic or therapeutic templates.

5.2.2. The Genus Conus: Hawaii’s Killer Cone Snails

The genus Conus (Family: Conidae, Subfamily: Coninae) has maintained a position of predatory superiority within tropical marine ecosystems for some 50 million years. The genus attributes its evolutionary success to the development and delivery of a venomous cocktail of peptides, proteins, amino acids and small organic molecules. The cone snail injects this venom through a radula harpoon that is similar to a hypodermic needle, which is capable of penetrating deep into the dermis of its target prey species (2). These venoms comprise a potent pharmacopeia of individual bioactive peptide constituents, commonly referred to as conotoxins or conopeptides. New estimates surpassing half-a-million discrete, biologically active molecules have been suggested for this genus (3-6), making it an invaluable domestic resource of medically relevant template molecules.
Stemming from their intended predatory use as potent neurotoxic agents for prey immobilization (7-9), the chemical complexity and functional side-chain variability of conopeptides has been studied extensively, providing tools for probing ion channel function and structure-activity relationships. The majority of research however has focused on the clinical significance and therapeutic potential of conopeptides, evident by the number of conopeptide derived molecules in pre-clinical development or clinical phase trials for the treatment of a broad spectrum of conditions ranging from neuropathic pain and Alzheimer’s, to Parkinson’s and Epilepsy (10-15).

Early investigations fuelled the rapid identification of a number of novel bioactive peptides including αA-conotoxin OIVA from Conus obscurus [50] and ω-conotoxin SIIIA from Conus striatus (16). Most notably, in 2004, the United States Food and Drug Administration (FDA) approved Prialt™ (ω-Conotoxin MVIIA), a peptide naturally expressed in the venom of Conus magus, for the treatment of chronic neuropathic pain (17, 18). Unfortunately, due to this heightened medical interest, in conjunction with intensive exploitation by the ornamental shell-trade industry, the over-harvesting of Conus has the potential to result in the depletion of population densities (19, 20). Consequently, a rapid and biosustainable approach to identify novel, clinically significant conopeptides, while simultaneously reducing pressure on native snail populations, must be employed.

5.2.3. Conus textile

The current study was initiated to demonstrate the identification of novel, clinically relevant peptide from a domestic Hawaiian species that can be reintroduce into the previously described bioengineering strategy discussed at length in Chapters 2-4. This can be achieved by performing a comprehensive investigation into the venomic multiplicity of Conus textile using Reverse-Phase High Performance Liquid Chromatographic (RP-HPLC) profiling and mass spectral analysis of C. textile venom. This widely distributed tropical species at present, represents one of the most well-studied cone snail venoms, with countless documented molecular constituents including 77 fully characterized conopeptides. By harnessing advances in proteomic technology,
the conovenomic approach to peptide prospecting, facilitates the identification of
previously uncharacterized peptides despite the comprehensive knowledge of *C. textile*
venom, successfully demonstrating the power and utility of this technique.

Analysis of milked venom samples collected from a large geographic area, revealed an
unprecedented level of venomic diversity from the *C. textile* species. A conovenomic
comparison led to the identification, isolation and pharmacological characterization of a
previously unknown clinically significant peptide, Tx2081, which selectively targets the
nicotinic acetylcholine receptor (nAChR), a well documented therapeutic target for the
treatment of chronic neuropathic pain (21-25).

**5.3. EXPERIMENTAL PROCEDURES**

**5.3.1. Geographic Biodiversity - RP-HPLC Profiling**

**5.3.1.1. Snail Milking**

Milked Venom (MV) samples from non-captive specimens of *C. textile* were obtained
within 24 hours of field collection. Envenomation was stimulated by the presence of live
gastropods, *Cypraea caputserpentis*. On extension of the cone snail's proboscis, the
plunger of a 5000 μL pipette was depressed and the tip placed near the upturned aperture,
close to the foot of the prey. On subsequent firing of the radula, envenomation was
observed by the release of excess venom, typically as a visible 'cloud', which was
carefully aspirated to avoid dispersion. The collected MV was acidified (1% v/v TFA)
and either frozen (-20°C) for further analysis. Aliquots of seawater (blanks) were
collected and processed in an identical manner.

**5.3.1.2. Chromatographic Separation and Analysis, RP-HPLC**

MV samples were prepared and analyzed using one of three conditions, described as follows:

- **System 1:** Capillary scale chromatography (Phenomenex; C<sub>18</sub>, 5 μm, 300 Å, 1.0 x
  250 mm, flow 100 μL min<sup>−1</sup>) used for comparative RP-HPLC/UV profiling,
quality control of peptide purity, peptide quantification and peptide co-elution experiments.

- **System 2:** Analytical scale chromatography (Vydac; C$_{18}$, 5 μm, 300 Å, 4.2 x 250 mm, flow 1 mL min$^{-1}$) used for the isolation and purification of native peptides for MALDI-TOF-(TOF)-MS.

- **System 3:** Preparative scale chromatography (Vydac; C$_{18}$, 10 μm, 300 Å, 22 x 250 mm, flow 5 mL min$^{-1}$) used for desalting and scaled separation of native MV peptides for sequencing and pharmacological assay.

Systems 1 and 2 used a Waters 2695 Alliance RP-HPLC System interfaced with a 996 Waters Photo Diode Array Detector for automated sample analysis and detection. Data was acquired and analyzed using Waters Millennium$^{32}$ (v3.2) software. Samples were eluted using a linear 1% min$^{-1}$ gradient of organic Solvent B, against aqueous Solvent A (Table 1) for 65 min, followed by a terminating high organic wash (80% Solvent B for 5 min), and pre-equilibration step (5% Solvent B) for 10 min prior to subsequent injections. Eluent was monitored from 200–300 nm and extracted at 214 nm in order to monitor the peptide backbone (amide bond). The preparative RP-HPLC/UV system (System 3), used a 625 Waters HPLC pump and controller interfaced with a 996 Waters Photo Diode Array Detector. Both gradient control and data acquisition were controlled by Waters Millennium$^{32}$ software. Filtered (Nylon 0.22 μm) crude MV venom extracts were manually loaded and eluted from the preparative scale column using the same 1% gradient at 5 mL min$^{-1}$ and monitored at 214 and 280 nm. Fractions were collected manually, lyophilized and stored at –20°C for future analysis.

Table 1. RP-HPLC buffers, aqueous solvent A and organic solvent B.

<table>
<thead>
<tr>
<th>RP-HPLC BUFFERS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLVENT A</td>
<td>0.1% (v/v) TFA/Aqueous</td>
</tr>
<tr>
<td>SOLVENT B</td>
<td>90/10 (v/v) CH$_3$CN/Aq. (0.08% v/v TFA)</td>
</tr>
</tbody>
</table>
5.3.2. Geographic Biodiversity - Molecular Mass Profiling

5.3.2.1. Direct Electropray Infusion-Mass Spectrometry (ESI-MS)
AB/MDS-Sciex API 3000 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) was used in this investigation under conditions described previously by Chun et al. 2012 (26). The ESI-MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve <5-ppm mass accuracy, as per manufacturer’s protocol.

5.3.2.2. MALDI-TOF MS Venom Analysis
ZipTip™ or RP-HPLC/UV purified venom fractions in Solvent A (Table 1) were mixed 1:1 with matrix solution (40 g L⁻¹ 2,5-dihydroxybenzoic acid (DHB) in 1:1 0.1% v/v aq. TFA: CH₃CN). Small volumes (1 μL) was spotted onto a MTP 384 polished steel target plate (Bruker Daltonics) and dried under a stream of N₂ gas. Mass spectra were acquired on a Ultraflex III (Bruker Daltonics), controlled by the Compass 1.2 SR1 software package (Bruker Daltonics), in positive reflector mode from m/z 500 to 5000. Mass spectra were summed (400 to 1200 laser shots) until no further improvement in the signal to noise ratio of peaks was possible. Peptide II Calibration Mix (Bruker Daltonics) was used for external calibration, with a 5-ppm mass accuracy. Analysis of the spectra was completed using FlexAnalysis v3.0 (Bruker Daltonics).

5.3.3. Peptide Sequencing

5.3.3.1. Collision Induced Dissociation (CID)
The reduced venom peptide, Tx2081, was desalted via RP-HPLC/UV (System 3), isolated and spotted onto the target plate with DHB matrix, as described above (Section 5.3.2.2). Tandem mass spectra (MS/MS) were acquired in reflector positive LIFT mode on the UltraflexIII (Bruker Daltonics), externally calibrated with Peptide Calibration Mix II (Bruker Daltonics) with a MS/MS accuracy of 0.04 Da. FlexAnalysis v3.0 (Bruker Daltonics) was used for manual inspection and annotation of the LIFT-spectra. The RapiDeNovo module in BioTools (Bruker Daltonics) was used to make additional assignments to the amino acid sequence.
5.3.3.2. **Edman Degradation**

Non-alkylated and Maleimide alkylated derivatives were applied to Polybrene-treated glass fiber support filters for automated Edman degradation on a gas-phase sequencer (Model 470A; Applied Biosystems, Foster City, CA, USA). Assignment of the amino acid sequence was essentially as described by Atherton *et al.* 1993 (27) and Matsudaira, 1989 (28).

5.3.4. **Solid Phase Peptide Synthesis**

5.3.4.1. **Fmoc Synthesis**

A non PTM variant of the Tx2081 peptide [RPQC^4CSHPAC^10NVDHPEIC-NH₂:] was manually assembled on a 0.5 mMole scale using 10–30 min coupling steps with 2 mMole Fmoc-amino acid, as described previously in from Schnölzer *et al.*, 1992 (29) and Kapono *et al.*, 2012 (18). An Fmoc-Cys(Trt)-Rink-Amide MBHA Resin (0.55 meq. g⁻¹; Peptides International, Louisville, KY, USA) was used for peptide assembly to provide the required C-terminal chemistry (amide). Side chain protecting groups were: Cys(Trt), Cys(Acm), Asp(tBu), Arg(Pbf) and Gln(Trt). Glu(otBu), His(Trt), Asn(otBu) and (as supplied by Peptides International, Louisville, KY, USA).

5.3.4.2. **TFA Cleavage**

Fmoc peptidyl-resins were subjected to a cleavage by reagent K as described previously (Chapter 2). In short, TFA was used in the presence of the scavengers thioanisole, Phenol, H₂O and TIPS (82.5%, 5%, 5%, 5%, 2.5% v/v), for 2.5 h at 24°C. The resulting cleaved peptide material was recovered by filtration, cold *t*-butyl ether precipitation followed by centrifugation. The resulting crude peptide was lyophilized and stored at –20°C, for future use.

5.3.4.3. **Random Disulfide Bond Formation**

Refolding of synthetic peptide was achieved by air oxidation using 15 mg of material that had previously undergone TCEP reduction and RP-HPLC purification (System 3). Oxidation was carried out in 0.1 M NH₄HCO₃ buffer at pH 8.7 for 5 days stirring at
5.3.4.4. Directed Disulfide Bond Formation

Cleaved Tx2081 peptide containing Cys(Acm) in positions 4 and 10, were RP-HPLC/UV purified (System 3), and then air oxidized, as described above (Section 5.3.4.3). Partially oxidized materials, as confirmed by ESI-MS, were then subjected to spontaneous thiol deprotection and disulfide bond formation. Deprotection was achieved by dissolving the partially folded peptide in 50% v/v acetic acid (1 mg mL$^{-1}$) followed by the addition of a freshly saturated I$_2$ solution in 50% acetic acid (v/v) to the stirring peptide (25% reaction vol.). After 5 min, the reaction was quenched with the addition of 10 μL aliquots of Na$_2$S$_2$O$_3$ (1 M) until the stirring solution became clear. Finally, TFA was added (200 μL) and the resulting acidified material was centrifuged (12,000g, 5 min) before direct purified by RP-HPLC/UV (System 2). The final, oxidized product was mass confirmed by ESI-MS.

5.3.5. Pharmacology

5.3.5.1. Expression of Voltage-gated Ion Channels in *Xenopus Oocytes*

The following nAChR isoforms (α1, α3, α4, α5, β2, β4, γ; δ; ε) in were expressed in *Xenopus* oocytes using linearized plasmids, transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion®, Carlsbad, CA, USA). The harvesting of stage V–VI oocytes from anaesthetized female *Xenopus laevis* frog was described previously by Liman *et al.*, 1992 (30). Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng nL$^{-1}$ using a micro-injector (Drummond Scientific®, Broomall, PA, USA). The oocytes were incubated in buffer (pH = 7.4) containing NaCl (96 mM), KCl (2 mM), CaCl$_2$ (1.8 mM) MgCl$_2$ (2 mM) and HEPES (5 mM), supplemented with 50 mg L$^{-1}$ gentamycin sulfate.
5.3.5.2. Electrophysiological Recordings

Two-electrode voltage-clamp recordings were performed at room temperature (18–22°C) using a Geneclamp 500 amplifier (Molecular Devices®, Downingtown, PA, USA) controlled by a pClamp data acquisition system (Axon Instruments®, Union City, CA, USA). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution (pH 7.4) was composed of NaCl (96 M), KCl (2 mM), CaCl$_2$ (1.8 mM), MgCl$_2$ (2 mM) and HEPES (5 mM). Voltage and current electrodes were filled with KCl (3 mM). Resistances of both electrodes were kept between 0.5–1.5 MΩ. During recordings, the oocytes were voltage-clamped at a holding potential of −70 mV and superfused continuously with ND96 buffer via gravity-fed tubes at 0.1–0.2 mL min$^{-1}$, with 5 min incubation times for the bath-applied peptides. Acetylcholine (ACh) was applied via gravity-fed tubes until peak current amplitude was obtained (1–3 sec), with 1–2 min washout periods between applications. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured before and following incubation of the peptide.

To assess the dose-response correlation, data were fitted with the Hill equation: $y = \frac{100}{[1 + (EC_{50}/[\text{toxin}])^h]}$, where $y$ is the amplitude of the toxin-induced effect, $EC_{50}$ is the toxin concentration at half maximal efficacy, $[\text{toxin}]$ is the toxin concentration and $h$ is the Hill coefficient. A comparison of sample means was performed using a paired Student’s $t$ test ($p<0.05$). All data are presented as mean ± standard error (S.E.M) of at least 4 independent experiments ($n \geq 4$). All data was analyzed using pClamp Clampfit 10.0 (Molecular Devices®, Downingtown, PA, USA) and Origin 7.5 software (Originlab®, Northampton, MA, USA).

5.3.5.3. Whole Animal Bioassay

Standardized concentrations (1 mg mL$^{-1}$ body mass) of native of Tx2081 (2.5, 5, 10, 20, 40, 80, 160, 320 and 640 pMol g$^{-1}$) in 10 µL volumes (in PBS), were injected intramuscularly (IM - foot) into Hawaiian snakehead cowries (Cypraea caputserpentis) using a Hamilton 10 µL syringe at a depth of 2 mm. Following injection, animals were placed in glass Petri dishes filled with fresh aerated seawater, where paralysis was
confirmed by the inability of the animal to cling to the substrate. Dosage and paralysis were recorded and plotted according to Reed and Muench, 1938 (31), and the PD$_{50}$ was extrapolated using GraphPad Prism Software (v5.02). All dose experiments were repeated in triplicate or greater (n ≥ 3).

5.4. RESULTS

5.4.1. Geographic Biodiversity - RP-HPLC Profiling

Venom samples from geographically diverse specimens of *C. textile* demonstrate an abundance of chromatographic peaks, 20–70 per venom profile (Figure 1). Venom samples from Hawaii (n=30) and the Great Barrier Reef, Australia (n=50) displayed similar chromatographic profiles, with variations in relative peptide concentration observed in the moderately hydrophilic region, 25–40% CH$_3$CN or 25 to 40 min (Figure 1A & 1C respectively). No observable patterns could be correlated to cone snail sex or collection/milking season from the venom profiles regardless of location.
The moderate to hydrophobic RP-HPLC/UV profile region of 40–80% CH₃CN (40 to 80 min) accounted for >30% of venom profile variability, with the most diverse venom profiles were observed from samples collected in American Samoa (n=20 specimens) (Figure 1B). The hydrophobic C. textile δ-conotoxin TxVIA (Rt 65.3 min) was observed in both Hawaiian and American Samoan samples, confirming that the analyzed venom profiles were indeed that of the target species (see arrow in Figure 1A & 1B respectively). The δ-conotoxin TxVIA peak was absent in Australian venom samples further highlighting venomic diversity as a function of geographic location (Figure 1C), however one previously uncharacterized peak (underlined), with a retention time (Rt) of 26.6 min, was observed with commonality to all three locations (Figure 1A-C). The observed geographic overlap of this peak hints at its importance in predation strategies, and distinguishes this particular conopeptide for advance characterization studies. It's sequence has been deduced subsequently in Section 5.4.3.
5.4.2. Geographic Biodiversity - Molecular Mass Profiling

Venom samples from geographically representative specimens *C. textile* contained conopeptides with an *m/z* of 744.9 to 4930.7 (examined *m/z* range: 500 – 5000).

Typically, venom samples from the Australian specimens contained the highest number of molecular masses, compared to other locations (Figure 2A-C). In the venom samples analyzed from three distinct geographic locations, Hawaii, American Samoa and the Great Barrier Reef, Australia, a combined total of 178 individual peaks were observed. Of these *m/z*, 42 peaks (~24%) were common to two collection sites, with only 11 (~6%) being common to all three. Again, the commonality of the 11 peptides to all three sample sites indicates and importance in the predator/prey relationship.

![Figure 2. Molecular mass (MALDI-TOF MS) profile of milked venoms from individual, non-captive Conus textile specimens. (A) The island of O‘ahu, Hawaii, United States of America. (B) The island of Tutuila, American Samoa. (C) Gould Reef in the Great Barrier Reef, Australia.](image)

When all the highly abundant and resolvable molecular masses were tabulated, a total of 178 distinct masses were observed, independent of location. Twenty seven (n=27) of the 178 peptide constituents (~15%) correlate to known *C. textile* peptides, excluding post-
translationally modified (PTM) variants. An abbreviated list of the characterized peptides corresponding to known *C. textile* molecules has been compiled in Table 2. Importantly, the peptide identified during RP-HPLC profiling was again observed in Mass Profiling experiments, in its decarboxylated form, further confirming the molecule as a venomic heavyweight.

**Table 2.** Previously characterized conopeptides independent of location.

<table>
<thead>
<tr>
<th>Conopeptide</th>
<th>Observed Molecular Mass (Da)</th>
<th>Calculated Molecular Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convulsant Peptide</td>
<td>2488.04</td>
<td>2487.99</td>
</tr>
<tr>
<td>TxIA</td>
<td>1657.75</td>
<td>1657.65</td>
</tr>
<tr>
<td>TxIIIC</td>
<td>1305.41</td>
<td>1305.37</td>
</tr>
<tr>
<td>Tx10b/Tx10c</td>
<td>1357.49</td>
<td>1357.46</td>
</tr>
<tr>
<td>TxIIIA</td>
<td>1712.48</td>
<td>1711.46</td>
</tr>
<tr>
<td>TxMLKLT1-0111</td>
<td>3067.31</td>
<td>3068.18</td>
</tr>
</tbody>
</table>

### 5.4.3. Peptide Sequencing

#### 5.4.3.1. Collision Induced Dissociation (CID)

The sequence of the novel peptide discovered during RP-HPLC profiling (Section 5.3.1) has been deduced. The parent sequence was assigned as: R-[I/L/O]$^2$-Q-C-C-S-H-[I/L/O]$^8$-A-C-N-V-D-H-P-E-[I/L/O]$^{17}$-C (Error! Reference source not found.). The ambiguities at positions 2, 8 and 17 resulted from the isobaric residues (Iso)leucine (I and L) and 4-*trans*-hydroxylproline (O), which have residue masses of 113.084 and 113.048 Da, respectively. The final sequence was confirmed through traditional Edman degradation (Section 5.4.3.2). The CID fragment ions, together with mass assignment and sequence confirmation indicated an amidated C-terminus.
5.4.3.2. Edman Sequencing

A RP-HPLC/UV purified sample of Tx2081 was thiol alkylated (N-phenylmaleimide), ~300 nMole, and subjected to 20 cycles of automated Edman degradation. Sequential degradation provided an unambiguous 18 amino acid sequence: RO\textsuperscript{2}QCCSHO\textsuperscript{8}ACNVDHP(-)\textsuperscript{16}IC. Cycle 16 indicated a low recovery level PTH-Glu (<1 nMole) and was designated as a 'blank cycle' based on the expected sequential cycle, with observed yields within the normal degradative range.

Seventeen (17 of the 18) amino acids were conclusively assigned by Edman degradation. Positions 2 and 8 were assigned as 4-\textit{trans}-hydroxylproline and position 17 was described as isoleucine. Low levels of glutamic acid in cycle 16, combined with the observed mass difference of 44 Da (-CO\textsubscript{2}), under various mass spectrometric conditions, further corroborated the presence of a carboxylated glutamic acid at position 16 within Tx2081.
5.4.4. **Solid Phase Peptide Synthesis**

Based on the assigned C-terminal amide sequence above, a synthetic, non-post translationally modified version of Tx2081, Tx2005 was synthetically constructed and folded by random air oxidation. Upon completion, the correct target molecular mass was observed by ESI-MS: Obs. [$\text{M+3H}]^{3+}$ 668.8 Da. (Calc. $\text{MH}^+$ 2005.1 Da.). RP-HPLC revealed the presence of a dominant peak at Rt=24.7 min (Figure 4).
Figure 4. Solid Phase Peptide Synthesis of a non PTM version of Tx2081 known as Tx2005. (A) RP-HPLC UV/Vis. chromatogram of Tx2005 at $\lambda=214\text{nm}$. (B) ESI-MS of synthetic Tx2005 observed predominantly at the $[\text{M}+3\text{H}]^{3+}$ charge state.
5.4.5. Pharmacology

5.4.5.1. Electrophysiological Recording

Biological activity was observed with synthetic Tx2005. This analogue inhibited the human AChR subtypes (\(\alpha_3\beta_2\), \(\alpha_3\alpha_5\beta_2\), and \(\alpha_3\beta_4\)), with the \(\alpha_3\beta_4\) isoform demonstrating the highest sensitivity (IC\(_{50}\) 2.1±0.2 \(\mu\)M) and exhibiting a 77% maximum inhibition (Figure 5). While the isoform \(\alpha_3\beta_2\) demonstrated the highest level of inhibition (96%) with an IC\(_{50}\) of 5.4±0.5 \(\mu\)M. No significant inhibition was observed at the remaining isoform targets (\(\alpha_4\beta_2\), \(\alpha_4\beta_4\), \(\alpha_7\), \(\alpha\beta\delta\gamma\), \(\alpha\beta\gamma\varepsilon\)) at concentrations up to 100 \(\mu\)M.

![Figure 5. Pharmacology of synthetic Tx2005. (A) Resulting effect of 10 \(\mu\)M toxin application upon the action potential of different nAChR isoforms. (B) Resulting dose dependent inhibition of various nAChR isoforms.](image)

5.4.5.2. Whole Animal Bioassay

The synthetic Tx2005 analogue demonstrated an inability to cause paralysis at same PD\(_{50}\) concentration (n=15). Total paralysis was achieved with 10.24 nMol g\(^{-1}\) (n=7), this being \(~16\)x that of native Tx2081. A dose-dependent trend was observed, however maximum dose did not induce lethality in the test animals. A PD\(_{50}\) of 3.6 nMol g\(^{-1}\) (3.6 \(\mu\)Mol Kg\(^{-1}\); whole snail weight) was calculated, this PD\(_{50}\) concentration being \(~100\)x more than observed with native Tx2081.
5.5. DISCUSSION

Using techniques in peptide prospecting, a comparison of venom from a domestic Hawaiian *conus* species, *C. textile*, revealed extreme venomic variability and allowed for the identification of a novel, previously uncharacterized peptide, Tx2081. The species-specific ‘biomarker’ δ-conotoxin TxVIA (32-36), confirmed parallel analysis of venom for a single, target species. The data clearly demonstrates the extent of intraspecies venom variability that has been documented previously within other members of the genus (37-40) – a characteristic that now extends to *C. textile*. This in turn possibly increases earlier estimates of bioactive venom constituents available domestically in Hawaii (3, 4, 41), and effectively expands the bioengineering potential of *Conus* peptides.

Within *C. textile*, chromatographically, the diversity was most pronounced within the hydrophobic region of the RP-HPLC/UV venom profiles, and most prominently in specimens from American Samoa. It would be easy to surmise that the plasticity observed in the venom from this *conus* species may be attributed to the tailoring of venom, based primarily on localized prey species. Conversely, mass spectral analysis revealed a level of molecular mass consistency across all locations indicating that certain venomic constituents are crucially valuable in the envenomation strategy and could potentially be important in bioengineering strategies as well.
The newly characterized peptide, Tx2081 was produced synthetically in order to build
upon the biosustainable approach to peptide prospecting. Alternatively, it would have
been necessary to sacrifice many animals to allow the isolation of sufficient amounts of
peptide for pharmacological characterization. Voltage-clamp analysis, combined with a
bioassay conducted in a prey species, confirmed that target of Tx2081/Tx2005 as the
nicotinic acetylcholine receptor (nAChR), and important receptor medically for the study
and treatment of chronic neuropathic pain. Additionally, the described approaches to
peptide prospecting, identification on novel, valuable peptide toxins, and finally probe
bioengineering could repeated in an effort to establish a molecular toolbox of clinically
relevant peptide calipers to study a wide array of disease processes. These peptides could
also lead the way as template molecules for bioengineering new treatments strategies, or
provide the knowledge to produce the next generation of therapeutic molecules.

5.6. CONCLUSION

The current prospective on peptide prospecting from *C. textile* venom using RP-HPLC
and molecular mass profiling, has provided greater insight into the peptide complexity of
the venom from this species. Moreover, this approach revealed the presence of a novel,
conopeptide, Tx2081, which demonstrates experimentally, a high level of differentiated
specificity to human isoforms of neuronal type nAChR, a well-documented target for the
treatment of chronic neuropathic pain (21-24). The bioengineering of this peptide could
produce both a therapeutic peptide to potentially replace currently used narcotic
painkillers, but could also be fluorescently bioengineered to investigate the expression of
nAChR's in neuronal surfaces, and further contribute to the compilation of pain models
which could help to create new, more efficient treatments for chronic neuropathic pain in
the future.
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Title: The Use of Novel Peptide Probes in Determining Ion Channel Distribution in Native Human Cardiac Tissues