DEVELOPMENT AND OPTIMIZATION OF SCREENING ASSAYS FOR TARGETED MINIPROTEIN THERAPEUTICS: FOCUS ON KV1.3 AND MMP-2

Ph.D thesis

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1 INTRODUCTION

Miniproteins are small (1–10 kDa), highly stable proteins with rigid tertiary structures making them promising tools for drug development, especially against targets considered "undruggable" by traditional modalities like ion channels and highly homologous extracellular enzymes. Their size and structural rigidity allow them to bind with high affinity and specificity, while maintaining good tissue penetration and low immunogenicity.

This thesis focuses on two therapeutically relevant targets: Kv1.3 potassium channels, which are involved in chronic inflammatory diseases through their role in T cell activation and proliferation, and MMP-2, a matrix metalloproteinase linked to tumor invasion and glioblastoma progression. Venom-derived miniproteins, such as chlorotoxin (CTX), have demonstrated strong potential for targeting MMP-2, while other miniprotein scaffolds have been shown to inhibit the Kv1.3 potassium channel.

Despite their potential, miniprotein drug development is limited by the lack of robust and scalable screening tools. This work aims to develop and optimize assay systems that enable the efficient screening of miniprotein therapeutics. Two model systems were used: one for CTX-based MMP-2 targeting, and another employing engineered KcsA-Kv1.x chimeras for identifying Kv1.3-binding ligands. These platforms support both ligand characterization and high-throughput screening, helping to advance targeted miniprotein-based drug discovery.

2 OBJECTIVES

The main goal of this work was to develop and refine screening assays to support the discovery of miniprotein therapeutics for two distinct targets: MMP-2 and Kv1.3.

Model system I.: CTX targeting MMP-2

- Develop bead-based assays to characterize CTX and its variants.
- Resolve conflicting data on CTX target specificity.
- Adapt the system for high-throughput screening and phage display applications.

Model system II.: Miniprotein scaffolds targeting Kv1.3

- Design and express KcsA-Kv1.x chimeras, including both turret-only and turret-filter (T+F) versions.
- Identify suitable miniprotein scaffolds for Kv1.3 targeting.
- Validate the chimera-based assays for binding predictivity and HTS compatibility.
- Demonstrate selection of Kv1.3-binding ligands from phage libraries.
- These efforts aim to provide a robust foundation for future miniprotein drug discovery programs targeting ion channels and tumor-associated enzymes.

3 METHODS

3.1 Production of CTX, mCTX, Bs-Tx7, CTX-Cy5 and CTX-A488

3.1.1 Design and expression

CTX and its variants (mCTX, Bs-Tx7) were produced recombinantly in E. coli using fusion constructs with DsbC to support correct folding and disulfide bonding. Expression was carried out under autoinducing conditions, and constructs included a His-tag for purification. Fluorescent versions (CTX-Cy5 and CTX-A488) were generated by site-specific labeling with NHS-ester dyes, followed by purification via HPLC.

3.1.2 Protein purification

Purification involved IMAC, cleavage with SplB protease, and further polishing by ion exchange or size exclusion chromatography.

3.2 Flow cytometry-based binding assays

3.2.1 IgG coated bead binding test

Target proteins were captured using antibody-coated magnetic beads. Labeled CTX variants were incubated with the beads, and binding was measured via flow cytometry.

3.2.2 Cobalt-coated bead binding test

His-tagged target proteins were immobilized on cobalt-coated beads. Binding of fluorescently labeled ligands, including CTX-Cy5, was measured directly by flow cytometry. In the case of phage-displayed variants, detection was performed using an anti-M13 primary antibody followed by a Cy5-labeled secondary antibody.

3.3 Kv1.x targeting miniprotein scaffold representatives

Selected miniprotein scaffold representative peptide toxins known to bind Kv1.3—ShK, Vm24, HgTX1, KTX1, and MTX—were used to test and benchmark the screening assays.

3.4 Recombinant production of KcsA-Kv1.x screening targets

3.4.1 Design and expression

Chimeras combining KcsA with human Kv1.1, Kv1.2, or Kv1.3 turret (T) or turret + filter (T+F) regions were cloned into expression vectors and expressed in E. coli (XL1-Blue or C41 strains).

3.4.2 Purification and quality control

Membrane fractions were isolated and chimeras purified using IMAC and detergent solubilization. Purity and

proper assembly were verified by SDS-PAGE and size exclusion chromatography.

3.5 Cells for electrophysiology recordings

Kv1.1 and Kv1.2 currents were recorded from CHO cells transiently transfected with plasmids encoding the respective channels, while Kv1.3 currents were measured in activated human peripheral blood mononuclear cells (PBMCs), which naturally express high levels of Kv1.3 following phytohemagglutinin-induced activation.

3.6 Patch clamp measurements with Kv1.x channels

Whole-cell recordings were performed to validate miniprotein activity and selectivity against Kv1.3, Kv1.1, and Kv1.2.

3.7 Recombinant production of phages

Recombinant phages were generated by ligating annealed oligonucleotides encoding the peptide constructs into a linearized pAS62 phagemid vector. The final construct contained a signal sequence, the miniprotein of interest, a GSASSATR linker, and the C-terminal portion of the M13 pIII coat protein. The ligated vector was transformed into chemically competent E. coli XL1-Blue cells, and phage production was initiated using M13KO7 helper phage infection. Phages were harvested, PEG-precipitated, and purified for downstream binding assays and biopanning applications

3.8 Microplate-based ELISA binding assays

3.8.1 Phage binding assay

Phage solutions were prepared in resuspension buffer containing 2 mM DDM. ELISA plates (Ni^+-coated) were coated with various KcsA-Kv1.x constructs in 50 µL per well and incubated for 1 hour at room temperature. After blocking with TBS-BSA (1 mM DDM), wells were washed and incubated with phage-displayed miniproteins for 1 hour. Following washes, anti-M13 HRP-conjugated antibody was added and incubated for another hour. Signal was developed using TMB substrate, stopped with HCl, and absorbance was read at 450 nm to quantify phage binding

3.8.2 Phage competition assay

To evaluate relative binding affinity, ligand-displaying phages were co-incubated with increasing concentrations of unlabeled competitor toxins on chimera-coated plates. Binding was detected as above, and competition was assessed by calculating percent displacement. IC_{50} values were determined from dose-response curves fitted with a variable slope model using GraphPad Prism.

3.9 Phage-display based solid phase biopanning

Biopanning was performed on Ni^+-coated plates pre-coated with 5 µg/mL of T+F KcsA-Kv1.3. After blocking with TBS-BSA + 1 mM DDM, phage mixtures (e.g. Vm24:DDDKTX1 at varying CFU ratios) were added and

incubated. Wells were washed stringently with TBS + 0.1% Tween-20, and bound phages were eluted using 0.01 M HCl, neutralized with Tris (pH ~11), and quantified by infecting E. coli and titering via colony count. Enrichment was calculated relative to BSA-coated control wells.

4 RESULTS

4.1 Characterization of CTX and its variants by bead binding assays

4.1.1 Ig-coated bead binding test to measure MMP-2 binding

The Ig-coated bead assay was used to measure and compare the binding affinities of CTX and its variants to MMP-2 through competitive displacement of fluorescently labeled CTX (either CTX-Cy5 or CTX-A488). The assay confirmed that CTX, rCTX, mCTX, and CTX-Cy5 all exhibited similar affinities for MMP-2, with estimated IC_{50} values between 0.62 and 0.75 μM, as determined through dose-response fitting. Additionally, Bs-Tx7—a CTX-like toxin—was found to bind the same site on MMP-2 but with approximately 2.6-fold lower affinity than CTX.

4.1.2 CTX's target confirmation and HTS preparation

A cobalt-coated bead binding assay was used to assess the binding profile of fluorescently labeled CTX (CTX-Cy5) across a panel of His-tagged proteins: MMP-2, NRP1, MMP-9, TIMP-2, MMP-14, ANX2, CLC-3, αvβ3 integrin, and HSA. CTX-Cy5 showed strongest binding to MMP-2 and NRP1, with lower but detectable signals for

TIMP-2 and CLC-3. No significant binding was observed to MMP-9, ANX2, αvβ3, or HSA.

The same assay was then applied to phage-displayed CTX, and the resulting binding pattern closely matched that of CTX-Cy5. This confirmed that the phage format preserved native-like binding properties and that both ligand formats identified the same subset of targets.

These results demonstrated the reproducibility and consistency of the cobalt-coated bead assay across different ligand types, supporting its use in future high-throughput screening (HTS) and ligand optimization efforts.

4.2 Scalable KcsA-Kv1.x chimera expression and purification: T-only and T+F version

KcsA-Kv1.x chimeras containing either turret-only (T) or turret+filter (T+F) regions were successfully expressed in E. coli, with higher yields achieved using the C41(DE3) strain. Membrane proteins were isolated and purified via IMAC and size-exclusion chromatography. Functional tetramers were confirmed by native PAGE and phage ELISA. Although T+F constructs were harder to express, they showed superior functional mimicry of native Kv1.x channels in downstream binding assays.

4.3 Selection of Kv1.x targeting scaffolds for chimera characterization and future screening

To identify suitable miniprotein scaffolds for Kv1.x targeting, a custom database of K^+ channel-binding peptides was compiled using literature and curated online resources (e.g. KaliumDB, ConoServer, UniProt). Sequences were classified based on sequence similarity and disulfide bridge connectivity into 26 distinct structural scaffold families.

From this dataset, four scaffolds were selected based on developability, structural diversity, and phage dsiplay feasibility. Representative peptides — Vm24, MTX, ShK, and KTX1 — were chosen for experimental testing, and HgTX1 was also included as a variant within the same scaffold as KTX1.

These toxins were used to validate T-only and T+F KcsA-Kv1.x chimeras via phage ELISA and competition assays. Their performance confirmed that the selected scaffolds were appropriate for future library design and phage display-based HTS targeting Kv1.3.

4.4 Assessment of T-only and T+F KcsA-Kv1.3 chimeras

The binding performance of three Kv1.3-blocking toxins—Vm24, HgTx1, and KTX1—was evaluated using both T-only and T+F KcsA-Kv1.3 chimeras. Results from phage-binding and competition assays were compared to

blocking potencies measured via patch-clamp in native hKv1.3-expressing cells.

The T-only chimera showed inconsistent binding profiles: affinities did not match the functional potency ranking of the native channel. In contrast, the T+F chimera reproduced the correct rank order (Vm24 > HgTx1 > KTX1), aligning with patch-clamp data. ELISA and competition assay IC_{50} values also reflected these trends.

4.5 Assessment of T-only and T+F KcsA-Kv1.1 and Kv1.2 chimeras

To evaluate the predictive accuracy of Kv1.1 and Kv1.2 chimeras, the binding of MTX, ShK, and KTX1 was assessed using phage ELISA and competition assays.

For Kv1.2, the T-only chimera showed poor correlation with known potencies: MTX, a strong blocker of native Kv1.2, showed no detectable binding. ShK and KTX1 also exhibited inconsistent ranking. In contrast, the T+F chimera restored the expected rank order of toxin affinities, consistent with electrophysiological data. For Kv1.1, both T-only and T+F chimeras produced binding patterns that aligned with native Kv1.1 blocking potencies. ShK showed the strongest binding, followed by KTX1, with minimal binding from MTX — in agreement with functional assays.

4.6 Functional assessment of miniprotein displaying phages

The inhibitory activity of phage-displayed miniproteins (Vm24, HgTX1, KTX1) was evaluated using whole-cell patch-clamp recordings in activated human PBMCs, which naturally express high levels of Kv1.3. All three phage-displayed toxins were able to block Kv1.3 currents, with blocking potencies correlating with their reported IC_{50} values for the native peptides.

As a control, a non-binding mutant (DDDKTX1) displayed on phage showed no inhibition, confirming that current suppression was due to specific peptide-channel interaction and not the phage particle itself.

Blocking efficiency was inversely related to the phage concentration required for ~50% inhibition: Vm24 showed the highest potency, followed by HgTX1, and KTX1 was the weakest, mirroring native peptide rankings. These findings validate that phage-displayed ligands maintain functional activity, supporting their use in downstream HTS applications.

4.7 Developing a biopanning protocol using the T+F KcsA-Kv1.3-based screening assay for HTS application

A solid-phase biopanning protocol was established using the T+F KcsA-Kv1.3 chimera coated onto Ni^{+}-microplates.

Phage mixtures containing Vm24 and DDDKTX1 in a 1:9 ratio—comprising 90% DDDKTX1-displaying phages—were prepared at a concentration of 10^{10} CFU/well. This mixture was screened against T+F KcsA-Kv1.3-coated wells, with BSA-coated wells serving as controls. Vm24-displaying phages were successfully recovered, showing a 62.5-fold enrichment over the BSA control after a single round of panning

5 CONCLUSIONS

This. This research highlights the pivotal role of screening assay development and optimization in advancing targeted miniprotein therapeutics. By focusing on two drug discovery projects at VRG Therapeutics—targeting MMP-2 for cancer therapy and Kv1.3 for chronic inflammatory diseases—this work establishes scalable and reliable screening platforms that streamline the identification of novel miniprotein-based drugs for future applications.

For MMP-2, a bead-based flow cytometry assay was successfully developed, resolving discrepancies regarding CTX binding specificity. Similar binding results obtained with native CTX and phage-displayed CTX underscore the reliability of the cobalt-coated bead assay, providing a strong foundation for future HTS campaigns to further improve MMP-2 affinity and selectivity. If successful, these efforts could lead to new treatments and more precise surgical tools for glioblastoma and other cancers where MMP-2 is overexpressed.

The Kv1.3 project presented additional challenges due to the membrane-bound nature of the target. In this study, these challenges were addressed by designing chimeric constructs that incorporated both the turret and filter regions (T+F) of Kv1.x, enabling structurally and functionally reliable models.

The summary of comparative predictivity assessments is presented in **Table 1.** The displayed IC50 values from competition assays represent toxin affinities. These values are compared with previously reported channel-blocking potencies for the corresponding native hKv1.x channels.

Table 1. Summary of chimeric protein-binding results studies in the light of IC50 values from literature.

	T-only chimera		T+F chimera		Kv1.x blocking	
Toxin ligand	IC_{50} (nM)	Rank	IC_{50} (nM)	Rank	IC_{50} (nM)	Rank
Kv1.3						
Vm24	0.577 (±0.431)	2	0.036 (±0.004)	1	0.003	1
HgTx1	0.671 (±0.090)	3	0.108 (±0.022)	2	0.086	2
KTX1	0.416 (±0.062)	1	98.2 (±81.6)	3	0.650	3
Kv1.2						
MTX	>1000 (NQ)	3	0.129 (±0.050)	1	0.8	1
ShK	0.070 (±0.026)	1	15.9 (±4.4)	2	9.0	2
KTX1	0.185 (±0.031)	2	205.3 (±59.4)	3	>1000	3
Kv1.1						
ShK	0.317 (±0.069)	1	0.271 (±0.037)	1	0.007–0.09	1
KTX1	1.270 (±0.308)	2	1.487 (±0.260)	2	1.1–41	2
MTX	>1000 (NQ)	3	>1000 (NQ)	3	>100	3

Toxin binding to T+F KcsA-Kv1.x chimeras strongly correlates with their blocking potency, supporting their superiority over T-only variants for screening toxin-phage libraries. These chimeras were successfully applied in two microplate-based phage-ELISA assays—direct phage binding and competition assays—demonstrating their suitability for HTS by successful biopanning experiments. Their strong predictivity and scalability make them ideal tools for identifying highly selective Kv1.3 inhibitors, which could lead to novel treatments for chronic inflammatory diseases and overcome the limitations of previous attempts (e.g. Dalazatide) to develop highly selective Kv1.3 inhibitors.

Beyond these model systems, this research establishes a broader guidance for investigating miniproteins in drug discovery. The combination of scalable production,

predictive chimeric screening, and optimized biopanning provides a strong pipeline for accelerating ligand identification. Future applications could extend these methodologies to other ion channels, proteases, and structurally complex drug targets, expanding the scope of miniproteins as a promising therapeutic modality. By integrating these approaches into early-stage drug development, this work contributes to more efficient screening strategies, paving the way for next-generation selective therapeutics.

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