Characterization and inhibition of the dimer interface in bacterial small multidrug resistance proteins

by

Bradley E. Poulsen

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Biochemistry
University of Toronto

© Copyright by Bradley E. Poulsen 2012
Characterization and inhibition of the dimer interface in bacterial small multidrug resistance proteins

Bradley E. Poulsen

Doctor of Philosophy

Department of Biochemistry
University of Toronto
2012

Abstract

As one of the mechanisms of antibiotic resistance, bacteria use several families of membrane-embedded α-helical transporters to remove cytotoxic molecules from the cell. The small multidrug resistance protein family (SMR) is one such group of drug transporters that because of their relative small size [ca. 110 residues with four transmembrane (TM) helices] must form at the minimum dimers to efflux drugs. We have used the SMR homologue Hsmr from Halobacterium salinarum to investigate the oligomerization properties of the protein family at TM helix 4. We produced point mutations along the length of the TM4 helix in the full length Hsmr protein and assayed their dimerization and functional properties via SDS-PAGE and bacterial cell growth assays. We found that Hsmr forms functionally dependent dimers via an evolutionarily conserved $^{96}$GLxLIxxGV$^{98}$ small residue heptad repeat. Upon investigation of the large hydrophobic residues in this motif by substituting each large residue to Ile, Leu, Met, Phe, and Val, we determined that Hsmr efflux function relies on an optimal level of dimerization. While some substitutions led to either decreased or increased dimer and substrate-binding strength, several Ile94 and Val98 mutants were equal to wild type dimerization levels but were
nonfunctional, leading to the hypothesis of a mechanistic role at TM4 in addition to the locus of dimerization. The functionally sensitive TM4 dimer represents a potential target for SMR inhibition using a synthetic TM4 peptide mimetic. Using exponential decay measurements from a real-time cellular efflux assay, we observed the efflux decay constant was decreased by up to ~60% after treatment with the TM4 peptide inhibitor compared to control peptide treatments.

Our results suggest that this approach could conceivably be used to design hydrophobic peptides for disruption of key TM-TM interactions of membrane proteins, and represent a valuable route to the discovery of new therapeutics.
Acknowledgments

To my family, friends, and colleagues: thank you all for your support and guidance over the years.
# Table of Contents

Abstract ................................................................................................................................. ii

Acknowledgments .................................................................................................................. iv

Table of Contents .................................................................................................................... v

List of Figures .......................................................................................................................... ix

List of Tables ........................................................................................................................... x

List of Appendices .................................................................................................................. xi

List of Abbreviations ............................................................................................................. xii

Chapter 1: Introduction .......................................................................................................... 1

1.1 Introduction to membrane proteins ............................................................................... 2
  1.1.1 The cellular membrane ............................................................................................. 2
  1.1.2 Amino acid composition and secondary structure of membrane proteins ............ 4
  1.1.3 Structural features of α-helical and β-barrel proteins ........................................... 5

1.2 Folding of α-helical membrane proteins ........................................................................ 8
  1.2.1 Forces driving membrane protein folding ............................................................... 8
  1.2.2 The two-stage folding model of α-helical membrane proteins ............................... 11
  1.2.3 Membrane protein insertion is mediated by the translocon ................................. 12

1.3 Oligomerization motifs of α-helical membrane proteins ............................................. 14
  1.3.1 GAS_{right}/GxxxG motifs ....................................................................................... 14
  1.3.2 GAS_{left}/Heptad motifs ....................................................................................... 16
  1.3.3 Polar residue motifs ............................................................................................... 17
  1.3.4 Transmembrane peptides targeting membrane protein oligomerization ............ 18

1.4 Bacterial antibiotic resistance ......................................................................................... 19
  1.4.1 The evolution of “superbugs” ................................................................................. 19
  1.4.2 The resistance rise and new drug decline ............................................................... 21
  1.4.3 Combinational antibiotic treatments: the future is here ..................................... 22
  1.4.4 Understanding resistance mechanisms: a key step for rational drug design ...... 24
1.5 Small multidrug resistance proteins ................................................................. 24
   1.5.1 The five families of multidrug efflux proteins ........................................... 24
   1.5.2 The small multidrug resistance family ...................................................... 27
   1.5.3 SMR drug binding and efflux mechanism ................................................. 30
   1.5.4 Structures of EmrE .................................................................................. 32
   1.5.5 Oligomerization studies of SMRs ............................................................. 33
1.6 Thesis Hypothesis and Outline ............................................................................. 34

Chapter 2: The assembly motif of a bacterial small multidrug resistance protein ......... 37
2.1 Introduction........................................................................................................ 38
2.2 Results ............................................................................................................... 39
   2.2.1 Design of residue substitutions in Hsmr TM4 ........................................... 39
   2.2.2 Mutations at conserved sites in Hsmr TM4 compromise protein function ...... 41
   2.2.3 Disruptive TM4 mutants compromise Hsmr oligomerization .................... 41
   2.2.4 Modeling of Hsmr TM4-TM4 dimers ......................................................... 43
2.3 Discussion .......................................................................................................... 45
   2.3.1 The G\textsubscript{90}LxLIxxGV\textsubscript{98} sequence is crucial for Hsmr drug efflux activity and self-assembly .......................................................................................................................... 45
   2.3.2 TM4 mediated inter-monomer interactions are stabilized by close packing between residues ........................................................................................................................................ 45
   2.3.3 Implications for SMR inhibitor design .......................................................... 48
2.4 Materials and Methods ...................................................................................... 48
   2.4.1 Production of wild-type and mutant Hsmr proteins ..................................... 48
   2.4.2 Ethidium resistance assays .......................................................................... 49
   2.4.3 Gel electrophoresis and densitometry ........................................................ 49
   2.4.4 Modeling of Hsmr TM4 dimers .................................................................. 50

Chapter 3: Modulation of substrate efflux in bacterial small multidrug resistance proteins by mutations at the dimer interface ................................................................. 51
3.1 Introduction........................................................................................................ 52
3.2 Results ............................................................................................................... 54
   3.2.1 Large residue homology in the Hsmr GL\textsubscript{91}xL\textsubscript{93}I\textsubscript{94}xxGV\textsubscript{98} dimer motif ........................................ 54
   3.2.2 Large residue mutations can alter the strength of Hsmr oligomerization .......... 54
3.2.3 Several large residue substitutions in Hsmr TM4 alter protein function ............ 56
3.2.4 Oligomerization is correlated to ethidium binding........................................ 58

3.3 Discussion .............................................................................................................. 60
3.3.1 Minor side chain modifications modulate Hsmr efflux activity......................... 60
3.3.2 Dimer strength is correlated to substrate binding............................................. 61
3.3.3 Hsmr efflux activity is not exclusively related to dimer strength...................... 61
3.3.4 Implications for a functional role at TM4 in SMRs........................................ 64

3.4 Materials and Methods.......................................................................................... 64
3.4.1 Large residue analysis and mutagenesis at the TM4 dimer motif....................... 64
3.4.2 Protein expression, purification, and concentration determination............... 65
3.4.3 Oligomerization measurements........................................................................ 65
3.4.4 Ethidium resistance activity assay..................................................................... 66
3.4.5 Hsmr binding to ethidium bromide via fluorescence spectroscopy ............... 66
3.4.6 Modeling of Hsmr TM4 Dimers ...................................................................... 67

Chapter 4: Drug efflux by a small multidrug resistance protein is inhibited by a
transmembrane peptide............................................................................................. 68
4.1 Introduction............................................................................................................. 69
4.2 Results .................................................................................................................... 71
4.2.1 Peptide design and characterization................................................................. 71
4.2.2 Assay for antimicrobial activity ...................................................................... 72
4.2.3 Secondary structures of designed peptides..................................................... 73
4.2.4 Ethidium efflux of peptide-treated cells........................................................... 77
4.3 Discussion ............................................................................................................. 78
4.3.1 Synthetic transmembrane peptides display full-length Hsmr properties......... 78
4.3.2 TM4 peptide prevents ethidium efflux in vivo ................................................. 80
4.3.3 Conclusion ....................................................................................................... 81
4.4 Materials and Methods......................................................................................... 82
4.4.1 Peptide synthesis ............................................................................................. 82
4.4.2 Antimicrobial peptide assay ............................................................................ 82
4.4.3 Circular dichroism spectroscopy and SDS-PAGE ........................................... 83
4.4.4 Ethidium bromide efflux assay.......................................................................... 83
Chapter 5: Discussion ........................................................................................................ 85

5.1 Summary of Contributions ...................................................................................... 86

5.1.1 Identifying the oligomerization motif used by bacterial small multidrug resistance
proteins at TM4 ........................................................................................................... 86
5.1.2 An added role for TM4 in small multidrug resistance protein efflux ................. 87
5.1.3 A TM4 peptide inhibits Hsmr function .............................................................. 87

5.2 Peptides as antibiotics .......................................................................................... 88

5.2.1 Antimicrobial peptides targeting the bacterial membrane .............................. 89
5.2.2 Peptides targeting bacterial multidrug efflux protein TM-TM interactions .... 90
5.2.3 Improving TM inhibitor peptide efficacy ......................................................... 91
5.2.4 Designing TM peptides toward other bacterial targets ................................. 92

5.3 Concluding Remarks ............................................................................................ 93

References .................................................................................................................. 95

Appendices .................................................................................................................. 106

Copyright Acknowledgements .................................................................................. 120
List of Figures

| Figure 1.1 | The biological membrane structure | 3 |
| Figure 1.2 | Distribution of residues in a membrane-embedded $\alpha$-helix | 5 |
| Figure 1.3 | Representative $\alpha$-helical and $\beta$-barrel membrane proteins | 7 |
| Figure 1.4 | Forces stabilizing membrane protein folding | 10 |
| Figure 1.5 | The two-stage folding model of $\alpha$-helical membrane proteins | 11 |
| Figure 1.6 | Model of translocon-mediated membrane insertion of an $\alpha$-helix | 13 |
| Figure 1.7 | GAS$_{\text{right}}$ oligomerization motif | 15 |
| Figure 1.8 | GAS$_{\text{left}}$/heptad oligomerization motif | 16 |
| Figure 1.9 | Polar residue oligomerization motif | 17 |
| Figure 1.10 | Examples of some common antibiotic families | 20 |
| Figure 1.11 | The rise of antibiotic resistant bacterial strains and the decline of new drug compounds | 22 |
| Figure 1.12 | Combinational drug treatment of $\beta$-lactam antibiotics | 23 |
| Figure 1.13 | The X-ray crystallographic structures of the five bacterial multidrug transporter families | 25 |
| Figure 1.14 | Sequence, efflux mechanism, and structures of EmrE | 29 |
| Figure 1.15 | Structures of some small multidrug resistance protein substrates | 32 |
| Figure 2.1 | TM4 residue conservation in the SMR protein family | 40 |
| Figure 2.2 | Resistance activity and dimerization profile of WT and TM4 mutant Hsmr proteins | 42 |
| Figure 2.3 | Model of SMR assembly mediated by TM4-TM4 contacts | 44 |
| Figure 2.4 | Sequence alignment of Hsmr TM4 and TM segments that associate via GG4 or small residue heptad repeats | 47 |
| Figure 3.1 | Sequence and TM4 dimer model of the SMR homologue | 53 |
| Figure 3.2 | Dimerization analysis of Hsmr TM4 mutants | 55 |
| Figure 3.3 | Resistance activity of WT and TM4 mutant Hsmr proteins | 57 |
| Figure 3.4 | Ethidium binding propensities of Hsmr TM4 mutants | 59 |
| Figure 3.5 | Dimer-activity relationships in Hsmr TM4 mutants | 63 |
| Figure 4.1 | Antimicrobial peptide assay | 73 |
| Figure 4.2 | Characterization of peptides related to Hsmr TM4 | 75 |
| Figure 4.3 | TM4 peptide inhibition of Hsmr ethidium bromide efflux | 76 |
| Figure 4.4 | Dose-dependence of TM4 inhibition of ethidium bromide efflux | 78 |
| Figure 4.5 | Proposed inhibition mechanism of drug efflux | 79 |
List of Tables

**Table 1.1** The five multidrug efflux proteins and substrates in four pathogenic bacteria  

**Table 3.1** Occurrence of large hydrophobic residues at positions 91, 93, 94, and 98 in TM4 within the small multidrug resistance protein family  

**Table 4.1** Sequences and characterization of designed Hsmr TM4 peptides
# List of Appendices

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix 1</td>
<td>List of oligonucleotides used in this work</td>
<td>106</td>
</tr>
<tr>
<td>Appendix 2</td>
<td>Supplementary data for Chapter 2</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Figure A2.1. Mass spectrometry analysis of purified Hsmr</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Figure A2.2. Antiparallel TM4 models</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Figure A2.3. Parallel TM4 models</td>
<td>110</td>
</tr>
<tr>
<td>Appendix 3</td>
<td>Supplementary data for Chapter 3</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Table A3.1. ClustalW sequence alignment of SMR family members</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Figure A3.1. Wild type and representative mutant TM4 dimer models</td>
<td>117</td>
</tr>
<tr>
<td>Appendix 4</td>
<td>Supplementary data for Chapter 4</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Figure A4.1. Purification and MS of the synthetic TM4 peptide</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Figure A4.2. MS results of the synthetic TM4 peptide variants</td>
<td>119</td>
</tr>
</tbody>
</table>
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette superfamily</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CHI</td>
<td>CNS searching of helix interactions</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CNS</td>
<td>Crystallography and NMR software suite</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Electron cryomicroscopy</td>
</tr>
<tr>
<td>DAGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DDM</td>
<td>Dodecyl maltoside</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EmrE</td>
<td><em>Escherichia coli</em> multidrug resistance protein E</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FQRP</td>
<td>Fluoroquinolone-resistant <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>GpA</td>
<td>Glycophorin A</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>Hsmr</td>
<td><em>Halobacterium</em> small multidrug resistance protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and cytotoxic extrusion family</td>
</tr>
<tr>
<td>MFP</td>
<td>Membrane fusion proteins</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>MHB</td>
<td>Mueller-Hinton Broth</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Mut</td>
<td>Mutant</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OMF</td>
<td>Outer membrane factor</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Pasmr</td>
<td><em>Pseudomonas aeruginosa</em> small multidrug resistance protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFO</td>
<td>Sodium perfluorooctanoate</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem 1</td>
</tr>
<tr>
<td>QAC</td>
<td>Quaternary ammonium compounds</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance nodulation cell division</td>
</tr>
<tr>
<td>Sar</td>
<td>Sarcosine (N-methyl glycine)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SL</td>
<td>Sphingolipid</td>
</tr>
<tr>
<td>Smr</td>
<td><em>Staphylococcus aureus</em> multidrug resistance protein</td>
</tr>
<tr>
<td>SMR</td>
<td>Small multidrug resistance family</td>
</tr>
<tr>
<td>Tbsmr</td>
<td><em>Mycobacterium tuberculosis</em> small multidrug resistance protein</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TPP</td>
<td>Tetraphenylphosphonium</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin-resistant <em>Enterococci</em></td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Portions of Chapter 1.1 to 1.3 have been published, in part, by Ng, D.P.*, Poulsen, B.E.*, and Deber, C.M., *Biochimica et Biophysica Acta* (2012); * denotes equally contributing authors.

Author Contributions: BEP, DPN, and CMD wrote the BBA review. Parts of the review contributed by BEP and CMD have been included in this chapter.
1.1 Introduction to membrane proteins

Biological membranes, consisting of a bilayer of hydrophobic lipids with polar head groups, protect and surround the aqueous contents of cells and their internal compartmentalized organelles. The membrane is selectively permeable to polar molecules because of its hydrophobic core, which allows the cell to create concentration gradients that can drive cellular processes. Membranes also contain proteins that can be categorized as peripheral (non-covalent or covalent interactions with lipid polar head groups) or integral (transmembrane, TM). These proteins have a variety of functions, from controlling the transport of molecules across the membrane to acting as recognition elements involved in signal transduction and providing structural features maintaining cell adhesion and morphology.

The hydrophobic nature of the membrane and the proteins that it contains has proven challenging in the determination of high-resolution protein structures (White, 2009). At this time, a mere 1.4% of the 73156 protein structures available in the Protein Databank (http://www.rcsb.org/pdb) belong to membrane-associated proteins, of which only 312 are unique structures. Although this number is low, the rate of novel structure determination is growing exponentially which has led to an increasing understanding of membrane protein folding (White, 2009).

1.1.1 The cellular membrane

The notion of a distinct hydrophobic/hydrophilic division by the plasma membrane does not convey the actual complexity of its structure. The ~60 Å membrane consists mainly of phospholipids with a ~30 Å hydrophobic core of lipid acyl chains of varying lengths and a ~15 Å interfacial region on either side containing polar head groups and water molecules (Fig. 1.1). The relative amounts of phospholipids that comprise the membrane vary substantially between species and cell-type in their head group moiety as well as and the lipid chain length and its level of saturation.
Eukaryotes, bacteria, and archaea all use glycerol as the backbone for the majority of their membrane lipids (van Meer and de Kroon, 2011). The shared lipid head groups between bacteria and eukaryotes are phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL), with the latter two located only in mitochondria. Additionally, eukaryotic membranes contain phosphatidylcholine (PC) and phosphatidylinositol (PI) as glycerophospholipids, as well as sphingolipids (SL) and sterols.

In *E. coli*, the relative amounts of the aforementioned lipids are generally reported to be 75% PE, 20% PG, and 5% CL (Goldfine, 1984). Bacterial membrane lipid acyl chain length can vary as a function of growth temperature to allow membrane fluidity in extreme conditions; as temperature increases, acyl chains become longer and less saturated, and *vice versa* (Goldfine, 1984). In higher animals, lipid chains consist predominantly of 16 or 18 carbons with between 0 and 3 *cis*-double bonds. The mammalian plasma membrane consists of 40% PC, 23% PE, 4% PI, 8% PS, and 25% SL (van Meer, Voelker *et al.*, 2008). Furthermore, the presence of cholesterol in eukaryotes modulates the fluidity of the membrane. Interestingly, lipid composition varies in a tissue-specific and organelle-specific manner and likely plays a significant role in protein folding which makes the detailed understanding of membrane protein folding more challenging.
1.1.2 Amino acid composition and secondary structure of membrane proteins

A TM domain does not explicitly contain hydrophobic residues: approximately 20% of residues in the membrane are polar (Stevens and Arkin, 1999). In this case, introducing a polar or charged residue into a hydrophobic environment is likely satisfied either by interactions with another polar residue or the peptide backbone (as is the case in some oligomerization motifs), formation of water- or ion-lined pores, or deformation of the membrane to interact with the aqueous phase (Perrin and Nielson, 1997). To insert such polar residues into the hydrophobic lipid environment, the remaining residues on the TM segment are often correspondingly elevated in hydrophobicity in order to yield an overall threshold hydrophobicity that is permissive for membrane insertion. Although several in vitro hydrophobicity scales for the 20 amino acids have been presented (Kyte and Doolittle, 1982; Degli Esposti, Crimi et al., 1990; Liu, Li et al., 1996), seminal work by von Heijne has led to the identification of the positional insertion requirements using an in vivo translocon assay (Hessa, Kim et al., 2005; Hessa, Meindl-Beinker et al., 2007). The residues that are decidedly hydrophobic and will promote insertion into the membrane when placed in the middle of the helix are (from most to least favourable): Ile, Leu, Phe, Val, Met, and Cys. As a general trend, polar residues are typically located near the ends of the helix and are likely to interact with the polar head groups and water molecules in the interfacial region of the membrane (Fig. 1.2).

Two protein secondary structures are commonly found in the hydrophobic membrane: α-helices and β-sheets. Soluble proteins generally consist of a mélange of secondary structures including α-helices, β-sheets, and random-coiled regions, yet the TM domains of membrane proteins typically form α-helices due to the enthalpic requirements of the hydrophobic environment. An α-helix, unlike a β-sheet or coiled structure, can self-satisfy the H-bonding potential of its own polar peptide backbone. Hydrogen bonds form between the oxygen from the carbonyl (C=O) at amino acid position $i$, and the hydrogen from the amide (N-H) at position $i + 4$. β-sheet structures, albeit rare compared to α-helices, are mainly present in the outer membranes of mitochondria and Gram-negative bacteria, and require several strands to satisfy the hydrogen bonding requirements of the backbone, forming large β-barrel proteins as a result.
Figure 1.2. Distribution of residues in a membrane-embedded α-helix. A representative helix is shown from the single-pass membrane protein GpA (PDB ID 1AFO). The α-helix structure is formed to self-satisfy the hydrogen-bonding potential of the polar backbone, with hydrogen bonds forming between the oxygen from the carbonyl (C=O) at amino acid position $i$ and the hydrogen from the amide (N-H) at position $i + 4$. Although hydrophobic amino acids are found throughout the helix, the majority of residues in the central portion of the helix are solely hydrophobic while the presence of polar and aromatic residues occurs mainly near the termini of the helix. This and subsequent structural figures in this chapter, were produced with PyMOL (Delano Scientific).

1.1.3 Structural features of α-helical and β-barrel proteins

α-helical proteins are the predominant type of membrane protein, as they are found in all membrane types across all species. The principal requirement for an α-helical membrane protein
TM domain is a stretch of ~18-25 amino acids that is energetically satisfied in the hydrophobic lipid bilayer. The α-helices, with the polar backbone shielded from the membrane hydrophobic core, allow the non-polar side chains to project and interact with the hydrophobic lipid acyl chains. The α-helices can traverse the membrane either once, in the case of single pass membrane proteins, or several times, termed multi-spanning. Soluble loop regions range in size from only a few residues to large extra- or intra-cellular soluble domains. The varying number of helices and loops, coupled with the added feature of oligomerization, allows for the tremendous diversity of α-helical membrane proteins. This diversity results in extremely diverse functions.

α-helical membrane proteins include receptors such as bacteriorhodopsin (Fig. 1.3A) and the b2 adrenergic G-protein-coupled receptor (Luecke, Schobert et al., 1999; Rasmussen, Choi et al., 2007); membrane pores such as aquaporin (Murata, Mitsuoka et al., 2000); ion channels such as the CorA Mg\(^{2+}\) transporter (Eshaghi, Niegowski et al., 2006; Payandeh and Pai, 2006); and metabolite transporters such as the lactose transporter LacY (Abramson, Smirnova et al., 2003). Other α-helical membrane proteins are involved in energy accumulation and transduction in the case of the electron transport chain member cytochrome c oxidase (Yoshikawa, Shinzawa-Itoh et al., 1998), as well as proteins responsible for cell adhesion.

The second type of membrane proteins is the β-barrel structures that are found in the outer membranes of bacteria, mitochondria, and chloroplasts (Wimley, 2003). Unlike an α-helix, the β-sheet is not a stand-alone structure that can self-satisfy its polar backbone hydrogen bonding potential in the membrane. β-barrel proteins are closed structures that are typically formed from a single large strand that folds into several antiparallel β-sheets in which the first strand is hydrogen bonded to the last. In this cylindrical barrel conformation, the polar backbone of each β-strands is laterally hydrogen bonded in a circular pattern that creates a stable structure in the membrane (Wimley, 2003). β-barrel structures have short loops on the periplasmic side of the membrane and elaborate loops on the extracellular side (Tamm, Hong et al., 2004), as exemplified by the ferric enterobactin receptor (Fig. 1.3B). This is achieved using an even number of β-strands with alternating tight turns and longer loops producing an asymmetric structure (Wimley, 2003). β-barrel proteins utilize an alternating sequence of hydrophobic and polar residues; this allows the hydrophobic amino acids to face the lipid acyl chains in the membrane while the polar residues can face the aqueous interior of the barrel. The number of residues in each strand ranges from 9-11 to traverse the membrane, while the number of strands per β-barrel ranges widely from 8-22 (Wimley, 2003; Tamm, Hong et al., 2004). Like an α-
helical membrane protein, β-barrel proteins also have the ability to remain monomeric or oligomerize into dimers and trimers.

The relative sizes, as well as the varying complexity in the loop regions of β-barrels, give them the opportunity to undertake numerous functions. For example, some porins have a more ‘hollow’ centre that allows various molecules to diffuse freely, while other β-barrels have selectivity filters in the barrels to allow only specific molecules to pass. In bacteria, β-barrel proteins are classified into five families according to their function: general porins (i.e. OmpC), passive transporters (i.e. FadL), active transporters (i.e. FepA), enzymes (i.e. OmpLA), and structural proteins (i.e. OmpA) (Koebnik, Locher et al., 2000).

![Figure 1.3. Representative α-helical and β-barrel membrane proteins.](image)

**Figure 1.3. Representative α-helical and β-barrel membrane proteins.** Secondary structure of the proteins are coloured as follows: α-helices, red; β-sheets, yellow; random coil, green. **(A)** Bacteriorhodopsin (PDB ID 1C3W) contains seven α-helices that form a bundle with tertiary contacts, and relatively short loop regions; within the loop a small amount of β-sheet is present. α-helical membrane proteins can also contain a single helix with larger intra and/or extracellular regions, and/or form oligomeric quaternary structures via helix-helix interactions. **(B)** β-barrel membrane proteins are typically found in the outer membranes of mitochondria or Gram-negative bacteria, such as the ferric enterobactin receptor FepA (PDB ID 1FEP). The structure is formed from a large β-sheet that coils to form a closed barrel structure where the first strand forms hydrogen bonds with the final strand. The individual strands are typically arranged in an antiparallel formation and they, like α-helices, satisfy the hydrogen-bonding potential of the polar backbone in the membrane. The loop regions are usually larger on the extracellular side of the membrane, and can extend back into the ‘pore’ of the barrel to act as selectivity filters.
1.2 Folding of α-helical membrane proteins

The insertion of a TM α-helix in the membrane by the translocon is the first determinant of a membrane protein, yet the fate of the protein is dependent on the helix stability in this environment: the helix will either remain solvated by lipid as a single-spanning monomeric membrane protein, or the helix will only be partially solvated, driving two or more helices together. Although membrane protein helices are solvated by lipid, acyl chains are not the ideal solvent for hydrophobic side chains that protrude irregularly into the cross section of the lipid bilayer. This relative instability promotes helix-helix interactions that will form at faces that are the least lipid-accessible (Johnson, Rath et al., 2006). These interactions occur largely via van der Waals or polar/electrostatic interactions (Fig 1.4), and involve specific sequence motifs that either fold the protein into its tertiary structure or allow for quaternary oligomerization.

1.2.1 Forces driving membrane protein folding

Of the several forces used by proteins to stabilize their fold, van der Waals interactions play a major role in membrane proteins, especially since the majority of residues are largely hydrophobic, relying on what is often referred to as ‘knobs into holes’ packing wherein two TM helices align with their residues interacting along a helix-helix interface. Hydrophobic side chains along this interface can interact via induced dipoles. Due to rapidly moving electrons, an induced dipole can occur when two molecules come within close contact and the electron density in one molecule becomes redistributed in proximity to the other molecule (also termed London dispersion forces). Several of van der Waals interactions are discussed in Section 1.3 below, with one of the most famous examples being the result of the GG4 motif in the human erythrocyte protein GpA, where a GVxxGV sequence on each helix is the locus of dimer formation due to the close approach of helices allowed by the smaller Gly residues, thereby creating ‘holes’ and the larger Val residues acting as ‘knobs’ (MacKenzie, Prestegard et al., 1997; Senes, Gerstein et al., 2000; Cunningham, Poulsen et al., 2011).

Hydrogen bonding and electrostatic interactions are also very common in membrane proteins. In addition to the obvious H-bonding that occurs within the polar peptide backbone in the formation of α-helices and β-sheets as discussed above, hydrogen bonding also occurs between pairs of polar amino acids within the membrane. Hydrogen bonding requires a H-donor
(such as an amine or hydroxyl) and a H-acceptor (such as the lone pair of electrons from the oxygen on a carbonyl or hydroxyl, or the nitrogen from an imine). Electrostatic interactions in proteins (also called salt bridges) between amino acids also utilize hydrogen bonding but have an added attractive force between two oppositely charged ions, for example between hydrogens on cationic lysine and oxygens on the anionic glutamate.

Other less common forces involve aromatic residues that utilize π-orbitals, which protrude perpendicular to the plane of the ring allowing a delocalized electron density. This delocalized electron density gives a partial negative character at the center of the ring and positive character at the outside, which allow for cation-π interactions between cationic residues and the negative centre of the aromatic ring, or π-π interactions between two aromatic residues in either a parallel stacked or perpendicular interaction with the positive exterior of one ring oriented with the negative interior of the other.
Figure 1.4. Forces stabilizing membrane protein folding. (A) Molecules without permanent dipoles (purple C-H ellipses) can achieve non-covalent bonding (black dashed line) upon the induction of a dipole (red/blue C-H ellipses) by the electron density in each molecule becoming redistributed in proximity to the other molecule. (B) In protein folding, these van der Waals interactions can become induced between hydrophobic residues such as Val and Ala, and (C) occur along the length of the helices in the GpA dimer (red line), with each monomer coloured orange and blue. (D) Hydrogen bonding and electrostatic interactions occur in polar molecules between an H-donor and an H-acceptor such as the lone pair of electrons from oxygen. (E) Hydrogen bonding between the polar Asn residues occurs between the H-donor amines and the H-acceptor oxygen carbonyls, and (F) between hydrogens on the cationic Lys and oxygens on the deprotonated Glu. (G) Aromatic rings such as the benzene moiety in Phe (left) have π-orbitals (red teardrops, centre) protruding perpendicular to the plane of the ring allowing a delocalized electron density (red spheres, right). This gives a partial negative character at the center of the ring (red) and positive character at the outside (blue), which allow for (H) cation-π interactions between cationic Lys and aromatic Trp, or (I) π-π interactions between two aromatic residues such as His and Phe.
1.2.2 The two-stage folding model of α-helical membrane proteins

In 1990 Popot and Engelman outlined a simplified two-stage folding model of α-helical membrane proteins (Popot and Engelman, 1990) (Fig. 1.5). The first stage of this model involves the insertion of hydrophobic segments into the lipid bilayer, which occurs largely as a result of the hydrophobic effect. By this model, the overall hydrophobicity of a potential TM segment is the dictating factor of membrane insertion. In order for a segment to insert, a polypeptide stretch must be of the appropriate length to form an α-helix that spans the bilayer, and it must contain amino acids of sufficient hydrophobicity to be solvated by the lipid alkyl chains. This hydrophobicity threshold value has been determined via in vitro (Liu, Li et al., 1996) and in vivo (Nilsson, Johnson et al., 2003) techniques and is approximately equivalent to a poly-alanine stretch. The length of any given α-helix can vary tremendously between membrane proteins, with a range deduced from high-resolution structures to be 14-39 residues, although 20 residues has been considered optimal to span the membrane bilayer (Bowie, 1997). When α-helices are longer than 20 residues, a helix may tilt in the membrane or acyl chains can become ordered to thicken the bilayer; contrarily, shorter helices can be accommodated by acyl chain disorder allowing the thinning of bilayer thickness (Killian and Nyholm, 2006).

Figure 1.5. The two-stage folding model of α-helical membrane proteins. The first stage of membrane protein folding is the efficient insertion of a hydrophobic segment into the membrane (mediated by the translocon), and the formation of α-helical secondary structure. The second stage is comprised of the specific lateral association of these helices within the bilayer, which are driven by residue motifs on the α-helix surfaces.
The second stage of the α-helical membrane protein-folding model occurs upon helix-helix tertiary and/or quaternary interactions. Tertiary contacts occur within helices from multi-spanning membrane proteins; quaternary contacts occur between helices from separate chains, either from multi- or single-spanning membrane proteins, to form oligomers. These helix-helix contacts are precise: minor changes can cause abrogation in structure and function and have disease-phenotypic implications (Ng, Poulsen et al., 2011). Tertiary and quaternary helix packing is highly organized, occurs via sequence specific motifs (section 1.3), and is driven by protein-protein forces outlined above as well as protein-lipid and lipid-lipid interactions. Several examples of protein-protein interactions are exemplified in Figure 1.4, including van der Waals, hydrogen bonding and electrostatic interactions, and aromatic π interactions. While van der Waals interactions are often stabilized by the creation of a long helix-helix interface, a single polar residue in the middle of two α-helices is sufficient to drive association of the TM segments to satisfy the hydrogen bonding potential of the residue (Zhou, Merianos et al., 2001; Johnson, Heslop et al., 2004; Rath and Deber, 2008). Although the hydrophobic alkyl chains comprising the bilayer core are a more favourable solvent for the hydrophobic α-helices than the aqueous alternative, lipids can also contribute to the folding and association of membrane proteins. Lipid-lipid preferential interactions have been reported to assist in the stability of an α-helical protein bundle, and lipid-facing residues of a helix are optimized for protein-lipid interactions (Johnson, Rath et al., 2006; Cunningham, Poulsen et al., 2011).

1.2.3 Membrane protein insertion is mediated by the translocon

Although the two-stage folding model provides the framework for α-helical membrane protein folding, the insertion of α-helices is not a spontaneous process in vivo. The translocon is itself a membrane-bound α-helical protein and acts as a channel to either funnel a polypeptide through the membrane in the case of secreted proteins, or it traps the polypeptide segment, opening laterally and inserts the α-helix into the membrane bilayer (Fig. 1.6) (Osborne, Rapoport et al., 2005). This unique property allows the translocon to act as a selectivity filter, discerning the fate of a polypeptide in both bacteria (SecYEG) and eukaryotes (Sec61) (Dalbey, Wang et al., 2011). The mechanism used by the translocon to determine the fate of the polypeptides as secreted or membrane-embedded is an active area of research (Cunningham, Rath et al., 2009; Norholm, Cunningham et al., 2011). The proposed model for this mechanism is that while inside
the translocon pore, a direct protein-lipid interaction during an open state of the translocon dictates whether the polypeptide is of sufficient hydrophobicity for membrane-insertion based on the free energy of interaction between the TM segment and the lipid (Hessa, Kim et al., 2005; Hessa, White et al., 2005; Hessa, Meindl-Beinker et al., 2007; Mulvihill and Deber, 2010). The overall hydrophobicity of this segment, coupled with position-dependent polar residues along the α-helix, are the major factors that dictate the fate of the segment, but marginally hydrophobic TM segments and α-helical segments from the interior of globular proteins with high hydrophobicity (termed δ-helices) have also been shown to insert into the membrane. This latter situation demonstrates the complexity of this process, leading to the suggestion that perhaps several helices are held inside the translocon before membrane insertion (Hessa, Meindl-Beinker et al., 2007; Cunningham, Rath et al., 2009; Seppala, Slusky et al., 2010).

![Figure 1.6. Model of translocon-mediated membrane insertion of an α-helix.](image)

A schematic representation of the helix insertion process in vivo is shown in side view. The translocon from *T. thermophilus* SecYE (PDB ID 2ZJS) is coloured in red. An ideal helix (blue) is shown in the water-exposed translocon pore after translation (blue shading, left), as it partitions into the hydrophobic membrane environment upon interacting with lipids (coloured yellow, right). Adapted from (Mulvihill and Deber, 2010).
1.3 Oligomerization motifs of α-helical membrane proteins

Oligomerization of membrane proteins is often required for function. The forces driving two or more detached helices together in the membrane is essentially the same as in tertiary contacts outlined above. Quaternary contacts are slightly easier to decipher experimentally than tertiary contacts, and this experimental work coupled with conservation analysis has led to the identification of several oligomerization motifs, or specific sequences, that have been optimized by evolution to allow for stable helix-helix interactions in the membrane.

1.3.1 GAS\textsubscript{right}/GxxxG motifs

It has been noted that in nature, there is an abundance of Gly residues in TM α-helices that are found in GxxxG sequence motifs (Senes, Gerstein et al., 2000). The right-handed packing of helices is mediated by glycines that are separated along the sequence four residues apart (hence GG4), placing them on the same helix face; in a side view of such a helix, the Gly residues are located essentially one above the other due to the 3.6 residues per turn geometry of an α-helix (Walters and DeGrado, 2006) (Fig. 1.7).

The prototypical dimer, glycophorin A (GpA), is a single-spanning human erythrocyte glycoprotein associated with blood group determination that employs a LIxxGVxxGVxxT dimerization sequence (Lemmon, Flanagan et al., 1992a; Lemmon, Flanagan et al., 1992b; MacKenzie, Prestegard et al., 1997; Fleming and Engelman, 2001). The Gly residues appear as ‘holes’ in the helix surface and dimerization occurs due to ‘knobs-into-holes’ packing, with the adjacent Val ‘knob’ residues supporting the structure through van der Waals interactions. Recent work suggests that GG4 interactions may be more complex than previously thought, with the identities of the ‘knob’ residues playing an important role in the oligomerization strength of this interaction (Cunningham, Poulsen et al., 2011). In the latter study, it was determined that the adjacent Val residues can be replaced with Ile, but not Leu or Ala, in order to maintain dimerization, a result which suggests a requirement for β-branched residues at this location. Other possible interactions in the motif are weak H-bonding between Cα-H groups on one helix and the carbonyls of the opposite helix (Senes, Ubarretxena-Belandia et al., 2001; Arbely and Arkin, 2004). Nevertheless, the specific role(s) of Leu and Ile residues in this GpA local sequence are not fully understood, yet likely form further van der Waals interactions as mutation
of these residues affects dimerization. The remaining Thr forms polar interactions with the backbone of the opposite monomer.

These sequences are also referred to as GAS\textsubscript{right} since they have right-handed crossing angles and other small residues such as Ala and Ser can replace the Gly in the GG4 motif (Walters and DeGrado, 2006). Although only a handful of GG4 motifs have been structurally characterized, these oligomerization sequences likely play a major role in both tertiary and quaternary helix-helix interactions in membrane proteins due to the many possible combinations of Gly, Ala, and Ser that occur four residues apart in TM sequences.

**Figure 1.7. GAS\textsubscript{right} oligomerization motif.** (A) The Glycophorin A dimer (GpA, PDB ID 1AFO) utilizing a LxGxGxGxT motif, with the Gly residues (red) allowing for the close approach of helices. (B) An enhanced view of the GpA GVxxGV motif with the adjacent Val residues acting as ‘knobs’ (Val residues on each monomer coloured green or yellow) interacting with the Gly ‘holes’ (red).
1.3.2 GAS\textsubscript{left}/Heptad motifs

As with the GG4 motif, Gly, Ser, and Ala residues can accommodate the close approach of helices in a typical heptad repeat which leads to left-handed crossing angles (Lear, Stouffer \textit{et al.}, 2004; Walters and DeGrado, 2006). Examples of the GAS\textsubscript{left} motif are not as common as the GAS\textsubscript{right} motif. However, due to the increased tendency of small residues to situate in heptad motifs, it is likely that these motifs are commonly found in nature, especially in tertiary contacts.

The heptad repeat can also contain large residues, as in the case of the Leu-Ile zipper, which drives the oligomerization of the phospholamban pentamer (Arkin, Adams \textit{et al.}, 1994; Simmerman, Kobayashi \textit{et al.}, 1996) (Fig. 1.8). Phospholamban is found in cardiac sarcoplasmic reticulum and is associated with the regulation of calcium pump activity. This membrane protein utilizes an IxxxLxxLxxIxxxLxxIxxxL heptad repeat (typified with letters \textit{abcdefg}), with the Leu residues at the \textit{a} positions and the Ile at the \textit{d} positions, resulting in the large hydrophobic residues lining a single face of a helix while mediating helix-helix contacts via van der Waals packing interactions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{GAS\textsubscript{left}/heptad oligomerization motif. (A) Side view of the phospholamban pentamer (PDB ID 1ZLL) utilizing a IxxxLxxLxxLxxIxxxLxxIxxxL heptad repeat (typified with letters \textit{abcdefg}). (B) An enhanced top view of these interactions with the Leu residues at the \textit{a} positions rendered in green the Ile at the \textit{d} positions in red, resulting in the large hydrophobic residues lining a single face of a helix while mediating helix-helix contacts via van der Waals packing interactions.}
\end{figure}
1.3.3 Polar residue motifs

Polar residues in the membrane often lead to hydrogen bonding between the side chains of two or more helices. These categories of oligomerization sequences are highly variable and can arise from an interaction between a single polar residue on each helix or from interactions between multiple residues on each helix face. An example is the T-cell receptor ζ-chain, which serves as a portion of the T-cell receptor complex that is responsible for recognizing antigens bound to the major histocompatibility complex (Call, Schnell et al., 2006). The T-cell receptor ζ-chain utilizes polar residues in a DxxLxxYxxxLT motif to form an H-bonded dimer in the membrane that includes an Asp-Asp inter-monomer interaction and Tyr-Thr, Thr-Tyr interactions (Call, Schnell et al., 2006) (Fig. 1.9). Other interactions that have been determined to mediate helix-helix interactions in the membrane via polar/electrostatic mechanisms include cation-π and π-π between aromatic and/or basic amino acids (Lear, Gratkowski et al., 2003; Hong, Park et al., 2007; Johnson, Hecht et al., 2007; Bocharov, Mineev et al., 2008).

Figure 1.9. Polar residue oligomerization motif. (A) The T-cell receptor ζ-chain dimer (PDB ID 2HAC) utilizing a DxxLxxYxxxLT motif with Asp rendered in blue, Tyr in red, and Thr in green. (B) An enhanced view of one of the hydrogen bonding pairs, Thr and Tyr. An H-donor from Thr forms a hydrogen bond (black dotted line) with the lone pair of electrons on the oxygen in Tyr. Side chain atoms are coloured: C, green; O, red; and H, white.
1.3.4 Transmembrane peptides targeting membrane protein oligomerization

Misassembled proteins that successfully traffic to the membrane can lead to disease by a loss or gain of function and/or oligomerization. Small molecules that could interact with either the TM domains or the loop regions of such proteins would be ideal in correcting this misassembly. A further possibility would be the use of TM peptide fragments that could interact with the helix of a naturally occurring defective membrane protein. Endogenous TM segments often modulate function of membrane proteins, and peptides that mimic this control could be employed in disease treatment. A study of the Na/K-ATPase sodium pump determined that its enzymatic activity - which is normally regulated by a γ-subunit - was modulated by synthetic peptides mimicking the γ-subunit (Zouzoulas, Therien et al., 2003). Similarly, the calcium channel γ6 subunit inhibits Cav3.1 low voltage-activated calcium current in human embryonic kidney cells and cardiomyocytes using a critical GxxxA motif in TM1. Thus, with an eight-residue peptide where the native sequence contains this GG4 motif, specific inhibition was obtained of the Cav3.1 calcium current. This finding has implications for future use as in a therapeutic context after myocardial infarctions (Chen and Best, 2009).

Inhibiting homo-oligomerization by peptides may also be an area of disease treatment. As proof of principle, this approach has been demonstrated in vitro for the bacterial protein diacylglycerol kinase (DAGK) (Partridge, Melnyk et al., 2003). As a three-TM protein that forms a trimer centered at TM2, DAGK peptide fragments were created with identical sequences of each of the three TM helices. Upon incubation of each peptide with purified full-length DAGK in detergent micelles, the authors observed a visible interaction between the TM2 peptide and DAGK by SDS-PAGE, as well as a significantly reduced level of enzyme activity in DDM in the presence of the TM2 peptide.

Peptide inhibition has been accomplished in vivo using peptides that target the Class II G-protein coupled secretin receptor, which functionally oligomerizes at TM4. It was found that by synthesizing TM4 peptides of an identical sequence to the native GPCR and treating cells expressing the GPCR with such peptides, the function of the GPCR was reduced (Harikumar, Pinon et al., 2007). Furthermore, the single-spanning membrane protein ErbB2 is a tyrosine kinase receptor for the epidermal growth factor (EGF) that via a mutation can be constitutively activated as a dimer leading to cancer. Both synthetic peptides and short vector-encoded fusion peptides with the native TM sequence specifically inhibit the ErbB2 dimerization, thus
abolishing the signaling pathway (Bennasroune, Fickova et al., 2004). Although the examples of peptides targeting TM domains are few, it is a promising area of study if the proper conditions for treatment by largely hydrophobic peptides can be devised.

1.4 Bacterial antibiotic resistance

1.4.1 The evolution of “superbugs”

The 1928 discovery of the first antibiotic penicillin by Sir Alexander Fleming quickly led to the observation of bacterial resistance to the drug by the enzyme penicillinase in 1940, before penicillin even reached clinical use. In turn, scientists developed new potent semi-synthetic penicillin-based molecules, which some bacteria also developed resistance to (Walsh, 2000). This is a classic example of predator versus prey co-evolution, where bacteria have developed resistance mechanisms to fight off the toxicity of these molecules that are given to treat bacterial infections by specifically inhibiting a bacteriological process resulting in bacterial death (see Figure 1.10 for some common antibiotics and their general mechanisms of action). Through evolution, bacteria “superbugs” have developed several mechanisms to combat antibiotics: production of antibiotic altering or degrading enzymes, altering the antibiotic target protein to prevent inhibition, the development of new biochemical pathways to avoid the drug, and finally, the production of membrane-bound drug efflux proteins (Walsh, 2000; Nikaido, 2009).

The aforementioned penicillinase was the first example of an antibiotic degrading or altering enzyme. In this case, penicillinase, a member of the β-lactamase family, hydrolyses the β-lactam ring that is present in several families of antibiotics including the penicillins, cephalosporins, and carbapenems. This β-lactam ring is the key to the efficacy of these antibiotics: it mimics the D-alanyl-D-alanine amino acid precursor during the final step of cell wall synthesis by the enzyme transpeptidase (Walsh, 2000). The β-lactam binds to the active site of transpeptidase, irreversibly inhibiting the protein and preventing cell wall formation, leading to bacterial cell death. As an added resistance mechanism, some transpeptidases have evolved to prevent β-lactam binding by subtly changing the active site while maintaining its functional activity.
Figure 1.10. Examples of some common antibiotic families. Cell wall synthesis is inhibited by (A) penicillins, cephalosporins, and carbapenems utilizing the four-membered $\beta$-lactam ring as exemplified by penicillin G to inactivate cell wall synthesis enzymes; (B) vancomycin, a member of glycopeptide antibiotics, prevents crosslinking of peptidoglycan by binding to the elongating peptide chains. Bacterial DNA replication and transcription is prevented by (C) fluoroquinolones such as ciprofloxacin by inhibiting DNA gyrase or the topoisomerase II enzyme; (D) sulfonamides such as sulfamethoxazole, that inhibit folic acid formation, thus preventing nucleotide synthesis. Several groups of antibiotics target protein synthesis such as: (E) chloramphenicol and (F) the macrolide erythromycin (both target the 50S ribosomal subunit); and (G) tetracycline and (H) the aminoglycoside gentamicin (both target the 30S ribosomal subunit).
1.4.2 The resistance rise and new drug decline

The National Institute of Health estimates that 70% of pathogenic bacteria have developed resistance to at least one of the antibiotics commonly used to treat the bacterial infection. The ability to combat infections with antibiotics is crucial, especially in children, the elderly, and those who are immuno-compromised: of the 1.7 million hospital-acquired infections in the United States per year, ~99,000 cases result in death (Klevens, Edwards et al., 2007). One of the major causes of hospital-acquired infections is by the Gram-positive bacterium *Staphylococcus aureus* (Arias and Murray, 2009).

As outlined above in section 1.4.1, bacterial resistance to antibiotics has been observed since the development of these drugs. Penicillin degradation by the enzyme penicillinase was one of the first mechanisms of antibiotic resistance determined. To combat this degradation, methicillin was developed, a “drug of last resort”, since the β-lactamase group of proteins did not degrade it. However, a methicillin-resistant *Staphylococcus aureus* (MRSA) strain of bacteria developed through evolution and became more prevalent. A new antibiotic, vancomycin, became a subsequent “drug of last resort”, and until 1996 was effective as such until MRSA strains also developed vancomycin resistance. Over the last 30 years MRSA, along with other pathogenic bacteria such as vancomycin-resistant *Enterococci* (VRE) and fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), has increased dramatically (Fig. 1.11).

The rise in antibiotic resistant bacteria coincides with the decline in novel antibiotics available to treat bacterial infections, with only four novel antibiotics approved between 2003-2007 (Fig. 1.11; Spellberg, Guidos et al., 2008). With this in mind, the discovery of new antibiotics will be a crucial factor in the treatment of fatal infections. A multifaceted approach could be taken to achieve this: high-throughput screening of current drug libraries, collection of novel organisms for natural antimicrobial discovery, rational drug design towards a specific target, and using combinational therapy to re-sensitize bacteria to current antibiotics using added compounds.
1.4.3 Combinational antibiotic treatments: the future is here

In the absence of new antimicrobials (Fig. 1.11), a method of overcoming drug-resistant bacteria infections is to treat extreme cases with multiple drugs. This could, however, exacerbate the problem of antibiotic resistance, but in some cases this combinational drug therapy is inevitable. An example of treatment with a combination of antibiotics is tuberculosis, caused by *Mycobacterium tuberculosis*. Due to its slow doubling time, complex cell wall structure and its resistance to various antibiotics, tuberculosis infections are typically treated with four
compounds: isoniazid (fatty acid synthase inhibitor), rifampicin (transcription inhibitor), pyrazinamide (fatty acid synthase inhibitor), and ethambutol (cell wall synthesis inhibitor) (1973; 1976). Despite this multidrug treatment, tuberculosis strains have been identified to be resistant to these drugs, and additionally, extensively resistant strains are resistant to fluoroquinolones and aminoglycosides (Leibert and Rom, 2010).

Another example of combination drug treatments is addition of a β-lactamase inhibitor clavulanic acid with the penicillin family of antibiotics (Fig. 1.12). β-lactamases are able to bind to these antibiotics, permanently break open the β-lactam ring after nucleophilic attack from the hydroxyl of a serine residue, and release the inactive β-lactam (Drawz and Bonomo, 2010). Clavulanic acid, which also contains a β-lactam (although without inherent antibiotic activity), competes for the active site of β-lactamases. In doing so, upon nucleophilic attack by the enzyme at the β-lactam ring, clavulanate is not released from the binding site, effectively causing permanent inhibition of the β-lactamase while being consumed (Drawz and Bonomo, 2010). Other compounds have also been developed as inhibitors for β-lactamases, thereby re-sensitizing antibiotic resistant bacteria to this drug class. These mechanisms of combination therapeutics can be valuable as models to design such treatments for other classes of drugs that no longer are effective on their own.

Figure 1.12. Combinational drug treatment of β-lactam antibiotics. Due to the high prevalence of β-lactamases that degrade antibiotics that inhibit cell wall synthesis such as amoxicillin (left), infections are treated with a combination of the antibiotic and clavulanic acid (right). Clavulanic acid also contains the four-membered β-lactam ring and is recognized by β-lactamases and results in suicide inhibition after a covalent linkage at the active site of the enzyme is formed.
1.4.4 Understanding resistance mechanisms: a key step for rational drug design

High-throughput screens of small molecule libraries against drug-resistant bacteria can be rewarding but remain largely empirical. A more focused approach is to design a small molecule or peptide targeting a specific site. To accomplish this, knowledge of key interaction or enzymatic sites will be required for targeting. The example of β-lactamase inhibition by clavulanic acid outlined above (Section 1.4.3) demonstrates how this can be achieved. Realization was made that the β-lactam ring in the penicillin family was the key to their efficacy and its hydrolysis by bacteria rendered it ineffective; thus, other β-lactam-containing molecules were added with the penicillins as inhibitors (Reading and Cole, 1977; Drawz and Bonomo, 2010). Targeting enzymatic and drug binding sites with inhibitors has clearly yielded positive results, but the adaptive nature of rapidly reproducing bacteria allows them to develop resistance to these techniques as well. Altering drug binding sites is key for bacterial survival against antibiotics. Even with the addition of clavulanic acid to penicillin treatments, bacteria are beginning to overcome the penicillins in a new way: they have altered the β-lactamase binding site to reject clavulanic acid binding (Drawz and Bonomo, 2010). Since β-lactamases represent only one mechanism of bacterial antibiotic resistance, furthering our understanding the other enzymatic drug-evasion processes can subsequently lead to drug design towards high-probability targets.

1.5 Small multidrug resistance proteins

1.5.1 The five families of multidrug efflux proteins

Drug efflux transporters represent an effective method of evading the toxic effects of antibiotics; as the antibiotic enters the bacterial cell, the membrane-embedded transporter quickly removes the molecule before any toxicity to the bacteria is possible. To overcome the diverse structures of antibiotic molecules, each protein is able to efflux multiple drugs, and bacteria often have multiple types of multidrug efflux proteins, thus exacerbating the problem of antibiotic resistance. The multidrug transporters are classified into five families of membrane proteins (Nikaido, 2009): the major facilitator superfamily (MFS), the resistance nodulation cell division
(RND), the ATP-binding cassette superfamily (ABC), the multidrug and cytotoxic extrusion family (MATE), and the small multidrug resistance family (SMR) (Fig. 1.13 and Table 1.1).

**Figure 1.13. The X-ray crystallographic structures of the five bacterial multidrug transporter families.** All drug efflux proteins are located in the inner membrane and remove cytotoxic compounds from the bacterial cell. The monomeric multidrug and cytotoxic extrusion family (MATE) transporter NorM from *Vibrio cholerae* is rainbow coloured to distinguish the 12 TM helices (PDB ID 3MKT). Each six-TM helix monomer in the dimeric ATP-binding cassette superfamily (ABC) transporter Sav1866 from *S. aureus* is coloured red and blue (PDB ID 2ONJ). The monomeric major facilitator superfamily (MF) drug efflux protein EmrD from *E. coli* is rainbow coloured to distinguish the 12 TM helices (PDB ID 2GFP). EmrE, the small multidrug resistance protein (SMR) from *E. coli* forms an antiparallel dimer, with each four-TM helix monomer coloured red and blue (PDB ID 3B5D). Also from *E. coli* is AcrB of the resistance nodulation cell division (RND) that forms a trimer, with each 12-TM helix monomer coloured red, blue, and green (PDB ID 1IWG). By far the largest complex, the RND family is able to efflux drugs from the cytosol to the exterior of the outer membrane through the β-barrelled outer membrane factor (OMF), coloured yellow (TolC, PDB ID 1EK9), which is in complex with the RND family through the hexameric membrane fusion proteins (MFP), rendered grey (AcrA, PDB ID 2F1M). Only two MFP monomers are shown on the sides for clarity. RND proteins have the added ability to remove periplasmic compounds that the other four families have removed from the cytosol.
Table 1.1. The five multidrug efflux proteins and substrates in four pathogenic bacteria.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>MFS</th>
<th>MULTIDRUG EFFLUX FAMILY$^{abcd}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>QAC, FQ, AG, CM, TET</td>
<td>β, QAC, AG, CM, FQ, ML, TET</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>β, QAC, AG, CM, FQ, ML, SF, TET</td>
<td>QAC, AG</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>QAC, FQ</td>
<td>N/A</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>FQ, AG</td>
<td>Isoniazid$^{e}$</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations of multidrug efflux families are as follows: MFS, major facilitator superfamily; RND, resistance nodulation cell division; ABC, ATP-binding cassette superfamily; SMR, small multidrug resistance family; MATE, multidrug and cytotoxic extrusion family.

$^b$ Abbreviations of drug classes are as follows: AG, aminoglycosides; β, beta-lactams; CM, chloramphenicol; FQ, fluoroquinolones; ML, macrolides; QAC, quaternary ammonium compounds; SF, sulfonamides; TET, tetracyclines.

$^c$ The table is left blank where no information is available; N/A signifies “Not Applicable” as the pathogen is not known to harbor that protein family.

$^d$ Sources of information are: (De Rossi, Branzoni et al., 1998; Li, Poole et al., 2003; Nikaido, 2009; da Silva, Von Groll et al., 2011)

$^e$ Isoniazid is a tuberculosis treatment to inhibit the mycolic acid waxy coat production in mycobacterium.

The MFS proteins are subdivided into 17 distinct families throughout prokaryotic and eukaryotic species, two of which are drug efflux proteins with either 12 or 14 TM helices (Pao, Paulsen et al., 1998). They act as H$^+$ antiporters and in bacteria they extrude various antibiotics, both neutral and positively charged (Table 1.1), and are found in several pathogens such as Staphylococcus aureus and Escherichia coli (NorA and MdfA, respectively) (Yin, He et al., 2006; Nikaido, 2009). Each species often has several homologs (S. aureus has the 14-TM QacA and QacB, as well as the 12-TM NorA, NorB, NorC), each extruding its own subset of compounds. For example, EmrD from E. coli (Fig. 1.13) is able to efflux uncouplers, quaternary ammonium compounds (QAC), and SDS (Nikaido, 2009).

The RND efflux pumps are found in Gram-negative bacteria such as P. aeruginosa and E. coli since they interact with outer membrane factor (OMF) β-barrel proteins (Paulsen, Park et al., 1997) through periplasmic ‘adapter’ membrane fusion proteins (MFP) (Dinh, Paulsen et al.,
The benefit of this inner membrane to outer membrane interaction is that the drugs that are removed from the cytosol can be removed directly from the periplasm as well. Furthermore, the RND-MFP-OMF complex can remove drugs located in the periplasm, especially vital to the bacteria, as this is the site of action of β-lactam and other peptidoglycan synthesis antibiotics. The RND family, along with the ABC and SMR families, require oligomerization for function. RNDs function via proton antiport and form trimers, with each monomer consisting of 12 TM segments that have an independent drug/proton binding pocket, using residues Asp407, Asp408 and Lys940 (Murakami, Nakashima et al., 2002; Murakami, Nakashima et al., 2006). The trimeric structure has been proposed to function with each monomer in a distinct state: drug accessible, drug bound, and drug extrusion. Along with the binding pockets in the TM domain, RNDs also have binding pockets in the periplasmic domain that allow for drug molecules to be transferred either from the transmembrane domain (drugs originating in the cytosol), or acquired from the periplasmic space (drugs originating in the periplasm) (Murakami, Nakashima et al., 2006).

ABC transporter drug efflux in bacteria has a more limited role in comparison to eukaryotes, but they are still found in many pathogenic bacteria such as Sav1866 from S. aureus (Dawson and Locher, 2006). The transporters couple the efflux of various drugs to ATP hydrolysis (Table 1.1). The MATE family also has a limited role in bacterial resistance, as only a handful of homologs of this distinct family have been identified. The most characterized is NorM from Vibrio parahaemolyticus, and contains 12 TM helices to aid in the antiport of Na⁺ with fluoroquinolones and QACs (He, Szewczyk et al., 2010; Nikaido, 2009). The SMR family of proteins are by far the smallest multidrug efflux proteins yet they are capable of effluxing mainly large QACs using proton antiport. SMRs are found in all bacterial types, with the homolog EmrE from E. coli being the most characterized among them (Fig. 1.14) (Bay, Rommens et al., 2008).

1.5.2 The small multidrug resistance family

The small multidrug resistance proteins (SMRs) are a family of membrane-bound efflux transporters by which bacteria are able to extrude molecules from the cell and thereby confer resistance to cytotoxic compounds (Nikaido, 2009). SMRs have a high prevalence and ~50% of bacteria sequenced to date have at least one SMR family member with species including the Gram-positive Staphylococcus aureus (Smr), Gram-negative Escherichia coli (EmrE),
mycobacteria such as *M. tuberculosis*, and the archaea *Halobacterium salinarum* (Hsmr) (Bay, Rommens *et al.*, 2008; Schuldiner, 2009). Although the mechanistic details of efflux remain to be clarified, SMRs facilitate the removal of a broad variety of cationic sanitizing agents, dyes, and antibiotics from the bacterial cell through use of the proton motive force (Littlejohn, Paulsen *et al.*, 1992; Paulsen, Littlejohn *et al.*, 1993; Grinius and Goldberg, 1994; Yerushalmi, Lebendiker *et al.*, 1995; Heir, Sundheim *et al.*, 1999; Masaoka, Ueno *et al.*, 2000; Rotem, Sal-

Members of the SMR family are relatively small proteins compared to the other multidrug efflux families (often having the same substrates), and are comprised of ~110 residues of which most are membrane embedded. Consisting of four transmembrane (TM) $\alpha$-helices with short loops connecting these membrane spanning regions (Fig. 1.14), SMR monomers must self-assemble into higher order oligomers for function, likely due to their relatively small size vs. the large substrate sizes that the small proteins must efflux (Tate, Kunji *et al.*, 2001; Soskine, Steiner-Mordoch *et al.*, 2002; Tate, Ubarretxena-Belandia *et al.*, 2003; Ubarretxena-Belandia, Baldwin *et al.*, 2003; Butler, Ubarretxena-Belandia *et al.*, 2004; Elbaz, Steiner-Mordoch *et al.*, 2004; Ubarretxena-Belandia and Tate, 2004). The exact size of a fully assembled SMR structure remains an area of active investigation; however, SMR dimers are thought to represent the minimal drug binding or functional unit (Rotem, Sal-man *et al.*, 2001; Ubarretxena-Belandia and Tate, 2004; Chen, Pornillos *et al.*, 2007; Bay, Rommens *et al.*, 2008; Schuldiner, 2009; Morrison, Dekoster *et al.*, 2011).
Figure 1.4. Sequence, efflux mechanism, and structures of EmrE. (A) EmrE consists of 110 residues forming four TM helices with relatively short loops. The first and last residue, and the estimated helix boundaries are numbered. The TM region mainly consists of hydrophobic residues with a few polar and aromatic amino acids. A single conserved, membrane-embedded Glu14 residue coordinates proton entry and the cationic substrates. (B) SMRs utilize the proton motive force to effectively remove cytotoxic compounds from the cell in a 2H⁺ in to 1 molecule out ratio. (C) Cryo-EM structure of EmrE at 7 Å resolution bound to tetraphenylphosphonium (TPP) demonstrates an asymmetric dimer with each monomer displayed in rainbow colour to show similar helices with TM4 proposed at the top. (D) X-ray structure of EmrE at 4 Å resolution bound to TPP demonstrates an antiparallel dimer with monomers coloured blue or gold. Red spheres highlight residues that had been demonstrated to be required for function at the time of the structure publication in 2007. The model shows TMs 1-3 of each monomer surrounding TPP, coordinating the molecule with Glu14 and various aromatic, hydrophobic, and polar residues. TM4 appears to be removed from the binding pocket. Adapted from (Chen, Pornillos et al., 2007). (B) and (C) were produced using PDB IDs 3B5D and 2I68, respectively.
1.5.3 SMR drug binding and efflux mechanism

SMRs are able to bind and efflux a variety of quaternary ammonium compounds (QACs) (Fig. 1.15) (Korkhov and Tate, 2008). Although these are not the conventional antibiotics given for bacterial infections due to their toxicity, hospital surfaces and surgical instruments are sanitized with benzalkonium, and dequalinium is used in throat antiseptics. Furthermore, the SMR homologs from \textit{P. aeruginosa} and \textit{M. tuberculosis} (Pasmr and Tbsmr, respectively) have been identified to extrude the macrolide erythromycin (De Rossi, Branzoni \textit{et al.}, 1998; Li, Poole \textit{et al.}, 2003). This suggests the SMR family is not far from evolving an even more clinically significant role in antibiotic resistance.

QACs share common features: they have a quaternary ammonium group which carries a positive charge and the groups bonded to this nitrogen atom are often large, hydrophobic, and aromatic. Both the number of positive charges and aromatic rings varies amongst substrates. Although the majority of SMR substrates have a single positive charge, some QACs such as dequalinium (Fig. 1.15) and propidium (not shown) have two quaternary ammonium groups yielding an overall charge of +2. Several SMR substrates, such as the large fluorescent dye ethidium contain four aromatic rings, while the sanitizing agent benzalkonium contains only a single aromatic ring (Fig. 1.15). The other major substrate that is especially used for structural and biochemical characterization is tetraphenylphosphonium that contains a phosphorus atom in place of nitrogen, also yielding a positive charge with four individual phenyl groups. A final difference among substrates is shape: tetraphenylphosphonium employs a tetrahedral orientation of the phenyl groups, while ethidium is mostly a planar molecule with the exception of a single phenyl moiety which can rotate freely around its bond to the larger planar portion of the structure.

Several key residues have been identified for SMR efflux function via mutagenesis experiments, mainly characterized in the homolog EmrE. Namely, a single highly conserved membrane-embedded charged residue on TM1, Glu14, has been identified as the primary functional residue since it can coordinate both the positive character on substrates and protons (Muth and Schuldiner, 2000). Interestingly, mutation of Glu14 to its closest relative Asp renders the protein non-functional (Yerushalmi, Mordoch \textit{et al.}, 2001). Upon further characterization it was determined that the pKa of Glu14 is pH 7.3 while that of the Asp mutant is 5.8 (Soskine, Adam \textit{et al.}, 2004). This difference explains the loss in activity of the Asp mutant at
physiological pH since protonation of Asp would not occur unless the bacteria were growing under acidic conditions. Furthermore, a heterodimer of EmrE containing one monomer with Glu14 and the other with Asp14 maintains function (Rapp, Seppala et al., 2007; Seppala, Slusky et al., 2010). The accepted general mechanism of transport, as determined by measurements of substrate-induced proton release using the substrate TPP (Rotem and Schuldiner, 2004; Soskine, Adam et al., 2004; Schuldiner, 2009; Morrison, Dekoster et al., 2011), is that two protons enter the cytosol for every drug molecule pumped out. A heterodimer of a monomer with Glu14 and another with Gln14 results in efflux activity of only monovalent substrates in a 1:1 H⁺: substrate ratio (Steiner-Mordoch, Soskine et al., 2008).

Although SMR substrates are aromatic and positively charged, there are several differences that require the SMR to be promiscuous in its abilities to bind and efflux each of these varying molecules. Structural studies of EmrE as outlined below, gave the first indication of how a single protein is able to efflux a diverse set of drug compounds. The helices of EmrE were visualized using cryo-EM and were determined to be uniquely re-oriented to coordinate each compound that was bound differently, revealing a certain level of ‘plasticity’ in a binding pocket (Korkhov and Tate, 2008). The ability of helices to orient and bind these molecules uniquely is likely achieved using several residues lining this substrate-binding pocket. This is supported by mutagenesis and biochemical studies, demonstrating several conserved residues on TMs 1-3 are required for the efflux function of various substrates. Some mutations of these residues led to loss of function of some QACs, supporting the idea that individual residues are utilized for a specific substrate (Chen, Pornillos et al., 2007; Korkhov and Tate, 2008).

Furthermore, not all SMR homologues display the same resistance properties: a comparison of EmrE and Hsmr revealed that while both proteins were resistant to the toxic effects induced by ethidium and acriflavin, only EmrE was resistant to methyl viologen (Ninio and Schuldiner, 2003).
1.5.4 Structures of EmrE

EmrE has been characterized structurally by electron cyromicroscopy (cryo-EM) (Tate, Kunji et al., 2001; Tate, Ubarretxena-Belandia et al., 2003; Ubarretxena-Belandia, Baldwin et al., 2003; Korkhov and Tate, 2008), X-ray crystallography (Chen, Pornillos et al., 2007), and most recently nuclear magnetic resonance spectroscopy (NMR) (Morrison, Dekoster et al., 2011) (Fig. 1.14). In all cases, EmrE was found to have an asymmetrical topology, a finding that has resulted in controversy (Schuldiner, 2009). The X-ray structure (Fig. 1.14D) of EmrE bound to the substrate TPP$^+$ at 4 Å resolution indicates an antiparallel homodimer (Chen, Pornillos et al., 2007). Although the resolution was inadequate to identify residue side chains, Se-Met labeling was used to estimate their approximate locations. This model of EmrE shows that TM helices 1...
through 3 of each monomer create a six-helix binding ‘pocket’ within the membrane bilayer that surrounds the substrate and utilize the conserved negatively charged residue Glu14 in TM1 to coordinate the protons and/or cationic portion of substrates (Chen, Pornillos et al., 2007). The remaining residues lining the substrate-binding pocket consist of hydrophobic amino acids that coordinate the hydrophobic substituents of the substrates to be extruded from the cell. This structure has recently been confirmed via solution NMR spectroscopy of EmrE in lipid bicelles (Morrison, Dekoster et al., 2011). The authors were able to demonstrate that asymmetric antiparallel EmrE exchanges between inward- and outward-facing states.

Cryo-EM studies have also aided in the elucidation of the SMR mechanism. In two-dimensional crystals at 7 Å resolution the crystallographic asymmetric unit consists of a SMR tetramer composed of two asymmetric dimers (Tate, Kunji et al., 2001). As previously mentioned, EmrE co-crystallized with ethidium, tetraphenylphosphonium, propidium, and dequalinium all displayed different helix projections (Korkhov and Tate, 2008). With each substrate, EmrE helices (proposed to be TMs 1-3) of each monomer uniquely re-orient themselves to coordinate each compound differently. This was the first structural indication of how a single protein is able to efflux a diverse set of drug compounds. This cryo-EM data also shows that two of the eight helices in the dimer remain rigid in all cases of drug binding. Based on all structural models, these rigid helices appear to be the TM4 helices and are removed from the drug-binding site composed of TMs 1-3. This, coupled with the X-ray structure, demonstrated that TM4 is removed from the substrate-binding site and is at the very least the site of dimerization.

1.5.5 Oligomerization studies of SMRs

The minimum substrate-binding unit of SMRs is decidedly a dimer (Rotem, Sal-man et al., 2001; Ubarretxena-Belandia and Tate, 2004; Chen, Pornillos et al., 2007; Bay, Rommens et al., 2008; Schuldiner, 2009; Morrison, Dekoster et al., 2011). Other than the evidence from structural studies as outlined above, biochemical studies using fluorescence have demonstrated the dimer as a minimal binding unit. As EmrE binds TPP, the fluorescence by tryptophan residues that line the substrate binding pocket becomes quenched to a saturated 2:1 EmrE:TPP ratio (Tate, Ubarretxena-Belandia et al., 2003; Elbaz, Tayer et al., 2005). Studies of EmrE in the detergent dodecyl maltoside (DDM) have also revealed oligomeric properties by chemical cross-
linking (Soskine, Steiner-Mordoch et al., 2002; Soskine, Mark et al., 2006), electrospray ionization mass spectroscopy analysis (Ilag, Ubarretxena-Belandia et al., 2004), and size exclusion chromatography and ultracentrifugation (Butler, Ubarretxena-Belandia et al., 2004).

Furthermore, co-expression of WT and non-functional EmrE monomers results in a lowered resistance phenotype (Yerushalmi, Lebendiker et al., 1996). In an attempt to identify possible GAS$_\text{right}$ and GAS$_\text{left}$ motifs, the Schuldiner group mutated all Gly residues in EmrE to Cys or Pro, and identified a potential GAS$_\text{left}$ motif using the residues Gly90 and Gly97 (Elbaz, Salomon et al., 2008). When mutated at these two positions, EmrE was unable to confer resistance and bind to substrates, and the dimerization propensity on SDS-PAGE was abolished.

Although a dimeric functional unit is apparent, the overall size of SMRs (eight $\alpha$-helices, four per monomer) is still smaller than the 12-36 TM complexes of the other families that efflux the same QAC substrates (Fig. 1.13). Other than the cryo-EM structures that were crystallized as a dimer of dimers (Tate, Kunji et al., 2001; Ubarretxena-Belandia and Tate, 2004), evidence for tetramerization has also been presented (Rath, Melnyk et al., 2006). TM peptides of the SMR homologue, Hsmr, were synthesized based on the appearance of Hsmr oligomers on SDS-PAGE (Ninio and Schuldiner, 2003). TM4 was determined to be a dimer in SDS, and a tetramer in the gentler detergent PFO using a ‘two-faced helix’ (Rath, Melnyk et al., 2006). Whether this tetramer at TM4 is biologically relevant is still unclear, although no other potential higher-order oligomerization motifs have yet to be identified. Furthermore, evidence of six distinct EmrE species up to decamers has been observed via SDS-PAGE (Bay, Budiman et al., 2010), albeit analysis of the precise oligomeric size of membrane proteins is not accurate via this method (Rath, Glibowicka et al., 2009).

### 1.6 Thesis Hypothesis and Outline

It has been accepted that the minimal functional unit of SMRs are dimers with evidence that the locus of oligomerization is at TM4 (Rath, Melnyk et al., 2006; Elbaz, Salomon et al., 2008; Korkhov and Tate, 2008). In an effort to further understand this oligomerization, we used the SMR homologue from *H. salinarum*, Hsmr. Our reasoning for choosing Hsmr versus the more widely-studied EmrE is that Hsmr, but not EmrE, retained its oligomeric properties in SDS
micelles (Ninio and Schuldiner, 2003). This could be envisioned as useful in mutagenesis studies to the full-length Hsmr molecule to monitor variations in SDS oligomerization.

As a logical continuation of the Hsmr peptide studies, we set forth to produce the key mutations found in this work to disrupt dimerization of the TM4 peptide in SDS or tetramerization of the peptides in PFO (Rath, Melnyk et al., 2006). Chapter 2 outlines this characterization of the oligomeric and functional properties of these mutants at TM4 in the full-length Hsmr protein. We made a library of point mutations along the TM4 helix in both evolutionarily conserved and non-conserved locations. The mutants were designed based on size of the side chain: large residues were mutated to Ala and small residues (Ala or Gly) were mutated to larger hydrophobic residues. After assaying these single point mutations for function and dimerization propensities, we noted that six of the twelve mutations, all of which are residues on the evolutionarily conserved helix face, led to a disruption of dimerization and function leading to the elucidation of a $^{90}$GLxLIxxGV$^{98}$ dimer motif (Poulsen, Rath et al., 2009).

At approximately the same time, the G90 and G97 positions were reported to be essential for dimerization and function in EmrE (Elbaz, Salomon et al., 2008). This GAS$_{\text{left}}$ heptad dimerization motif was intriguing since it did not completely fit with the typical heptad: an Ile94 was in place of a small residue. The roles of the four large residues in this six-residue motif were not understood. To address this, we continued in making modifications to the full-length Hsmr at TM4 by mutating each of these large dimerization residues in the $^{90}$GLxLIxxGV$^{98}$ motif to Ile, Leu, Met, Phe, and Val. Chapter 3 presents our findings that modification to residues of equal hydrophobicity and size with variations of shape could result in abolishment of function and altered dimerization propensity and substrate binding. In some cases, dimerization was weakened resulting in reduced binding while in other cases dimerization increased resulting in increased binding; either way, function was diminished. A third group of mutants led to the notion that TM4 may have an added role in SMR function as some mutants displayed WT dimerization levels yet were functionally disruptive (Poulsen, Cunningham et al., 2011).

Both sets of mutants in our oligomerization studies of Hsmr solidified the suggestion that SMRs oligomerize via a functionally dependent $^{90}$GLxLIxxGV$^{98}$ dimer motif that is very sensitive to minor changes. This led to the hypothesis that a synthetic peptide displaying the exact sequence of TM4 Hsmr could conceivably compete with and disrupt the native TM4-TM4 interactions of the full-length protein. In Chapter 4, we show through using a cellular efflux
assay of the fluorescent SMR substrate ethidium bromide, that bacterial cells containing Hsmr are able to remove cellular ethidium via first order exponential decay with a rate constant of \( k = 10.1 \pm 0.7 \times 10^{-3} \text{ s}^{-1} \). Upon treatment of the cells with the synthetic TM4 peptide, we observed a saturable and \( \sim 60\% \) decrease in the efflux rate constant for TM4 to \( 3.7 \pm 0.2 \times 10^{-3} \text{ s}^{-1} \). In corresponding experiments with various control peptides including scrambled sequences and a sequence of D-chirality, a decrease in ethidium efflux was either not observed or was marginal, likely from non-specific effects. Designed peptides did not evoke bacterial lysis, indicating that they act via the \( \alpha \)-helicity and membrane insertion propensities of the native TM4 helix.
Chapter 2: The assembly motif of a bacterial small multidrug resistance protein


Author Contributions: BEP and CMD designed the research. BEP performed the research and analyzed the data. AR performed computational modeling and homology analysis. BEP and CMD wrote the paper with editing assistance from AR.
2.1 Introduction

As outlined in Section 1.5, individual small multidrug resistant proteins must assemble into homo-oligomeric structures in order to transport substrates, a requirement likely related to the relatively small size of the individual four transmembrane segment-SMR molecule (Yerushalmi, Lebendiker et al., 1996; Masaoka, Ueno et al., 2000; Rotem, Sal-man et al., 2001; Kikukawa, Nara et al., 2006; Soskine, Mark et al., 2006). The exact oligomeric size of a fully assembled SMR structure remains an area of active investigation, with evidence for both dimeric (Rotem, Sal-man et al., 2001; Soskine, Steiner-Mordoch et al., 2002; Elbaz, Steiner-Mordoch et al., 2004; Ubarretxena-Belandia and Tate, 2004) and tetrameric (Ninio and Schuldiner, 2003; Tate, Ubarretxena-Belandia et al., 2003; Ubarretxena-Belandia, Baldwin et al., 2003) stoichiometry; however, SMR dimers are thought to represent the minimal functional unit (Schuldiner, 2009). Structural studies of dimers of the Escherichia coli SMR EmrE indicate that substrates bind a pocket formed by TMs 1-3 of two monomers (Fleishman, Harrington et al., 2006; Chen, Pornillos et al., 2007). This pocket is lined with aromatic and hydrophobic residues from TMs 1-3 that interact with substrates, and a conserved Glu residue in TM1 that coordinates protons and positive charges on substrate molecules (Chen, Pornillos et al., 2007).

To date, a role for residues in TM4 in substrate binding and translocation has not been recognized (Fleishman, Harrington et al., 2006), and these segments are paired and separated from the binding site (Chen, Pornillos et al., 2007). This pair of TM4 helices is in close contact along the entire helix length, and remains in approximately identical conformations and positions in both the free and substrate-bound dimer (Fleishman, Harrington et al., 2006; Korkhov and Tate, 2008). The TM4 helix pair has accordingly been proposed to represent a strong inter-monomer association that does not contribute to conformational change during ligand binding but instead stabilizes the dimer interface (Fleishman, Harrington et al., 2006). This potential dependence of SMR function on TM4 self-assembly suggests that inhibition of the self-interaction of this segment may provide a straightforward means of controlling drug efflux.

Although these EmrE structural studies have provided invaluable information regarding the overall geometry of SMR self-interaction, atomic-resolution data capable of identifying the specific side chains and interactions that assemble SMRs are not yet available. Mutagenesis data from our laboratory and others, however, is beginning to pinpoint the residues and interactions that could stabilize TM4 mediated inter-monomer contacts in SMR family members. In a
previous study of peptides that correspond to TM4 of the *Halobacterium salinarum* SMR protein, Hsmr, we noted that TM4 had a propensity to strongly self-interact *in vitro* (Rath, Melnyk *et al.*, 2006). We also found that the oligomerization of Hsmr TM4 peptides was sensitive to residue replacements of Gly 90 and Ile 94 that altered side chain volume. Disruption of dimerization by replacement of the TM4 residues Gly 90 and Gly 97 with Cys or Pro was also noted in the *Escherichia coli* SMR, EmrE (Elbaz, Salomon *et al.*, 2008). The six-residue separation of these Gly residues suggested that they form a GG motif (Senes, Gerstein *et al.*, 2000; Liu, Engelman *et al.*, 2002) that mediates ‘knobs-into-holes’ packing across the TM4-TM4 interface (Rath, Melnyk *et al.*, 2006; Elbaz, Salomon *et al.*, 2008). However, the “knob” residues involved in such an interaction have not yet been identified, although roles for Leu 93 and Ile 94 SMR self-assembly have been suggested (Elbaz, Salomon *et al.*, 2008).

With the goal of cataloguing the entire series of residues that may be required for TM4 mediated SMR oligomerization, in the present work we have constructed a library of twelve mutants that scan the central portion of TM4 segment of Hsmr. This SMR homolog was selected because it is capable of self-assembly in the presence of sodium dodecylsulfate (SDS) (Ninio and Schuldiner, 2003), allowing for rapid screening and quantitation of oligomerization on polyacrylamide gel electrophoresis (PAGE). We find that three key residues – Gly 90, Gly 97, and Val 98 – define an assembly ‘hot spot’ where replacements are highly disruptive to Hsmr-based drug resistance.

## 2.2 Results

### 2.2.1 Design of residue substitutions in Hsmr TM4

An inspection of the Hsmr sequence and comparison of TM4 sequences among family members (Fig. 2.1) was followed by mutagenesis to full-length Hsmr at twelve TM4 positions that were targeted for replacement based on their approximate localization to the central portion of the segment and their degree of conservation (Fig. 2.1). Since the intention was to disrupt any potential helix-helix interactions at TM4, the residue substitutions selected were designed to alter side chain volume: those with larger side chains (Val, Leu, Ile, Asn) were replaced with Ala;
conversely, small side chains (Gly and Ala) were mutated to Val or Met. These replacements were anticipated to disrupt any close-packed or ‘knobs-into-holes’ type interactions in which TM4 may participate.

Figure 2.1. TM4 residue conservation in the SMR protein family. (A) Topology diagram of Hsmr. Each TM segment and the intervening loops are indicated relative to their approximate position in the bilayer (shaded yellow). (B) Residue conservation in the SMR protein family. The TM4 sequences of Hsmr and the SMR family members EmrE (from *E. coli*) and Pasmr (the *Pseudomonas aeruginosa* SMR protein) are shown. Residues boxed in blue are conserved in the SMR family. The characteristics of the two most commonly observed residues at each position [at >60% conservation levels from (Bay, Rommens *et al.*, 2008)] are indicated by the schematic below the sequences. Small residues (Ala, Gly, Ser) are indicated by a small dot; large residues (Ile, Leu, Val) are indicated by a large dot; and polar residues (Asn, His) are indicated by an asterisk. Non-conserved positions are denoted with X, and positions where conservation data were not available are denoted by dashes. Hsmr residues indicated in bold were mutagenized in the present study.
2.2.2 Mutations at conserved sites in Hsmr TM4 compromise protein function

The ability of each TM4 mutant to confer resistance to cytotoxic compounds was evaluated by measuring bacterial growth in the presence of either 200 µM or 500 µM of ethidium bromide (EtBr). Here, cell survival depends on the ability of Hsmr to pump EtBr out of the cell, with the degree of resistance proportional to protein activity (Ninio and Schuldiner, 2003). EtBr resistance in the presence of Hsmr TM4 mutants ranged from 0% to 100% vs. WT at the two EtBr concentrations tested (Fig. 2.2AB). The changes in apparent Hsmr activity observed in the WT and mutant proteins were not expected to arise from altered SMR expression levels, as protein recovery from this *E. coli* strain was found to be consistent among Hsmr variants.

The Hsmr mutants were classified into groups based on resistance phenotype at the two EtBr concentrations tested; cells expressing the fully disruptive G90V, G97V, and V98A mutants could not grow at 200 µM EtBr; those expressing the disruptive mutants L91A, L93A, and I94A could not grow at 500 µM EtBr; those expressing the partially disruptive mutants V100A and L101A had less than 70% of WT activity at 500 µM EtBr; and those expressing the non-disruptive mutants V89A, A92M, V95A, and N102A retained at least 80% of WT activity at 500 µM EtBr (Fig. 2.2AB).

2.2.3 Disruptive TM4 mutants compromise Hsmr oligomerization

To determine whether the activity trends observed could be traced to disrupted helix-helix contacts, we exploited the ability of the Hsmr protein to self-associate in the detergent SDS (Ninio and Schuldiner, 2003), and separated monomeric and oligomeric Hsmr molecules using polyacrylamide gel electrophoresis (PAGE) (Fig. 2.2C). The two partially disruptive (V100A, L101A) and four non-disruptive (V89A, A92M, V95A, N102A) mutants ran as WT (A92M shown as an example). Conversely, each of the three fully disruptive mutants significantly disrupted the self-association of Hsmr, with the G90V- and G97V-substituted molecules dimerizing at ~60% WT levels, and the V98A mutant dimerizing with ~40% WT efficiency. Two of the three disruptive mutants, L91A and L93A, reduced Hsmr dimerization to ~50-60% of the WT value. However, the disruptive I94A replacement had no effect on Hsmr self-association on SDS-PAGE, even though this mutant exhibited reduced resistance to EtBr *in vivo*.
Figure 2.2. Resistance activity and dimerization profile of WT and TM4 mutant Hsmr proteins. The growth of E. coli expressing WT Hsmr or the indicated mutant is shown normalized to WT growth values in the presence of 200 µM (A) and 500 µM (B) ethidium bromide. The growth of untransformed BL21(DE3) cells (-) was used as a negative control. Mutants are arranged from left to right in order of decreasing resistance activity versus WT and were classified according to activity level as described under Section 2.4.2. Error bars represent differences propagated from the standard deviations of at least three experiments. Note that the resistance activities of the highly disruptive mutants at 200 µM EtBr and the highly disruptive and disruptive mutants at 500 µM EtBr were indistinguishable from the activity of untransformed cells. (C) Representative silver-stained SDS-PAGE and quantitation of dimerization efficiency of WT and mutant Hsmr proteins. Each lane contains 100 ng of purified protein. Positions of monomer and dimer are indicated to the left on the gel by single and double dots, respectively. The histogram displays the mean percentage of dimerization relative to WT on SDS-PAGE for each mutant. Error bars were propagated from the standard deviation of three experiments. The significance levels of unpaired t tests comparing the dimerization efficiency of each mutant with WT are: *, p < 0.05; **, p < 0.01. The gel migration patterns and dimerization efficiency of all partially disruptive and non-disruptive mutants did not differ from WT; a single non-disruptive mutant (A92M) is shown here as an example. (D) Helical wheel projection of the Hsmr TM4 sequence. Substituted residues are indicated as follows: red, all disruptive mutants, and green, all non-disruptive mutants. Note that all disruptive mutants localize to the evolutionarily conserved face of the TM helix (blue shaded region) and vice versa for the non-disruptive mutants.

Upon mapping these residue groups onto a projection of TM4 (Fig. 2.2D), it was noted that the fully disruptive, disruptive, and partially disruptive mutants all lie on the evolutionarily conserved face of the TM4 helix. Additionally, the fully disruptive and disruptive mutants
(G90V, L91A, L93A, I94A, G97V, and V98A) localize toward the central portion of the TM4 segment, while the two partially disruptive mutants (V100A, L101A) are positioned towards its C-terminal end. It can therefore be concluded that the minimum sequence on the TM4 helix required for SMR-mediated resistance at high toxicant levels could be represented as G90LxLIxxGV98.

2.2.4 Modeling of Hsmr TM4-TM4 dimers

Since structural and biochemical data suggest a role for TM4-TM4 interactions in SMR assembly, computational modeling of TM4 homodimers was used to visualize how the G90LxLIxxGV98 sequence might mediate Hsmr assembly. The modeling of Hsmr TM4 self-interaction was accomplished using CNS searching of helix interactions (CHI), an algorithm developed to model the self-assembly of the TM domains of glycophorin A (GpA) and phospholamban (Adams, Arkin et al., 1995; Adams, Engelman et al., 1996; Brunger, Adams et al., 1998). Hsmr TM4 dimers were modeled in both parallel and anti-parallel orientations since evidence exists to support each mode of SMR oligomerization [(Steiner-Mordoch, Soskine et al., 2008) and (Ubarretxena-Belandia, Baldwin et al., 2003; Ubarretxena-Belandia and Tate, 2004; Daley, Rapp et al., 2005; Chen, Pornillos et al., 2007; Nara, Kouyama et al., 2007; Seppala, Slusky et al., 2010; Morrison, Dekoster et al., 2011), respectively]. Independent parallel and anti-parallel simulations produced 24 TM4-TM4 association models (Appendix 2). Two of these (Fig. 2.3) were compatible with our present mutagenesis data and would not be expected to aggregate indefinitely (Rath and Deber, 2007).
Figure 2.3. Model of SMR assembly mediated by TM4-TM4 contacts. (A) Antiparallel and (B) parallel TM4 dimers compatible with mutagenesis data. The stationary monomers (A and B, left) combine with their paired monomers (center), which are then rotated 180° outwards from the plane of the figure to form TM4-TM4 dimers (right). Residues where substitutions were highly disruptive, disruptive, or partially disruptive to Hsmr drug resistance activity are colored red, pink, and light pink, respectively. Residues where mutations were non-disruptive and those that were not considered in the present study are rendered in green. (C) Top and side views of the antiparallel Hsmr TM4 dimer model (green helix) superimposed on the gray Cα backbone of the x-ray structure of the EmrE TM4 helix pair (PDB ID 3B5D). In the side view, Gly residues are rendered as red spheres and indicate the proximity of Gly90 to Gly97’ and of Gly97 to Gly90’ in the interface between the two helices. (D) Schematic top view of an SMR assembly model showing dimerization centered at TM4 along the conserved Gly face (red), with potential tetramerization along the non-conserved (green) face. TM helices 1–3 are rendered in gold. The figure was produced using PyMOL (DeLano Scientific).
2.3 Discussion

2.3.1 The G\textsubscript{90}LxLxxGV\textsubscript{98} sequence is crucial for Hsmr drug efflux activity and self-assembly

The six residues found to be individually essential for Hsmr drug resistance activity each localize to the conserved surface of the TM4 helix. These conserved residues, including Gly\textsubscript{90}, Leu\textsubscript{91}, Leu\textsubscript{93}, Ile\textsubscript{94}, Gly\textsubscript{97}, and Val\textsubscript{98}, are unable to confer resistance to high concentrations of ethidium when mutated to oppositely sized residues (Fig. 2.2AB). Mutants at Gly\textsubscript{90} and Gly\textsubscript{97} in the TM4 of the related EmrE SMR were similarly found to be defective in self-association as well as activity, implying that abrogated function arises from disruption of TM4 mediated inter-monomer interactions (Elbaz, Salomon et al., 2008). The SDS-PAGE results presented here also implicate the G\textsubscript{90}LxLxxGV\textsubscript{98} sequence in dimer assembly, with five of six individual mutants that exhibit impaired drug resistance also disrupting Hsmr oligomerization. We therefore conclude that the minimum TM4 sequence required for Hsmr drug resistance activity can be defined as G\textsubscript{90}LxLxxGV\textsubscript{98}. This sequence encompasses the Gly\textsubscript{90} and Gly\textsubscript{97} residues previously identified as important for TM4 mediated SMR self-assembly and function in EmrE (Elbaz, Salomon et al., 2008), and two of the residues key to strong self-association of Hsmr TM4 peptides (Rath, Melnyk et al., 2006).

2.3.2 TM4 mediated inter-monomer interactions are stabilized by close packing between residues

The pattern of large and small residues in the G\textsubscript{90}LxLxxGV\textsubscript{98} sequence of TM4 defined in this work by mutagenesis matches the conservation of residue size along the TM4 helix (Fig. 2.1), suggesting that ‘knobs-into-holes’ packing may stabilize SMR assembly via this segment. Two sequence motifs described in the literature are capable of mediating such interactions: the GG\textsubscript{4} motif (alternately termed GXXXG or GAS\textsubscript{Right}), and the small residue heptad motif (also known as GAS\textsubscript{Left}) [reviewed in (Moore, Berger et al., 2008)]. The former exhibits a characteristic \(i, i+3\) small residue periodicity, and is exemplified by the GpA homodimer, where two ‘small’ Gly residues create a shallow groove into which the ‘large’ Val residues of individual monomers intercalate at the dimer interface (Lemmon, Flanagan et al., 1992b; Smith and Bormann, 1995). Small residue heptad packing motifs, on the other hand, can be defined by
a seven-residue repeat (typically designated by the letters a – g), where the “a” and “d” positions most directly contact the opposing helix and are frequently occupied by small residues (Walters and DeGrado, 2006). Helix pairs packed by the GG₄ vs. heptad repeat motif also differ in their geometry [reviewed in (Moore, Berger et al., 2008)], with the former crossing in a right-handed manner at an angle of ~40°, and the latter in a left-handed fashion at ~20° (Walters and DeGrado, 2006).

Comparison of the patterning of the fully disruptive, disruptive, partially disruptive, and non-disruptive mutations in Hsmr TM4 to that of the interfacial residues of the GpA dimer (GG₄) or to that of sequences assembled via small residue heptad motifs (Fig. 2.4) suggests that the periodicity of the G₉₀LxLxIxGV₉₈ sequence required for Hsmr self assembly and/or function more closely approximates the latter, with the two key Gly90 and Gly97 residues occupying the “a” positions. The positioning of the Leu91, Leu93 and Val98 residues is also consistent with heptad repeat packing; analogous positions adjacent to small residues are occupied by large side chains and/or are in close contact in a helix pair packed via a small residue heptad repeat (Fig. 2.4). Placement of the Ile94 residue at position “d” in the TM4 heptad repeat, however, does not match the expected small residue pattern (Fig. 2.4), though we note that the functionally disruptive I94A mutation uniquely maintains dimerization relatively equal to WT (Fig. 2.2C). The large-to-small substitution at Ile 94 may therefore disrupt function by altering, rather than preventing, residue intercalation between Hsmr monomers.

Identification of the G₉₀LxLxIxGV₉₈ sequence defined in this work as a small residue heptad repeat is consistent with the left-handed geometry of antiparallel TM4-TM4 association observed in the EmrE crystal structure (Chen, Pornillos et al., 2007). Intercalation of the key small and large residues of the G₉₀LxLxIxGV₉₈ sequence additionally occurs in each of our parallel and anti-parallel orientations modeled in silico, and each is packed in a left-handed manner (Fig. 2.3). The geometry of the anti-parallel model, however, appears to more closely approximate the estimates of residue location along the TM segments provided by selenomethionine labeling of EmrE (Chen, Pornillos et al., 2007). For example, the crystallized anti-parallel EmrE dimer has its two Leu93 residues located adjacent to one another on the boundary between the dimer interface and the surrounding lipid; similar to the Leu93 positioning of the anti-parallel model of Hsmr TM4 presented in this work (Fig. 2.3A). This is inconsistent with our parallel model, where the Leu93 residues localize to opposite sides of the dimer (Fig. 2.3B). Superimposition of the anti-parallel Hsmr TM4 model with the X-ray structure of the
anti-parallel EmrE dimer indicates that the TM4 helices are appropriately oriented for
dimerization to occur at the G_{90}LxLxxGV_{98} sequence (Fig. 2.3C). For example, the two Gly
residues face one another across the interface between the TM4 helix pair. The Hsmr TM4
model and the EmrE structure show some deviation at the helix ends, potentially due to truncated
helix ends in EmrE and/or sequence differences such as the presence of Pro86 in EmrE.

<table>
<thead>
<tr>
<th>GG4: GpA</th>
<th>Hsmr TM4</th>
<th>Heptad: PSI B134-155</th>
<th>PSI B42-64</th>
<th>MS1-Gly</th>
<th>MHC α</th>
<th>MHC β</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITLIFI</td>
<td>VAGVG</td>
<td>H</td>
<td>YKIF</td>
<td>BQTLIL</td>
<td>KLSG</td>
<td></td>
</tr>
<tr>
<td>VMAGV</td>
<td></td>
<td>G</td>
<td>ASHE</td>
<td>LLGL</td>
<td>IGCV</td>
<td></td>
</tr>
<tr>
<td>IGL</td>
<td></td>
<td>L</td>
<td>HLAL</td>
<td>GGLL</td>
<td>CFLG</td>
<td></td>
</tr>
<tr>
<td>ILYG</td>
<td></td>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIYGI</td>
<td></td>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRL</td>
<td></td>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.4. Sequence alignment of Hsmr TM4 and TM segments that associate via GG4 or small
residue heptad repeats. The two small residues critical for GpA self-association (Lemmon, Flanagan et
al., 1992b) are indicated in bold. Small residues at positions a and d are indicated in bold for heptad
repeats in the Hsmr TM4 sequence (this work); an example of the GAS_{left} motif [chain B residues
134-155 and 42-64 of cyanobacterial PSI (Walters and DeGrado, 2006)]; the class II major histocompatibility
complex (MHC) α- and β-chains (Cosson and Bonifacino, 1992); and the de novo designed peptide MS1-
Gly with B = β-alanine (Lear, Stouffer et al., 2004). Residues in contact at the GpA dimer interface
(Lemmon, Flanagan et al., 1992b), in contact between the PSI helix pair [(Walters and DeGrado, 2006)
and this work], and key to Hsmr function (this work) are underlined. The mean propensity of small
residues (Gly, Ala, Ser) to occur at the indicated positions in the heptad GAS_{left} packing motif (Walters
and DeGrado, 2006) was aligned to the small residue periodicity of Hsmr TM4, where white (no shading)
indicates a propensity of <1; light gray indicates ≥1 to <1.25; dark gray indicates ≥1.25 to <1.5; and black
indicates ≥1.5. Note that the patterning of key Gly residues in the Hsmr TM4 sequence corresponds to
that of other small residue heptad repeat motifs and to the propensity values, whereas the patterns of large
residues required for function are similar to those in contact across the PSI helix pair. All sequences are
presented in an N- to C-terminal direction, with the exception of the PSI B134-155 sequence, which is
aligned in a C- to N-orientation.

The mutational analysis presented here does not specifically implicate oligomeric states
higher than dimer in Hsmr function; however, the TM4 surfaces exposed in each of our model
dimers are compatible with additional self-interaction. The potential of SMRs to form tetramers
via “two-faced” TM4-TM4 interactions has also been suggested to utilize some of the Ala and
Val residues on the non-conserved face of TM4 (Rath, Melnyk et al., 2006), and monomer
pairings that involve Ala92 and Val95 in helix-helix packing are observed in our parallel
computational models (Appendix 2). We note that a dimer-tetramer model of SMR assembly
mediated solely by TM4 could satisfy both parallel and anti-parallel assembly constraints, with anti-parallel contacts mediating dimerization at one surface and parallel at the other (Fig. 3.3D).

While our results cannot unequivocally exclude the possibility that the interactions mediated by the G<sub>90</sub>LxLxxGV<sub>98</sub> small residue heptad repeat motif on TM4 involve an Hsmr helix other than TM4, many lines of evidence support the role for TM4 self-interaction in SMR assembly. TM4 remains in a relatively constant conformation during SMR binding by a variety of substrates, acting as an anchor for the oligomer (Korkhov and Tate, 2008), and peptides corresponding to the Hsmr TM4 sequence self-associate strongly in a manner dependent on Gly90 and Ile94 (Rath, Melnyk et al., 2006).

2.3.3 Implications for SMR inhibitor design

Given the broad specificity and relatively inefficient binding of toxin substrates by small multidrug resistance proteins, strategies to inhibit their action that involve small molecule inhibitors targeted at the substrate binding pocket are unlikely to be consistently successful. The requirement of oligomerization for function in the SMR family, however, provides an additional opportunity for the design of inhibitors that disrupt this resistance pathway. Key to creation of such an inhibitor is knowledge of the SMR residues that must be targeted in order to reduce self-association. The SMR resistance/assembly motif defined in this work as the TM4 residues G<sub>90</sub>LxLxxGV<sub>98</sub> thus provides the starting point for the design of peptide or small molecules that inhibit SMR oligomerization through competition with critical TM4-TM4 interactions.

2.4 Materials and Methods

2.4.1 Production of wild-type and mutant Hsmr proteins

S. Schuldiner kindly provided the pT7-7 Hsmr-6His plasmid. Hsmr were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Plasmids were transformed into E. coli BL21(DE3) cells and grown at 37°C to A<sub>600</sub> ~ 0.7. Protein expression was induced by
adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to 0.5 mM while shaking for 1 hour. Cells were then pelleted by centrifugation and lysed with 4 mL/gram cells of 2% SDS, 6 M urea, 10 mM Tris, 10 mM imidazole, pH 8 (lysis buffer). Cell debris was pelleted by centrifugation at 10,000 x g and the supernatant was incubated with Ni-NTA beads for 1 hour. The beads were washed three times with lysis buffer and Hsmr was eluted with lysis buffer containing 400 mM imidazole. Eluted Hsmr was dialyzed against 0.3% sodium dodecylsulfate (SDS), 50 mM sodium phosphate buffer, pH 7. Purified Hsmr molecules were extracted from detergent for mass spectrometric analysis by incubation of a 10 μM dialyzed Hsmr protein solution with an equal volume of hexane in the presence of an appropriate amount of Bio-Beads SM-2 Adsorbent (as specified by Biorad) for 1 hour. The aqueous layer was collected and protein molecular weight (MW) confirmed by MALDI-MS analysis. Final protein concentrations were determined using the Micro BCA Protein Assay (Pierce) using manufacturer-supplied BSA as a standard.

2.4.2 Ethidium resistance assays

The ability of wild-type and mutant Hsmr proteins to confer resistance to the cytotoxic compound ethidium bromide (EtBr) was performed essentially as described (Ninio and Schuldiner, 2003). *E. coli* BL21(DE3) cells harbouring the Hsmr plasmid were grown on LB plates in the presence of 100 μg/mL ampicillin. Individual colonies were picked and added to 2xYT broth (w/v: 1.6% tryptone, 1% yeast extract, and 0.5% NaCl) containing 100 μg/mL ampicillin, 0.5 mM IPTG, and 0, 200, or 500 μM EtBr. Cell growth was assayed by OD₆₀₀ after 16 hours of growth with shaking at 37°C. Assays were performed in triplicate. Mutants with <20% of WT activity at 200 μM EtBr were classified as highly disruptive; those with <20% of WT activity at 500 μM EtBr disruptive; those with >50% but <70% of WT activity at 500 μM EtBr partially disruptive; and with >70% of WT activity non-disruptive.

2.4.3 Gel electrophoresis and densitometry

100 ng of purified Hsmr proteins were analyzed by SDS-PAGE on 4-12% NuPAGE gels (Invitrogen) in MES buffer according to the manufacturer’s directions. Unstained Mark-12 protein standards (Invitrogen) were used for MW estimation. Proteins were visualized by silver stain using SilverXpress Staining Kit (Invitrogen), and densitometry measurements performed
using ImageJ (NIH). Percent dimer was estimated by dividing the density of the dimer band by the total density of the dimer and monomer bands.

2.4.4 Modeling of Hsmr TM4 dimers

Potential dimerization sites for the TM4 sequence were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS) as described elsewhere (Adams, Arkin et al., 1995; Adams, Engelman et al., 1996; Brunger, Adams et al., 1998). Briefly, two identical α-helices were generated from the primary sequence of Hsmr TM4 (residues 85-105, inclusive) and their potential interactions identified via global computational searching. TM4-TM4 helix interactions were independently searched in parallel and anti-parallel orientations. All simulations were conducted in vacuo, a partial mimetic of the low dielectric of the membrane. Residues mediating the close approach of TM4 helices in each of the CHI-generated TM4 dimers as well as those in contact between chain B residues 42-64 and 134-155 of the cyanobacterial photosystem I (PSI; PDBID 1JB0) were determined by contact analysis in CNS; structures were queried for side chain and Cα atoms on each monomer found within 4.5 Å of side chain and/or Cα atoms on the other monomer. The 4.5 Å cutoff used represents ~one-half the center-to-center distance of the pair of EmrE TM segments that could represent TM4 (Korkhov and Tate, 2008), as well as ~one-half the average distance between helices packed by small residue heptad repeats (Walters and DeGrado, 2006). Cα atoms were included in residue contact calculations in order for Gly residues to be considered. CNS was also used to determine inter-monomer contact areas in the TM4 dimer models using a probe size of 1.88 Å. The contacting residue pairs in each model were compared to our present mutagenesis data and that of previous studies (Rath, Melnyk et al., 2006; Elbaz, Salomon et al., 2008). Dimer models were excluded from consideration if they did not include all of the residues identified as fully disruptive or disruptive at the TM4-TM4 interface, or if the interface encompassed one or more of the residues identified as non-disruptive in our present mutagenesis study.
Chapter 3: Modulation of substrate efflux in bacterial small multidrug resistance proteins by mutations at the dimer interface


Author Contributions: BEP and CMD designed the research. BEP performed the research and analyzed the data. FC performed homology analysis. KKYL assisted with mutagenesis. BEP and CMD wrote the paper with editing assistance from FC.
3.1 Introduction

The small multidrug resistance proteins consist of ~110 residues that are mostly membrane embedded as four TM α-helices, with short loops connecting these membrane-spanning regions (Fig. 3.1). SMR monomers must self-assemble into higher order oligomers for efflux function (Schuldiner, 2009), and have been shown to be functional while having a dual topology in the membrane (Seppala, Slusky et al., 2010). The most studied SMR family member, EmrE, has been crystallized as an asymmetrical homo-dimer bound to the substrate tetraphenylphosphonium (TPP⁺) (Chen, Pornillos et al., 2007). The model of EmrE shows that TM helices 1 through 3 of each monomer create a binding ‘pocket’ within the membrane bilayer that surrounds the substrate. A conserved charged residue in TM1, Glu14, coordinates the cationic character of SMR substrates and also facilitates proton entry into the cell upon efflux (Schuldiner, 2009). The remaining residues lining the substrate-binding pocket consist of hydrophobic amino acids that coordinate the hydrophobic substituents of the substrates to be extruded from the cell. Two-dimensional crystals of EmrE bound to a variety of substrates reveal that TMs 1-3 of each monomer are able to uniquely re-orient themselves to coordinate a broad variety of ligand structures (Korkhov and Tate, 2008).

The apparent stationary status of TM4 in SMR structural studies led to the notion that a TM4-TM4 interaction accounts for primary dimer formation, although other higher oligomerization contacts in SMRs are likely (Bay, Budiman et al., 2010). While the properties of TM4 have been relatively under-evaluated in comparison to the TM helices involved in the binding pocket, replacement of all Gly residues in EmrE with Cys or Pro identified Gly-90 and Gly-97 within TM4 to be required for activity and dimer formation (Elbaz, Salomon et al., 2008). In Chapter 2, we investigated the properties of TM4 in the related archaeabacterial Hsmr protein, which displays a unique ability to maintain its oligomeric properties in the commonly denaturing detergent sodium dodecyl sulfate (SDS) (Ninio and Schuldiner, 2003). This work identified a TM4-TM4 dimerization interface within a highly conserved ⁹⁰GLxLxxGV⁹⁸ segment (Fig. 3.1) (Poulsen, Rath et al., 2009), and identified the accompanying four large residues (L, L, I, V) as required to effectively mediate drug resistance and SMR self-association (Poulsen, Rath et al., 2009). However, the specific role(s) of the large residues within this GG7 motif are not fully understood; for example, they may be optimized as ‘knob’ residues to allow for favourable packing into the Gly ‘holes’ along the dimer interface. To approach this situation...
in a systematic manner, in the present work large residue replacements at the Hsmr TM4 interface are studied for their ability to efflux the cytotoxic compound ethidium bromide, and compared in the context of their relative abilities to form dimers and bind substrate. The overall results suggest a more dynamic role for TM4 beyond its participation in stabilizing the Hsmr dimer.

Figure 3.1. Sequence and TM4 dimer model of the SMR homologue Hsmr. (A) Topology diagram of Hsmr from the SMR homologue from *Halobacterium salinarum*. Each TM segment and adjoining loop region is indicated relative to their approximate anticipated positions in the bilayer (shaded beige). The amino acids are labeled in white circles, while the residues comprising the GG7 interface required for a TM4-TM4 interaction with another monomer are labeled in green circles. (B) CHI model of Hsmr TM4 monomer (left) and anti-parallel dimer (right) with Gly heptad interfacial residues labeled in green. Each monomer comprising the dimer is differentiated by grey (Leu93) or black (Leu93’).
3.2 Results

3.2.1 Large residue homology in the Hsmr GL$_{91}$xL$_{93}$I$_{94}$xxGV$_{98}$ dimer motif

A sequence comparison of the Hsmr TM4 dimer motif among SMR family members reveals that the Leu93, Ile94, and Val98 positions within the motif contain the residue in the dimerization sequence that is present in greater than 90% of occurrences throughout the SMR family (Table 3.1). Leu91 is the only residue in the dimer motif that is not strictly conserved, as there is a distribution amongst Leu, Ile, and Met of 42%, 32% and 25%, respectively. Since Hsmr naturally contains the residue representing the predominant side chain at each of these four positions, it is an ideal candidate for investigating the roles of the large residues in this dimer motif. Accordingly, Hsmr mutants were produced via site-directed mutagenesis at each of the four sites to include the large hydrophobic residues Phe, Ile, Leu, Met, Val, as well as the smaller Ala that had been previously studied (Poulsen, Rath et al., 2009) (see Section 3.4).

Table 3.1. Occurrence of large hydrophobic residues at positions 91, 93, 94, and 98 in TM4 within the small multidrug resistance protein family.

<table>
<thead>
<tr>
<th>Residue and Position$^a$</th>
<th>Occurrence (%) of the following large residue at the indicated position:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ile</td>
</tr>
<tr>
<td>Leu 91</td>
<td>32</td>
</tr>
<tr>
<td>Leu 93</td>
<td>0</td>
</tr>
<tr>
<td>Ile 94</td>
<td>95</td>
</tr>
<tr>
<td>Val 98</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$ Residue and position numbering according to the small multidrug resistance protein from *Halobacterium salinarum*.

3.2.2 Large residue mutations can alter the strength of Hsmr oligomerization

Dimerization at TM4 of purified WT and mutant Hsmr proteins in SDS micelles was measured using PAGE (Fig. 3.2) (Elbaz, Salomon et al., 2008; Poulsen, Rath et al., 2009).
Dimerization levels of WT and mutant Hsmr were measured by densitometry of the silver stained protein bands and dimer levels ranged between 34.6 – 50.2 +/- 0.4% between mutants (WT = 44.9 +/- 0.2%). The percent change in dimer formation of each mutant is displayed relative to WT (Fig. 3.2). Statistical analysis reveals that all mutations to Ala resulted in decreased dimerization relative to WT, with the L93A mutant yielding the largest decrease. Dimerization differs positionally among large-residue substitution mutants and WT: positions 91 and 93 yield lower dimer levels (except L91F and L93M), mutations at the 94 position results in no change in dimer formation, and the 98 position leads to higher dimerization levels than WT (with the exceptions of I94A and V98A). The changes in dimerization relative to WT are not extreme, suggesting the possibility that the Hsmr dimer may be stabilized by an additional interaction at another TM or in the loop regions.

Figure 3.2. Dimerization analysis of Hsmr TM4 mutants. Representative silver-stained SDS-PAGE and quantitation of 50 ng of purified Hsmr and mutant full-length proteins. The intact and disrupted TM4-TM4 interactions are indicated to the left of the gel by double and single circles, respectively (see Results for details). The black vertical line represents the division between two individual gels run and stained simultaneously. Dimerization levels were determined for each lane (see Section 3.4.3) and the histogram below the gel represents the percent difference in dimer levels between each mutant and WT Hsmr. Mutants are color-coded as: mutants at Leu91, red; Leu93, blue; Ile94, green; Val98, yellow. Error bars represent the propagated SEM of four experiments.
3.2.3 Several large residue substitutions in Hsmr TM4 alter protein function

To investigate whether the property of Hsmr TM4 mutants to form oligomers of varying stability is related to protein function, we measured the ability of each TM4 large residue mutant to confer resistance to a cytotoxic compound. Resistance activity was determined by measuring bacterial cell growth in the presence of 100 µg/ml ethidium bromide on solid agar media (Fig. 3.3A). Cell growth in this toxic environment is proportional to protein activity, as Hsmr is actively effluxing ethidium out of the bacteria (Ninio and Schuldiner, 2003; Seppala, Slusky et al., 2010). Data were normalized relative to the WT activity using equation 3.1, (Fig. 3.3BC) and TM4 mutants are subdivided based on activity levels from disruptive (< 33% of WT), partially disruptive (33% to 67% of WT) to functional (> 67% of WT). As previously reported, all large residue substitutions to Ala are functionally disruptive (Poulsen, Rath et al., 2009). Large residue mutations in conserved positions of the TM4 dimerization interface have varying effects on activity, with seven maintaining the efflux function of Hsmr (L91F, L91I, L91M, L93M, I94L, I94M, and V98I) and three becoming partially disruptive (L93F, L93I, and I94V). Leu to Val mutations at both the 91 and 93 positions, as well as I94F are the only disruptive substitutions at these three positions, whereas all mutations at the 98 position are disruptive (V98F, V98L, V98M), with the exception of V98I.
Figure 3.3. Resistance activity of WT and TM4 mutant Hsmr proteins. (A) Growth of *E. coli* cells on LB agar supplemented with 100 µg/ml ethidium bromide. Serially diluted overnight cultures (5 µl) were plated at each dilution factor (log$_{10}$) with the mutants grouped by each position along the dimerization interface alphabetically (mutants at Leu91, red; Leu93, blue; Ile94, green; Val98, yellow). *E. coli* cells without the Hsmr vector (-) were used as a negative control. (B) Cell growth density determined from the data in (A) are plotted against dilution factor. Shown are WT, I94L, and (-) with fitted lines extrapolated to the x-axis. (C) Ethidium resistance is plotted for each mutant relative to WT upon integration of the fitted line in (B) as described Section 3.4.4. At each position, large residue substitution to Ala results in loss of resistance activity. Error bars represent the propagated SEM of at least three experiments.
3.2.4 Oligomerization is correlated to ethidium binding

To determine if the strength of dimer strength was correlated to the substrate binding ability of Hsmr, ethidium bromide fluorescence was measured in DDM detergent micelles, an environment that allows the protein to be properly folded and bind substrate (Ninio and Schuldiner, 2003; Miller, Charalambous et al., 2009). The SMR substrate ethidium has a natural fluorescence that is readily quenched by surrounding water molecules, and thus the incorporation of ethidium into the hydrophobic substrate binding pocket of Hsmr yields a detectable fluorescence increase analogous to the effect seen in the ethidium-DNA interaction (Fig. 3.4A) (LePecq and Paoletti, 1967). The binding pocket is lined with the Phe, Trp, and Tyr residues that are able to coordinate the aromatic nature of ethidium, leading to a fluorescence increase which can be reversed upon the addition of an excess of an alternate substrate, TPP⁺ (Fig. 3.4A). The percent change in the fluorescence increase (as calculated using equation 3.2), reveals large differences ranging between -75% (L93V) and 60% (V98F) when compared to WT binding to ethidium (Fig. 3.4B). These changes in ethidium fluorescence plotted against the change in SDS-PAGE dimerization levels relative to WT yield a significant linear correlation (p < 0.01) with a Pearson coefficient of r = 0.61, which is improved (r = 0.88, p < 0.001) upon removal of the L93I and L93F outliers (Fig. 3.4C).
Figure 3.4. Ethidium binding propensities of Hsmr TM4 mutants. (A) Ethidium fluorescence emission spectra of 2 µM ethidium bromide (red), + 2 µM WT Hsmr (black), + 10 µM TPP⁺ (blue), in 0.08% DDM buffer upon excitation at 485 nm. (B) Percent fluorescence increase at 600 nm of each mutant shown as a histogram relative to WT. (C) Dimerization vs. ethidium binding data (Fig. 3.2 and Fig. 3.4B, respectively) relative to WT. Correlation analysis performed using Prism reveals a significant correlation (p < 0.01) and a Pearson coefficient of r = 0.61. Upon removal of the L93I and L93F outliers denoted by *, the correlation increases (r = 0.88, p < 0.001). Mutants are colour-coded as: Leu91, red; Leu93, blue; Ile94, green; Val98, yellow. Error bars represent the propagated SEM of at least three experiments.
3.3 Discussion

3.3.1 Minor side chain modifications modulate Hsmr efflux activity

Evaluation of the residues involved in helix-helix interactions in the Glycophorin A homodimer earlier suggested that the strength of dimerization can be regulated by the identities of large hydrophobic neighbouring residues in the small residue motif (Cunningham, Poulsen et al., 2011). Here we have extended this finding to systematic evaluation of the large residues in the conceptually analogous GL91xL93Ix4xGV98 motif in SMR proteins. In addition, while dimerization strength is anticipated to be a key variable in SMR function, no studies had yet been performed that correlate this strength with protein efflux activity. Focusing on conserved positions 91, 93, 94, and 98, in the present work we substituted the naturally occurring Hsmr residue for Ala, Phe, Ile, Leu, Met, and Val at each position, and determined both dimerization and efflux activity profiles.

Alanine (i.e., ‘small’ residue) replacements at interfacial positions along the SMR TM4-TM4 dimer interface had been shown to diminish ethidium resistance with the major reduction in side chain size resulting in the apparent inability of TM segments to properly pack and form the required functional TM4-TM4 dimer (Poulsen, Rath et al., 2009). The research presented here extends this initial analysis, and indicates that size and hydrophobicity are not the only determinants of proper dimer packing by van der Waals interactions: of the 16 novel ‘large’ residue substitutions generated in the current study, only seven were able to retain a functional activity status of at least 67% relative to WT.

The mutant activity profile at each position of the dimer interface suggests that the various interfacial residues do not contribute equally to ethidium efflux. The least conserved Leu91 residue is the most tolerant to substitution (Table 3.1), with only L91V leading to efflux disruption. Conversely, the Val98 position is the most vulnerable to mutation, with all but V98I leading to abolishment of function. It seems evident from experimental and statistical analysis that a structurally rigid β-branched residue at position V98 may be required for efflux, as either Val or Ile is present in 98% of SMR homologues (Table 3.1). This is similar to a recent finding in the dimeric membrane protein Glycophorin A that requires a large β-branched residue in the dimer interface to allow for proper packing/formation of quaternary structure (Cunningham,
Poulsen et al., 2011). At the L93 position, only a Met substitution retains full function of the protein, and at all positions in the TM4 interface, substitution to the smaller Val residue results in a decreased activity profile. It is remarkable that in a 112 amino acid protein, a single substitution of the isomeric Leu to Ile, or the removal of a single methyl group from Ile to Val, results in a significant disruption of activity. As discussed further below, the observed activity profile of Hsmr mutants highlights a complex balance between side-chain requirements in interfacial positions and protein function.

3.3.2 Dimer strength is correlated to substrate binding

Purified Hsmr was characterized for the ability to partially retain its oligomeric properties on SDS-PAGE gels. We determined that not only did some of our novel mutations result in even larger disruptions in dimer formation than previously observed with Ala substitutions, but for the first time we found mutations that increased dimerization strength (Fig. 3.2) (Poulsen, Rath et al., 2009). Using fluorescence as a measure of the ability of folded Hsmr mutants in DDM to bind ethidium (Miller, Charalambous et al., 2009), we found a significant linear dimer strength to substrate binding relationship (Fig. 3.4C); thus, as the strength of the TM4-TM4 interaction increases, the strength of ethidium binding also increases significantly (p < 0.01, r = 0.61). The relationship increases in significance to r = 0.88, p < 0.001 upon removal of two outliers, L93I and L93F, which appear to bind ethidium even while forming weak dimers. The different detergent system used in the oligomerization vs. the binding studies (SDS and DDM, respectively) likely accounts for variances in these relationships. Alternatively, the possibility exists of non-specific ethidium-Hsmr interactions at the Leu93 position, since this is the only position facing towards lipid in the SMR assembly model (Fig. 3.5B) (Chen, Pornillos et al., 2007; Poulsen, Rath et al., 2009).

3.3.3 Hsmr efflux activity is not exclusively related to dimer strength

The oligomerizing properties of TM4 - coupled with its distance from the TM1-3 substrate binding pocket - had led to the proposition that the TM4 helix is primarily responsible for forming the dimer required for both substrate binding and efflux activity (Rath, Melnyk et al., 2006; Chen, Pornillos et al., 2007; Elbaz, Salomon et al., 2008; Korkhov and Tate, 2008;
Poulsen, Rath et al., 2009). However, in the present work we have found that there does not seem to be a clear link between dimer strength and efflux capability (Fig. 3.5A). While the relative efflux activity of mutants does not correlate to dimer strength, we noted that all functional mutants lie within +/- 10% of dimerization strength relative to WT. This suggests that an active SMR relies on optimal dimer strength (Fig. 3.5B) to surround the substrate for binding and concomitantly to release it for efflux function. If the TM4-TM4 interactions in a given SMR mutant are weaker than a certain threshold, the SMR is unable to effectively bind substrate (i.e., L93V), whereas if the SMR binds ethidium too strongly due to a tight dimer, proper efflux will not occur (i.e., V98L). Interestingly, some mutants do not appear to affect dimer levels relative to WT, yet result in disruptive efflux activity (i.e., I94V). To determine if certain Ile94 mutants could be promoting a new dimerization face that coincidently has the same interaction strength as the native fold but propagates further conformational alteration, we performed CHI modeling of each of the mutants. Although the latter possibility is not excluded, we found that all mutants display virtually identical interactions to the WT dimer, with the V98L mutant displaying the largest deviation from WT (RMSD = 1.909 Å; Appendix 3). Due to the linear relationship of dimerization and binding, a plot of the ethidium binding capability of Hsmr mutants against activity appears virtually identical to Figure 3.5A, with the exception of the L93I and L93F mutants.
Figure 3.5. Dimer-activity relationships in Hsmr TM4 mutants. (A) Relative ethidium resistance against relative dimerization levels of WT and mutant Hsmr. The green shaded area above the dotted horizontal line represents the functional mutant group of activity $> 67\%$ relative to WT. Dashed vertical lines represent an imposed ‘threshold’ of $\pm 10\%$ of relative dimerization to WT; the red shaded region represents activity loss explained by dimer that is on either side of this threshold strength. Note that all functional mutants lie in the $\pm 10\%$ dimerization range relative to WT. The non-shaded region represents activity-reduced mutants that are unexplained by dimerization levels. (B) Top view of an SMR dimer-ethidium binding model of the representative activity conditions in (A), based on the EmrE x-ray structure (PDBID 3B5D). WT is able to form a dimer that has an optimal strength for activity. L93V forms a weak dimer that is unable to bind ethidium at levels required for an active form of the protein. V98L forms a strong dimer that increases ethidium binding yet is non-functional due to its inability to release the ethidium substrate. An I94V mutant does not affect dimer or substrate binding yet is non-functional, suggesting a possible mechanistic role at this position (see section 3.3.4 for a further discussion). (C) A CHI model of WT Hsmr TM4 anti-parallel dimer (side view) with the Ile94 and Val98 side-chain positions at the centre of the helix represented by green and yellow spheres, respectively.
3.3.4 Implications for a functional role at TM4 in SMRs

Structural studies have demonstrated that TMs 1-3 of each SMR monomer surround various substrates with a certain level of plasticity while the TM4-TM4 interaction remains relatively inactive in both the substrate-bound and -unbound states (Korkhov and Tate, 2008). Yet the present examples of dimeric non-functional mutants at the Ile94 or Val98 positions suggest an additional mechanistic involvement in substrate efflux that originates at the TM4-TM4 dimer interface. The Ile94 and Val98 positions are unique in this respect as several mutants do not affect dimer levels yet lead to disruptive function, while a β-branched residue appears to be required for function at the Val98 position. The CHI-produced Hsmr TM4 dimer that is highly similar to structural data from the E. coli homologue EmrE (Chen, Pornillos et al., 2007; Poulsen, Rath et al., 2009) shows that Ile94 and Val98 appear at the centre of the helix-helix interface and possibly protrude slightly towards TM3 in the folded protein (Fig. 3.5C). However, given that in vivo SMRs have the innate ability to efflux a variety of substrates – not limited, of course, to ethidium bromide – the exquisite sensitivity of the TM4-TM4 interaction to minor alterations in sequence raises the possibility that a ‘pivot point’ may be located at the centre of the TM4 helix in which a dynamic dimer of optimal strength is produced not only for binding, but potentially to aid in the mechanistic efflux requirements of each particular substrate in an interactive manner that optimizes the substrate binding pocket. As mutants that marginally strengthen or weaken dimerization were found here to produce functionally defective protein, our work identifies a potential target for inhibiting the ability of bacteria to evade the effects of cytotoxic compounds.

3.4 Materials and Methods

3.4.1 Large residue analysis and mutagenesis at the TM4 dimer motif

A BLAST-P search against the non-redundant protein database was performed to identify sequence similarity among the small multidrug resistance protein family members (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustalW (http://www.ebi.ac.uk/clustalw/index.html) was used to perform a multiple sequence alignment of closely related family members, and to
identify residues occurring at conserved positions in the TM4 dimerization interface (Appendix 3). The percent occurrence of the large, hydrophobic residues Phe, Ile, Leu, Met, and Val were calculated at positions 91, 93, 94, and 98, according to alignment with Hsmr. The Hsmr protein used in this study is linked to a C-terminal Myc and hexa-histidine tag on an ampicillin-resistant pT7-7 expression vector that was kindly provided by Dr. Simon S. Schuldiner (Ninio and Schuldiner, 2003). Site-directed mutagenesis at positions 91, 93, 94, and 98 was performed to obtain Ala, Phe, Ile, Leu, Met, and Val residues at each position using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

3.4.2 Protein expression, purification, and concentration determination

*E. coli* BL21(DE3) cells harbouring the pT7-7 vector were grown while shaking at 37°C to an O.D. 600nm ~0.6 in LB media supplemented with 50 µg/ml ampicillin, expression was induced with 0.5 mM IPTG for 1 h and cells were harvested by centrifugation. Cells were lysed by incubating with buffer containing 10 mM Tris HCl, 10 mM NaCl, 2% SDS, pH 8 for 2 h, followed by incubation with pre-equilibrated Ni-NTA Agarose (Qiagen) for 1 h. Resin was washed 3X with 25 bed volumes of buffer supplemented with 5, 10, and 20 mM imidazole, respectively. Purified Hsmr was eluted in buffer supplemented with 400 mM imidazole, which was subsequently removed by dialysis. Hsmr was dialyzed twice against 10 mM Tris HCl, 10 mM NaCl, pH 8 supplemented with 1% SDS for SDS-PAGE, or once with 1% n-dodecyl-β-D-maltopyranoside (DDM) followed by 0.08% DDM for fluorescence experiments. Protein concentration was determined by UV absorbance at 280 nm for all Hsmr mutants using molar extinction coefficients of 75000 and 25000 M⁻¹cm⁻¹ for buffer containing 1% SDS and 0.08% DDM, respectively. Extinction coefficients were determined for each environment for wild type (WT) Hsmr by taking the average of several BCA (Pierce) concentration measurements and the UV absorbance of the measured protein.

3.4.3 Oligomerization measurements

SDS-PAGE was performed 4 times using materials and protocols from Invitrogen. 50 ng of purified Hsmr was loaded onto a 4-12% NuPAGE Bis-Tris gel and stained using SilverXpress silver staining. Densitometry was performed using ImageJ (NIH) and the % dimer was
determined for each Hsmr construct as the density of dimer divided by the total protein for each lane. A student’s t-test was performed for all mutants comparing dimer levels relative to WT Hsmr at the p < 0.05 significance level. Linear correlation analysis of SDS-PAGE to ethidium binding was performed using GraphPad Prism software.

3.4.4 Ethidium resistance activity assay

A minimum of 3 overnight cultures of each mutant in E. coli BL21(DE3) cells (Invitrogen) were grown to saturation while shaking at 37°C in LB media containing 50 µg/ml ampicillin. The BL21(DE3) ampicillin-resistant test vector as supplied by Invitrogen was used as a negative control (−). The cultures were serially diluted 10-fold into LB media containing ampicillin and 5 µl of each dilution was spotted onto a BD Falcon XL BioDish containing LB agar supplemented with ampicillin and 100 µg/ml ethidium bromide (Sigma). The plates were incubated at 37°C for 20 h and were photographed with a Panasonic Lumix DMC-ZS1 camera. Densitometry of each colony was performed using ImageJ (NIH) and cell growth was determined as the integral of an extrapolated line fit to the cell density versus the dilution factor. The relative ethidium resistance activity of mutant Hsmr (mut) to the wild type (WT) Hsmr was determined using equation 3.1 (Seppala, Slusky et al., 2010):

\[
Normalized \ activity = \frac{Growth \ mut - Growth (-)}{Growth \ WT - Growth (-)}
\]

3.4.5 Hsmr binding to ethidium bromide via fluorescence spectroscopy

Fluorescence emission scans were performed using a Photon Technology International C-60 spectrofluorimeter and fluorescence intensities were measured using a Gemini EM Fluorescence Microplate Reader (Molecular Devices) in the 96-well format. Fluorescence of 2 µM ethidium bromide +/- 2 µM Hsmr (Fluor\textsubscript{EtBr}, and Fluor\textsubscript{WT EtBr} or Fluor\textsubscript{mut EtBr}, respectively) was measured in 0.08% DDM buffer with excitation and emission wavelengths of 485 and 600 nm respectively. The relative fluorescence increase of at least 3 measurements was determined using the emission intensity at 600 nm and the equation 3.2:
3.4.6 Modeling of Hsmr TM4 Dimers

Potential dimerization sites for the Hsmr TM4 and mutants were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS) as previously described (Brunger, Adams et al., 1998; Poulsen, Rath et al., 2009). The anti-parallel Hsmr WT structure of TM4 (residues 85-105, inclusive) was chosen based on its similarity to the EmrE TM4 crystal structure (Chen, Pornillos et al., 2007; Poulsen, Rath et al., 2009), and mutant structures were selected based on the proximity to the WT structure by measuring the intermonomer Gly90 to Gly97 Cα distances of each mutant. Comparisons of WT and mutant root-mean-square deviation (RMSD) values and imaging were performed using PyMOL (DeLano Scientific).
Chapter 4: Drug efflux by a small multidrug resistance protein is inhibited by a transmembrane peptide

The contents of this chapter have been published, in part, by Poulsen, B.E., and Deber, C.M., *Antimicrobial Agents and Chemotherapy*, (56): 3911-3916 (2012).

Author Contributions: BEP and CMD designed the research. BEP performed research and analyzed the data. BEP and CMD wrote the paper.
4.1 Introduction

As outlined in Section 1.5, bacteria effectively use membrane-bound efflux transporters to remove cytotoxic compounds as a mechanism of multidrug resistance (Nikaido, 2009). Among the five families of bacterial multidrug transporters, at least two have been shown to require oligomerization for function: the resistance nodulation division (RND) and the small multidrug resistance (SMR) proteins (Murakami, Nakashima et al., 2002; Chen, Pornillos et al., 2007; Nikaido, 2009). These families are both found in pathogenic Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, while SMRs are also found in Gram-positive and archeabacteria such as *Staphylococcus aureus* and *Halobacterium salinarum*, respectively (Bay, Rommens et al., 2008; Nikaido, 2009). Like many efflux proteins, SMRs use the proton motive force (PMF) to facilitate the removal of various cationic sanitizing agents, dyes, and antibiotics from the bacterial cell (Littlejohn, Paulsen et al., 1992; Paulsen, Littlejohn et al., 1993; Grinius and Goldberg, 1994; Heir, Sundheim et al., 1999; Masaoka, Ueno et al., 2000; Muth and Schuldiner, 2000; Nishino and Yamaguchi, 2001; Bay, Rommens et al., 2008; Schuldiner, 2009).

SMR proteins are relatively small in size compared to the other multidrug efflux families (which often efflux the same molecules, such as ethidium bromide). SMRs are comprised of ~110 residues forming four membrane-spanning TM α-helices with short connecting loops (Chen, Pornillos et al., 2007). The minimal functional unit of SMRs has been characterized as a dimer, although higher order oligomerization has also been proposed (Tate, Kunji et al., 2001; Soskine, Steiner-Mordoch et al., 2002; Tate, Ubarretxena-Belandia et al., 2003; Ubarretxena-Belandia, Baldwin et al., 2003; Butler, Ubarretxena-Belandia et al., 2004; Elbaz, Steiner-Mordoch et al., 2004; Rath, Melnyk et al., 2006; Schuldiner, 2009). EmrE from *E. coli* is the most extensively studied SMR family member, and has been characterized by both crystallographic and NMR methods as an antiparallel homodimer bound to the substrate tetraphenylphosphonium (Chen, Pornillos et al., 2007; Korkhov and Tate, 2008; Morrison, Dekoster et al., 2011). This model shows that TM helices 1 through 3 of each monomer surround the substrate, forming a six-helix binding pocket within the membrane bilayer, and utilize the conserved negatively charged residue Glu14 in TM1 to coordinate the protons and/or cationic portion of substrates (Muth and Schuldiner, 2000; Chen, Pornillos et al., 2007; Schuldiner, 2009). Several other hydrophobic residues on the interior of the binding pocket are able to
variably coordinate aromatic groups frequently present on substrates (Chen, Pornillos et al., 2007; Korkhov and Tate, 2008). However, the dimerization propensity per se of SMRs is centered at TM4 via a $^{90}$GLxLIxxGV$^{98}$ motif (Elbaz, Salomon et al., 2008; Poulsen, Rath et al., 2009).

Accordingly, upon considering strategies by which the drug efflux power of SMRs might be inhibited, we hypothesized that rather than attempting to inhibit SMR-mediated efflux by binding inhibitors into the substrate binding pocket – which itself is permissive to a wide variety of relatively weakly bound molecules – disruption of the TM4-TM4 helix-helix interaction employed by SMRs for dimerization may be an effective method (Caputo, Litvinov et al., 2008; Poulsen, Cunningham et al., 2011). This method of inhibiting oligomerization and function has been successfully achieved in a few instances [reviewed in (Fink, Sal-Man et al., 2012; Ng, Poulsen et al., 2012)] by using TM peptides that mimic and compete for the native TM-TM interaction. Notably, inhibition of dimer-dependent function has been accomplished using synthetic peptides to treat mammalian cells in the case of both the Class II G-protein coupled secretin receptor (Harikumar, Pinon et al., 2007), the ErbB2 tyrosine kinase receptor (Bennasroune, Fickova et al., 2004), and also the T-cell receptor (Quintana, Gerber et al., 2005; Cohen, Cohen et al., 2010). From bacteria, peptide inhibition of the dimeric aspartate receptor (Sal-Man, Gerber et al., 2004) and the trimeric diacylglycerol kinase has also been achieved (Partridge, Melnyk et al., 2003).

To implement this approach of inhibiting SMR-mediated substrate efflux by disrupting protein oligomerization, in the present work we utilize a synthetic TM4 peptide with the identical sequence of the SMR from H. salinarum (Hsmr). Using an ethidium fluorescence assay to visualize its efflux by Hsmr in intact cells, we report that treatment of these cells with the TM4 peptide shows a significant decrease in the rate of efflux. Our findings indicate the validity of this approach for specific peptide targeting of oligomeric membrane proteins.
4.2 Results

4.2.1 Peptide design and characterization

With the purpose of using synthetic peptides to specifically inhibit the oligomerization capabilities and subsequently the substrate efflux by Hsmr, five peptides of residue composition identical to wild type Hsmr TM4 were designed and synthesized. To aid in water solubility, these synthetic hydrophobic TM segments are supplemented during synthesis with poly-Lys residues at one or both termini of the peptide (Melnyk, Partridge et al., 2003); this design is typified by peptide K-TM4 (Table 4.1), with three Lys residues at each terminus. To achieve the possible benefit of an uncharged solubility tag at the N-terminus that may aid in peptide insertion into the cellular membrane, we further elaborated our tags to include an acetylated N-terminus containing AlaSar3, while retaining the three Lys residues at the C-terminus. Sarcosine residues have been characterized as aiding in the solubilization and maintaining the helicity of hydrophobic TM segments (Melnyk, Partridge et al., 2003). Further peptides consisted of a D-amino acid version of TM4 (D-TM4); and two scrambled peptides – one with a random sequence over the entirety of the segment (SCR), and the other where only the dimerization sequence encompassing residues 90-98 (GVSCR) is scrambled. In peptide SCR, the sequence was further chosen for omission of known oligomerization motifs (Ng, Poulsen et al., 2012).
Table 4.1. Sequences and characterization of designed Hsmr TM4 peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence&lt;sup&gt;abc&lt;/sup&gt;</th>
<th>MM (Da)</th>
<th>MIC&lt;sup&gt;d&lt;/sup&gt; (µM)</th>
<th>Oligomeric state&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Relative efflux rate with 1 µM peptide&lt;sup&gt;&lt;small&gt;fg&lt;/small&gt;&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-TM4</td>
<td>KKK-VAGVVGLALIVAGVVVLNAS-KKK</td>
<td>2689</td>
<td>12</td>
<td>dimer&lt;sup&gt;f&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>TM4</td>
<td>Ac(A(Sar),)-VAGVVGLALIVAGVVVLNAS-KKK</td>
<td>2632</td>
<td>&gt;25</td>
<td>dimer&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.37 +/- 0.02 **</td>
</tr>
<tr>
<td>SCR</td>
<td>Ac(A(Sar),)-LGLAVAVAVGLAVSVKGK</td>
<td>2632</td>
<td>&gt;25</td>
<td>monomer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.88 +/- 0.09</td>
</tr>
<tr>
<td>GVSCR</td>
<td>Ac(A(Sar),)-VAGVVLGVIAGVALVVLNAS-KKK</td>
<td>2632</td>
<td>&gt;25</td>
<td>monomer&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.65 +/- 0.04 *</td>
</tr>
<tr>
<td>D-TM4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ac(A(Sar),)-VAGVVGLALIVAGVVVLNAS-KKK</td>
<td>2632</td>
<td>&gt;25</td>
<td>dimer&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.79 +/- 0.03 *</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solubility tags (offset by hyphens) were added to each peptide. Ac signifies an acetylated N-terminus. All peptides contain an amidated C-terminus.
<sup>b</sup>Sar = N-methylglycine.
<sup>c</sup>Dimerization motif is underlined.
<sup>d</sup>Minimum inhibitory concentration defined by undetectable growth (see Fig. 4.1).
<sup>e</sup>Stoichiometry of peptides as determined by migration on SDS-PAGE gels (Fig. 4.2).
<sup>f</sup>Previously reported (Rath, Melnyk et al., 2006).
<sup>g</sup>Daily experiments were normalized to Hsmr (relative efflux = 1) after a rate constant (k) calculation using first order exponential decay. See Section 4.4.4 for details. The mean value of k for Hsmr efflux is 10.1 +/- 0.7 x 10^-3 s^-1, n = 10. Cells without the Hsmr vector have an efflux rate of 0.15 +/- 0.02 relative to Hsmr.
<sup>h</sup>Stoichiometry of peptides as determined by migration on SDS-PAGE gels (Fig. 4.2).
<sup>i</sup>Peptide D-TM4 contains all-D residues but is otherwise identical to TM4.

4.2.2 Assay for antimicrobial activity

Since hydrophobic segments with greater than three positively charged residues may have the propensity for causing bacterial membrane disruption – and hence categorized as cationic antimicrobial peptides (Hancock, 1997; Brogden, 2005) – we first assayed the bactericidal activity of each peptide to optimize our design for membrane insertion without disrupting its integrity (Fig. 4.1). Cells were grown in minimal media in the presence of up to 25 µM peptide, and among our designed peptides, only K-TM4 (with a +7 charge) caused cell death (measured as MIC) at 12 µM and greater. A known cationic antimicrobial peptide, 6kf17 composed of D-amino acids, was used as a control for lysis and displayed an MIC of 3 µM in the present system, similar to its previously reported value of 2 µM in MHB media (Stark, Liu et al., 2002). Cells were able to grow at all concentrations tested of the other TM4 peptide variants, and although the MIC of K-TM4 is at a concentration much higher than those used in the in vivo assays, it was excluded from further investigation. The assay was also performed using relatively enriched MHB media, resulting in virtually identical results.
Figure 4.1. Antimicrobial peptide assay. Cells were incubated in minimal media at 37°C for 20 h in the presence of 0-25 µM peptide and growth was measured by absorbance at 600 nm. Lines of best fit are shown. Cells were able to grow at all concentrations of the TM4 (red line), D-TM4 (blue), SCR (yellow), and GVSCR (green) peptides, while cell death occurred in the presence of the K-TM4 (grey) peptide at concentrations at and above 12 µM. A known antimicrobial peptide, 6k-f17 (black) (of all-D chirality) was used as a control and caused cell death at 3 µM, similar to the previously reported value (Stark, Liu et al., 2002). Error bars represent the SEM of at least three experiments.

4.2.3 Secondary structures of designed peptides

The TM4 and analog peptides were characterized for helicity and oligomerization by circular dichroism and SDS-PAGE, respectively (Fig. 4.2). All peptides display α-helical CD spectra in the SDS membrane mimetic environment with minima (or maxima as mirrored by D-TM4) at 208 and 222 nm – secondary structure consistent with the conformation of the TM
segments of native SMR proteins (Schuldiner, 2009) (Fig. 4.2A). A comparison of the absolute values of mean residue ellipticities (MRE) at 222 nm determined that only the SCR peptide is significantly different from the other three peptides (p < 0.05). The identical CD experiments of the peptides in buffer lacking SDS display spectra associated with a ‘random coil’ structure, as illustrated for the TM4 peptide. SDS-PAGE reveals that the two peptides that contain the native dimerization sequence of \textsuperscript{90}GLxLIxxGV\textsuperscript{98} (Poulsen, Rath \textit{et al.}, 2009) – TM4 and D-TM4 – are dimeric, while the two scrambled peptides GVSCR and SCR run as monomers (Fig. 4.2B). The SCR peptide migrates on SDS-PAGE more quickly than GVSCR, a finding consistent with its relatively lower helicity and thus reduced SDS binding (Rath, Glibowicka \textit{et al.}, 2009). Several attempts were made to dissociate purified dimeric Hsmr with the peptides in SDS, but changes in Hsmr and peptide gel migration were not observable, likely due to the ability of SDS to compete for tertiary and/or quaternary interactions, and of (at least some) peptides to form antiparallel dimers in micelles.
Figure 4.2. Characterization of peptides related to Hsmr TM4. (A) Circular dichroism spectra of samples of 20 µM peptide in the membrane mimetic detergent SDS. Spectra are D-TM4 (blue), SCR (yellow), GVSCR (green), and TM4 (red or black dotted, the latter without SDS present). As the D-amino acid version of TM4, D-TM4 displays the mirror image spectrum of TM4. (B) Representative Coomassie-stained SDS-PAGE of 0.5-1 µg purified peptides. The locations of Mark12 molecular weight standards are indicated to the left of the gel. The TM4 and D-TM4 peptides run as apparent dimers, while the GVSCR and SCR peptides are monomeric.
Figure 4.3. TM4 peptide inhibition of Hsmr ethidium bromide efflux. (A) Fluorescence measurements of *E. coli* cells harboring Hsmr actively effluxing ethidium with and without 1 μM TM4 (red and black lines, respectively) over 20 minutes. The curves shown represent the average of 8-10 measurements in each case. At t = 0 sec, the medium yields basal fluorescence levels, and as cells containing ethidium are added at t = 30 sec, the fluorescence signal becomes maximal and decreases proportional to ethidium efflux from the cell. (B) Representative single traces of ethidium efflux data fit to the first order exponential decay equation 4.1. Hsmr efflux is shown after incubation without peptide (black), and with 1 μM peptide (SCR, yellow; D-TM4, blue; GVSCR, green; TM4, red). Relative rate constants are given in Table 4.1.
4.2.4 Ethidium efflux of peptide-treated cells

Hsmr inhibition was measured using an *in vivo* ethidium efflux assay where ethidium fluorescence was measured over 20 minutes. The decrease in fluorescence is proportional to its efflux from the cell, as the emission intensity of ethidium is ~25-fold greater when interacting with intracellular DNA (LePecq and Paoletti, 1967). *E. coli* cells expressing Hsmr extrude ethidium (Fig. 4.3A) at a rate consistent with first order exponential decay ($R^2 > 0.99$) with a mean rate constant of $k = 10.1 \pm 0.7 \times 10^{-3} \text{s}^{-1}$. Upon treatment of Hsmr-containing cells with TM4 peptide, the rate of efflux is decreased to $k = 3.7 \pm 0.2 \times 10^{-3} \text{s}^{-1}$, while control cells lacking Hsmr have a basal decrease in fluorescence of $k = 1.5 \pm 0.4 \times 10^{-3} \text{s}^{-1}$. The normalized rate constants are displayed relative to the efflux by Hsmr (Table 4.1), with single representative decay curves shown in Figure 4.3B. Upon treatment with 1 µM additions of each peptide, only the SCR peptide displays a statistically equivalent efflux rate to Hsmr ($k = 0.88 \pm 0.09 \times 10^{-3}$). Importantly, the TM4 peptide has the most significant decrease in efflux rate, with a ~60% reduction of the decay rate constant ($k = 0.37 \pm 0.02 \times 10^{-3}, p < 0.0001$). Although the D-TM4 and GVSCR peptide treatments also reduce efflux by Hsmr ($k = 0.79 \pm 0.03 \times 10^{-3}$ and $0.65 \pm 0.04 \times 10^{-3}$, respectively, $p < 0.05$), they are both equivalent statistically to the SCR peptide and different from TM4 ($p < 0.0001$). The rate constant decrease was further found to be proportional to the concentration of TM4 (Fig. 4.4), with efflux inhibition becoming saturated at 1 µM peptide, although the relative rate constant does not reach the basal efflux rate of *E. coli* cells.
4.3 Discussion

4.3.1 Synthetic transmembrane peptides display full-length Hsmr properties

Because Hsmr and related SMRs are “small”, it is physically improbable that these proteins can function in vivo as drug efflux pumps in a monomeric form – particularly with their known activity against relatively “large” molecules such as ethidium bromide, tetraphenylphosphonium, and benzalkonium (Bay, Rommens et al., 2008). As well, attempts to inhibit their function by binding specifically designed inhibitors into a pocket prospectively formed by dimers must confront the fact that such pockets are dynamic, as they must transport – not permanently bind – their substrates, and must adjust to the demands of a wide variety of substrate structure and stereochemistry. It therefore seemed most plausible to address SMR inhibition at the source of its function, viz., by relegating the protein molecules to a monomeric
form. The SMR dimerization motif of $^{90}$GLxLIxxGV$^{98}$ within the TM4 sequence is both evolutionarily conserved and required for efflux function, as shown in the two family members EmrE and Hsmr (Elbaz, Salomon et al., 2008; Poulsen, Rath et al., 2009). Although SMR proteins are structurally flexible and use a variety of residues on TMs 1-3 to accommodate the various compounds that imbue to SMRs their multidrug efflux capabilities, TM4 appears rigid and sensitive to minor changes in the motif (Korkhov and Tate, 2008; Poulsen, Cunningham et al., 2011). Accordingly, we designed peptides to mimic and compete for this functionally dependent TM4-TM4 interaction between two Hsmr monomers, with the goal of causing inhibition. These principles underlie the present work, as shown schematically in Figure 4.5, where the initially dimeric SMR protein interacts with the designed TM4 peptide to form inactive SMR-TM4 1:1 complexes. Since SMRs function as antiparallel dimers (Chen, Pornillos et al., 2007; Korkhov and Tate, 2008; Morrison, Dekoster et al., 2011) the TM4 peptide (unable to form a dimer with itself due to its unidirectional N_in-C_out orientation in the membrane) could theoretically only interact with the N_in-C_in SMR subunit.

**Figure 4.5. Proposed inhibition mechanism of drug efflux.** The TM4 peptide (grey) converts from a random coil to an $\alpha$-helix upon insertion in the bacterial membrane with its neutral N-terminus and positively charged C-terminus shown. The TM4 peptide competes for the functional TM4-TM4 native SMR interaction (represented by the EmrE antiparallel dimer (Chen, Pornillos et al., 2007), each monomer colored red or blue; PDBID 3B5D), thereby preventing its efflux of ethidium bromide (molecule shown) by producing a TM4-EmrE monomer complex via antiparallel association with TM4 + an (inactive) EmrE monomer.
4.3.2 TM4 peptide prevents ethidium efflux *in vivo*

To test experimentally the ability of our TM4 peptide to inhibit Hsmr, we used an *in vivo* efflux assay that takes advantage of the fluorescence capabilities of an SMR substrate, ethidium bromide. Upon treatment of *E. coli* cells harboring Hsmr with ethidium bromide and the ionophore CCCP, the interior of cells become saturated with ethidium as the abolishment of the PMF prevents efflux, yielding a high fluorescence signal due to the cellular DNA-ethidium interaction (Fig. 4.3). As CCCP is removed, the cells are able to regain the PMF, resulting in the active removal of cellular ethidium, lowering the fluorescence signal (Ninio and Schuldiner, 2003). The efflux data are then fit to first order exponential decay, and the decay rate constant for Hsmr was determined to be $10.1 \times 10^{-3}$ s$^{-1}$. When the TM4 peptide was incubated with the cells before efflux, the activity of Hsmr was reduced to a decay constant of $3.7 \times 10^{-3}$ s$^{-1}$ ($p < 0.0001$). Importantly, the ‘control’ peptides D-TM4, SCR, and GVSCR, were significantly less effective in this assay (Table 4.1), reinforcing the notion that the presence of the native dimerization sequence motif and correct chirality underlie the specificity of TM4 inhibition. Interestingly, the control peptide treatments did result in a minor amount of efflux inhibition. Since the D-TM4 peptide should have the identical propensity as the TM4 peptide to insert into the bacterial membrane, such insertion may generally impede bacterial function and/or have a measurable effect on the membrane integrity. Although D and L TM peptides with an identical sequence from the bacterial aspartate receptor (Tar-1) have been shown to form heterodimers despite opposite chirality, structural models of the heterodimer demonstrated a modification to helix tilting was required to satisfy the hydrogen bonding of the native homodimer (Sal-Man, Gerber et al., 2004). A possible explanation for the lack of a D-TM4 interaction with Hsmr is the specific van der Waals interactions required for proper TM4-TM4 packing are not as permissive to modification as the polar interactions from the Tar-1 peptides, as demonstrated by a hydrophobic residue scan of Hsmr TM4 (Poulsen, Cunningham et al., 2011). Further design and investigation of effective D-peptide TM4 inhibitors would be useful since D-peptides are resistant to proteolytic degradation compared to L-peptides [as reviewed in (Brogden, 2005)]. Although effective at preventing Hsmr efflux over a 20-minute period (Fig. 4.3), at this point in our design the TM4 peptide is not yet able to significantly reduce cell growth over extended time periods in the presence of ethidium or benzalkonium due to the inevitable degradation of the L-peptide.
When a membrane protein dimerizes or assembles to higher quaternary structure, this self-association is mediated primarily by sequence motifs and van der Waals packing opportunities within the protein TM domain(s). For example, single-spanning membrane proteins such as glycoporphin A and bacteriophage M13 coat protein form strong TM-TM dimers by this pathway; the calcium channel regulator phospholamban assembles into pentamers in a similar manner (Rath, Tulumello et al., 2009). The observation that the designed wild type TM4 peptide and its D-TM4 analog migrate as dimers on SDS-PAGE (Fig. 4.2B) is a strong indication that these peptides retain their functional helix-helix interaction motifs. In contrast, the two scrambled peptides were unable to form dimers since they lack the native Hsmr dimerization sequence found in the TM4 and D-TM4 peptides. Furthermore, the design of this series of TM4 and analog peptides with uncharged N-termini would be expected to enhance their ability to insert into the bacterial membrane, perhaps in a ‘corkscrew’ manner, with the neutrally charged Ac-Ala(Sar)₃ N-terminal moiety acting as a membrane entry segment. All four peptides display a random coil structure in aqueous buffer and high amounts of α-helicity in the membrane mimetic SDS detergent system, with only SCR having a significantly lower helicity. The latter observation may be attributable to the mid-sequence Asn residue in SCR, as it has been shown that a polar side chain at this location may elicit reduced helicity and quicker gel mobility in vitro (Tulumello and Deber, 2011) as well as a reduced propensity to insert into mammalian cellular membranes (Hessa, Meindl-Beinker et al., 2007).

4.3.3 Conclusion

For the first time to our knowledge, specific TM peptide inhibition of a bacterial efflux protein (Hsmr) in vivo is demonstrated, through use of a peptide with sequence identical to its native TM4 that includes a deduced TM4-TM4 dimerization motif (Poulsen, Rath et al., 2009). Although the TM4 peptide does not achieve complete abolishment of Hsmr efflux function, further investigation and development of peptide analogs of TM4 could lead to a high-functioning SMR inhibitor. In the broader view, this approach could conceivably be used to design hydrophobic peptides for disruption of key TM-TM interactions of membrane proteins that arise in many organisms, and as such represent a valuable route to the discovery of new therapeutics.
4.4 Materials and Methods

4.4.1 Peptide synthesis

TM4 peptides containing the amino acids 85-105 of the full-length Hsmr protein (sequences shown in Table 4.1) were synthesized using a PS3 peptide synthesizer (Protein Technologies, Inc.) via Fmoc chemistry. Sarcosine (N-methyl-glycine) and/or lysine residues were added to both the N- and C-termini to increase the solubility of the peptide (Melnyk, Partridge et al., 2003). A 0.1-mmol scale synthesis was used for each peptide with the O-(7-azabenzotriazol-1-yl)-N,N,N’,N’-tetramethyl-uronium hexafluorophosphate and N,N-diisopropylethylamine activator pair, with 4-fold amino acid excess. A low load (0.18–0.22 mmol/g) FMOCPAL-polystyrene glycol-polystyrene resin yielded an amidated C-terminus upon peptide cleavage. To maintain a neutral charge at the N-terminus, peptides (with the exception of K-TM4) were acetylated on the resin prior to cleavage with a 1:1 solution of dichloromethane and acetic anhydride. Peptide cleavage and deprotection was carried out using a solution of 88% trifluoroacetic acid, 5% phenol, 5% ultrapure water, and 2% triisopropylsilane. The cleavage product was precipitated into diethyl ether at -80°C, washed twice with cold ether, dried, and resuspended into ultrapure water. Cleaved peptides were purified by reverse phase high performance liquid chromatography on a C4 preparative column (Phenomenex) with a water/acetonitrile gradient in the presence of 0.01% trifluoroacetic acid. Peptide molecular weights were confirmed by mass spectrometry (Appendix 4). Peptides were lyophilized, resuspended in ultrapure water, and concentration was determined using the BCA assay (Thermo).

4.4.2 Antimicrobial peptide assay

Bactericidal activity of the synthesized peptides was tested using the standard antimicrobial peptide assays (Stark, Liu et al., 2002; Glukhov, Stark et al., 2005). Minimal Media A (Ninio and Schuldiner, 2003) was used instead of MHB to maintain consistency with our efflux experiments. Briefly, 25000 colony forming units of E. coli BL21(DE3) cells (Novagen) harboring the pT7-7 Hsmr vector were grown in 100 µl Minimal Media A with 0-25
μM peptide in a 96-well clear cell culture plate (Nunc). Cell growth was measured by optical density at 600 nm on a Gemini EM microplate reader (Molecular Devices) after incubation for 20 h at 37°C. A D-amino acid peptide, D-6kf17, was used as a positive control for antimicrobial activity (Stark, Liu et al., 2002). All experiments were performed in triplicate and were background subtracted.

4.4.3 Circular dichroism spectroscopy and SDS-PAGE

CD spectra of peptides were recorded on a Jasco J-720 circular dichroism spectrometer at room temperature. Spectra in SDS (20 mM SDS, 10 mM Tris, 10 mM NaCl, pH 7.2) were recorded using a 1 mm path length cuvette at a peptide concentration of 20 mM. All spectra were background subtracted and converted to mean residue molar ellipticity (MRE [deg cm² dmol⁻¹ x10⁻⁴]). Mean residue ellipticities shown are the average of three separate scans, and statistical differences at 222 nm were determined between all peptides via t-tests. SDS-PAGE was performed using materials and protocols from Invitrogen. 0.5-1 µg of purified peptide was loaded onto a 12% NuPAGE Bis-Tris gel with Mark12 proteins standards and stained using Gelcode Blue Stain (Thermo).

4.4.4 Ethidium bromide efflux assay

Ethidium efflux was measured as previously described (Ninio and Schuldiner, 2003). E. coli BL21(DE3) harboring the pT7-7 Hsmr vector were grown at 37°C to OD 600 nm ~ 0.4 before expression was induced with 0.5 mM IPTG for 2 h while shaking. Cells were pelleted by centrifugation and resuspended in Minimal Media A, and treated with 40 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 5 min at room temperature before the addition of 1 µg/mL ethidium bromide and 0-1.5 µM peptide. Cells were incubated at 37°C while shaking for 1 h, pelleted by centrifugation, resuspended in Minimal Media A, and quickly measured for fluorescence while stirring on a Photon Technology International C-60 spectrofluorimeter for 1200 s at 1 s intervals (λex 530 nm, 2 nm slit width; λem 600 nm, 4 nm slit width). Data were normalized to the highest fluorescence point and was analyzed using GraphPad Prism fit to first order exponential decay equation 4.1:
\[ F = F_0 e^{-kt} \]

where \( F \) is fluorescence at any time, \( F_0 \) is the initial fluorescence value at \( t = 0 \), \( t \) is time in seconds, and \( k \) is the decay rate constant (s\(^{-1}\)).
Chapter 5: Discussion
5.1 Summary of Contributions

As outlined in Chapter 1, bacteria are able to confer resistance to various antibiotics via several mechanisms including efflux of the toxic compound from the cell. Of the five families of efflux proteins, the small multidrug resistance proteins (SMR) are not only found in a broad spectrum of bacterial species, but are also the smallest in size relative to the other families. Since the mechanism of efflux and the exact oligomeric state of this protein family is not fully understood, their relatively small size (4 TM segments versus 12 for the other families) makes SMRs great candidates for biochemical and structural investigation. Comprehending what may be a simpler efflux mechanism by SMRs might lead to a better grasp of drug efflux as a whole – the first step in rational drug design targeting the five efflux families. In this thesis we focused solely on SMRs, specifically the archeabacterial homolog Hsmr due to its ability to maintain its oligomeric properties in the detergent SDS. As a model system for all SMR proteins, Hsmr was studied extensively, from its functional and structural oligomerization to the early stages of inhibitor design.

5.1.1 Identifying the oligomerization motif used by bacterial small multidrug resistance proteins at TM4

As antiporter proteins of ~100 amino acids, SMRs must self-assemble into homo-oligomeric structures for efflux of drug molecules. Oligomerization centered at transmembrane helix four (TM4) has been implicated in SMR assembly, but the full complement of residues required to mediate its self-interaction remained to be characterized. Chapter 2 outlines how we used Hsmr, the 110-residue SMR family member of the archaebacterium Halobacterium salinarum, to determine the TM4 residue motif required to mediate drug resistance and SMR self-association. Twelve single point mutants that scan the central portion of the TM4 helix (residues 85–105) were constructed and were tested for their ability to confer resistance to the cytotoxic compound ethidium bromide. Six residues were found to be individually essential for drug resistance activity (Gly90, Leu91, Leu93, Ile94, Gly97, and Val98), defining a minimum activity motif of G90LxLxIxGV98 within TM4. When the propensity of these mutants to dimerize on SDS-PAGE was examined, replacements of all but Ile resulted in ~2-fold reduction of dimerization versus the wild-type antiporter. This portion of our work defined a minimum
activity motif of $G_{90}LxLIxxGV_{98}$ within TM4 and suggests that this sequence mediates TM4-based SMR dimerization along a single helix surface, stabilized by a small residue heptad repeat sequence.

5.1.2 An added role for TM4 in small multidrug resistance protein efflux

As outlined in Chapter 3, we further investigated the $G_{90}L_{91}xL_{93}I_{94}xxG_{97}V_{98}$ motif and upon analysis of sequence conservation of the dimeric TM4-TM4 interaction site, we found the Gly residues to be highly conserved. This coincides with the heptad repeat of small residues resulting on the same helix face, allowing the accommodation of the close approach of helices, analogous to the GG4 motif found in many TM proteins such as Glycophorin A. The large residue identities were of interest, since we hypothesized that they allow for optimal van der Waals interactions along the TM-TM interface. We continued our focus on the dimer motif, specifically at the large-residue positions 91, 93, 94, and 98, with all but the Leu91 having greater than 90% conservation. We substituted the naturally occurring Hsmr residue for Ala, Phe, Ile, Leu, Met, and Val at each of these positions in the Hsmr TM4-TM4 interface. Large-residue replacements were studied for their ability to dimerize on SDS-polyacrylamide gels, to bind the cytotoxic compound ethidium bromide, and to confer resistance by efflux. Although the relative activity of mutants did not correlate with dimer strength for all mutants, all functional mutants were within ±10% of dimerization relative to the wild type (WT), suggesting that the optimal dimer strength at TM4 is required for proper efflux. Furthermore, nonfunctional substitutions at the center of the dimerization interface that did not alter dimer strength suggested a dynamic TM4-TM4 “pivot point” that responds to the efflux requirements of different substrates. This functionally critical region represents a high-potential target for inhibiting the ability of bacteria to evade the effects of cytotoxic compounds, as demonstrated by its sensitivity to minor alterations to the motif.

5.1.3 A TM4 peptide inhibits Hsmr function

As a first step for inhibitor design targeting SMRs, we hypothesized that peptides mimicking the native TM4-TM4 interaction would compete for oligomerization resulting in a loss of function (Chapter 4). To test our hypothesis, we used a Lys-tagged synthetic peptide with
the exact sequence of TM4 of the full-length SMR Hsmr from *Halobacterium salinarum* (TM4 sequence: AcA(Sar)-VAGVGLALIVAGVVVLNVAS-KKK) (Sar = N-methyl-glycine) to compete with and disrupt the native TM4-TM4 interactions believed to constitute the locus of Hsmr dimerization. Using a cellular efflux assay of the fluorescent SMR substrate ethidium bromide, we determined that bacterial cells containing Hsmr were able to remove cellular ethidium via first order exponential decay with a rate constant of $k = 10.1 \pm 0.7 \times 10^{-3} \text{s}^{-1}$. Upon treatment of the cells with the TM4 peptide, we observed a saturable ~ 60% decrease in the efflux rate constant to $3.7 \pm 0.2 \times 10^{-3} \text{s}^{-1}$. In corresponding experiments with control peptides – including scrambled sequences and a sequence of D-chirality – a decrease in ethidium efflux was either not observed or was marginal, the latter likely from non-specific effects. Designed peptides did not evoke bacterial lysis, indicating that they indeed act via the $\alpha$-helicity and membrane insertion propensities of the native TM4 helix. Our overall results suggest that this approach could conceivably be used to design hydrophobic peptides for disruption of key TM-TM interactions of membrane proteins, and represent a valuable route to the discovery of new therapeutics.

5.2 Peptides as antibiotics

Small molecules have many advantages as drugs compared to the relatively larger peptides. Due to the small size, they are usually cheaper and easier to synthesize and generally have improved pharmacokinetics in that they are better absorbed, distributed, and are not metabolized as quickly (Pillarisetti, 2006). Despite their increased cost, more complex administration, and lower stability, peptides are generally more specific and have fewer adverse effects (Pillarisetti, 2006). Small molecule antibiotics generally target bacterial protein, DNA, or cell wall synthesis (Fig. 1.10) and have achieved high success in doing so. However, due to bacterial drug resistance even after several stages of modifications (i.e. $\beta$-lactams), it is clear that new viable classes of antibiotics are required to combat the increasing emergence of superbugs (Spellberg, Guidos *et al.*, 2008). One drug class that is becoming more popular is antimicrobial peptides, which specifically target the bacterial membrane. Our work in Chapter 4 of this thesis, although at early stages, defines a novel use of peptides targeting membrane protein TM-TM interactions in bacteria. As outlined below, these peptides have the potential for working in
conjunction with current antibiotics. Furthermore, with the elucidation of other bacterial membrane protein targets, can potentially be used independently. The benefit of these peptide inhibitors compared to small molecules peptides can be designed to target specific bacterial targets, removing any potential toxic side-effects that small molecules could have on the host eukaryotic cells (Pillarisetti, 2006).

5.2.1 Antimicrobial peptides targeting the bacterial membrane

Antimicrobial peptides (AMPs) are becoming increasingly popular in terms of future therapeutics for bacterial infections due to their broad specificity towards bacterial, and sometimes fungal and yeast, membranes. Several natural peptides have been isolated and studied for their antimicrobial activities [reviewed in (Brogden, 2005; Hancock and Sahl, 2006; Wimley and Hristova, 2011)]. These peptides are found in virtually all organisms including humans, and are part of the innate immune system. One of the general mechanisms of several AMPs is to employ their dual lipophilic and cationic properties to interact with the negatively charged bacterial membranes (rich in phosphatidylglycerol) and either form pores in the membrane (barrel-stave or toroidal pore models), or disrupt the membrane via global bilayer destabilization (carpet or detergent models) (Wimley and Hristova, 2011). Unfortunately, as with virtually all antimicrobials, bacteria resist them via mechanisms such as protease degradation (Gruenheid and Le Moual, 2012).

Attempts are in progress to turn the basic features of these natural peptides into more stable and viable therapeutics (Hancock and Sahl, 2006; Giuliani and Rinaldi, 2011). In terms of cost, designing these peptides to be as small as possible, and streamlining their production from recombinant and natural sources will be cost-effective. Improving the stability of the peptides to elongate their half-lives in vivo is also a crucial step for clinical use. Several unique approaches to alter chirality, side chain chemistry, and the peptide backbone are yielding promising results (Giuliani and Rinaldi, 2011). The use of D-amino acids has proven useful in preventing protease degradation, thus allowing the peptide to be more effective. Other methods of improving efficacy and bioavailability include modifications such as: unnatural amino acid side chains, lipidation of peptides, peptide cyclization, and the use of peptoids (side chain attached to N instead of Cα) (Giuliani and Rinaldi, 2011).
5.2.2 Peptides targeting bacterial multidrug efflux protein TM-TM interactions

In addition to general membrane disruption targeting bacteria, peptides can be designed as peptidomimetics targeting a protein interaction site. In these cases, a small peptide fragment can mimic a focused native interaction from a larger protein (see Section 1.3.4 for some examples). This could lead to weakened bacteria if the target is appropriate, or can potentially be used in conjunction with other drugs (Chapter 4). Perhaps this method of using peptidomimetics can be employed to inhibit proteins that confer resistance to antibiotics in bacteria by disrupting key structural components in their protein folds. This may be especially useful in the case of the multidrug efflux proteins (Fig. 1.13). Since these membrane-bound efflux proteins are able to bind and extrude compounds of varying shape, size, and net charge, the binding site may not be the ideal locus for inhibitor design. These proteins bind the drugs relatively weakly as they must be able to release the drug on the outside of the membrane. Furthermore, part of their multidrug properties arises from their ability to use different residues, and sometimes helices, depending on the drug they are extruding (Korkhov and Tate, 2008; Nikaido, 2009).

From a rational drug design viewpoint, this promiscuity of drug binding leads to the notion that inhibitors targeting these sites may be challenging, although much more detail into their mechanisms of efflux may be useful. These efflux proteins contain, at the very least, eight transmembrane helices including oligomerization, some of which are involved in translocation while others participate in more structural roles. By targeting these structural helices, and perhaps loop regions as well, the ability of these proteins to efflux drugs should be diminished, resulting in a lengthened time for the drug to take effect inside the bacterial cell. However, in order to design inhibitors to accomplish this, whether they are peptide-based or small molecules, we must first investigate which specific residues and helices to target based on their roles in efflux and structural function.

As a proof of principle, we used the SMR homolog Hsmr to determine the specific TM-TM interaction to target with peptides (Chapter 2). Based on evidence that TM4 was the site of SMR dimerization (Rath, Melnyk et al., 2006; Elbaz, Salomon et al., 2008), we constructed a series of single point mutations along the length of TM4. Upon characterization for activity and dimerization, we determined that Hsmr in fact dimerizes in an activity-dependent fashion using residues G90LxLIxxGV98 along its TM4 helix to allow proper packing with a partner TM4 helix from another monomer. Further characterization of this interface (Chapter 3) strengthened this
defined sequence as a high-affinity locus for inhibition by a peptidomimetic. Peptides with the exact sequence of Hsmr TM4 were subsequently synthesized in an effort to mimic and compete for this TM4-TM4 interaction (Chapter 4). Our hypothesis of disrupting this interaction was achieved: a significant decrease in the ability of Hsmr to efflux a substrate was observed upon bacterial treatments with the peptide. Although we were able to decrease the functional activity of Hsmr using our TM4 peptide inhibitor, the peptide alone was unable to restore the toxic effects by the ethidium substrate over extended periods of time. Regardless, this was a step in the right direction for the development of a new class of inhibitors designed as a combinational therapy with current antibiotics.

5.2.3 Improving TM inhibitor peptide efficacy

Designing peptides with the goal of targeting a specific TM-TM interaction requires several factors for effectiveness. Beyond the correct sequence in which the peptide is mimicking, the peptide must effectively insert into the membrane, form an α-helix, and compete for the native TM-TM interaction. In Chapter 4, a starting point for the TM4 peptide design was a sequence of L-amino acids with the exact sequence of the native TM4 from Hsmr. Three Lys residues on either side were added to the peptide to promote water solubility (Table 4.1; Melnyk, Partridge et al., 2003). The peptide at that point closely resembled a cationic antimicrobial peptide as described in Section 5.2.1, and actually had some antimicrobial activity (Figure 4.1). Although this could be a fruitful area to investigate, for the purposes of this study, modification was made to the N-terminus: three Lys residues were replaced by an acetylated Ala-Sar-Sar-Sar uncharged sequence. This was to remove any antimicrobial activity from the peptide, while maintaining its aqueous solubility for peptide delivery.

In order to achieve long-term goals of using this TM4 peptide – or any other peptide – for clinical use, several modifications would likely be required to improve the efficacy. The peptide would be required to be effective at such a level that would allow the effects of the antibiotic drug given in combination with the peptide. A modification that could be effective to achieve this is to design a peptide with an optimal sequence for membrane insertion and dimerization. To improve membrane insertion, note can be taken from residue membrane insertion scales to alter residues on the lipid-exposed helix face (Hessa, Meindl-Beinker et al., 2007). To improve dimerization, perhaps some minor modifications can be made to the TM4-TM4 dimer interface.
The work in Chapter 3 identified several TM4 point mutations at the Val98 position in the full-length Hsmr protein that resulted in an increased dimerization propensity on SDS-PAGE. Peptides with these substitutions could conceivably form stronger peptide-Hsmr hetero-interactions than the wild type sequence.

A major hurdle that must be overcome to promote efficacy of future TM peptide inhibitors is to design such peptides to be resistant to bacterial proteases. Countless modifications can be attempted for each peptide inhibitor of interest, such as: altering amino acid chirality and side chain chemistry, using unnatural amino acids and peptoids, and lipidating or cyclizing peptides (Giuliani and Rinaldi, 2011). Further studies of a shorter TM4 peptide targeting SMRs could also improve efficacy. For example, an uncharged peptide with the barebones dimerization sequence of GLxLIxxGV (with an acetylated N-terminus and amidated C-terminus) would clearly promote membrane insertion due to its high hydrophobicity. This could potentially increase inhibition, dependent on its ability to form a stable helix. However, such a peptide would require a more elaborate delivery method due to its likely aqueous insolubility.

5.2.4 Designing TM peptides toward other bacterial targets

Combinational therapy using current antibiotics with an antibiotic resistance protein inhibitor is a viable option to overcome superbugs. As demonstrated in Section 1.4.3, β-lactams are currently being administered in conjunction with the β-lactamase inhibitor clavulonic acid. Since multidrug efflux proteins are another major mechanism of antibiotic resistance, development of inhibitors for these families of membrane proteins will lead to the restoration of some antibiotic activities. The work in Chapter 4 of this thesis demonstrates early stages of such inhibitors in the form of transmembrane peptides. Upon improving the pharmacokinetics of TM peptides, given an appropriate bacterial membrane target protein, TM peptides could also be used in a more direct fashion without requiring a drug combination.

A single example of a single peptide targeting a bacterial membrane protein resulting in a deleterious effect has been shown (Sal-Man, Gerber et al., 2004). The bacterial aspartate receptor, Tar, is a dimeric membrane protein that mediates bacterial movement via chemotaxis upon signal binding. TM1 from Tar forms polar interactions to form a homodimer. Interestingly, both D- and L-amino acid TM1 peptides with an identical sequence to this helix were shown to
form interactions with the full-length Tar receptor in vitro, presumably since structural models of
the D-TM1 with L-TM1 heterodimer demonstrated a modification to helix tilting was required to
satisfy the hydrogen bonding of the native homodimer (Sal-Man, Gerber et al., 2004). This,
coupled with our findings presented in Chapter 4 showing the lack of a D-TM4 interaction with
Hsmr, suggests that a polar interaction may be more permissible for maintaining native
interactions using D-peptides. Subsequently, since the D-peptides could resist degradation, the
authors were able to utilize the Tar TM1 D-peptide to prevent chemotaxis in bacterial cultures.

Although the Tar receptor was successfully inhibited using peptides, the loss in
chemotaxis abilities by the bacteria did not prevent their growth, only their movement (Sal-Man,
Gerber et al., 2004). However, using this approach towards a more detrimental target in bacteria
could result in antibiotic effects. The ever-increasing number of new membrane protein
structures, along with other biochemical techniques defining transmembrane interactions, could
lead to more targets for rational-drug design. For example, ions, metabolites, and cofactors
required for biological processes often must be transported across the membrane by various
transport proteins. The bacterial Mg\(^{2+}\) transporter CorA forms a homopentamer (Eshaghi,
Niegowski et al., 2006; Payandeh and Pai, 2006) and the bacterial vitamin B12 transporter
BtuCD forms a dimer (Locher, Lee et al., 2002). Design of a TM peptide to successfully insert in
the bacterial membrane and specifically inhibit either of these transport proteins would
essentially starve the bacteria from these necessary compounds. Without these vital metabolites,
bacteria would be severely weakened, allowing for their successful eradication. Before such
peptides can be tested, the TM-TM interactions must first be determined. Using high-resolution
structures can aid in an estimate of which transmembrane helices interact, but other biochemical
methods of determining specific residue motifs will allow for a more refined peptide inhibitor
design (Rath and Deber, 2008; Rath, Tulumello et al., 2009).

5.3 Concluding Remarks

As the smallest of the five families of bacterial multidrug efflux proteins, the small
multidrug resistance protein family is a good candidate for investigation due to its minimalistic
nature. In this thesis, we focused on determining a locus for potential inhibitor design at key TM-
TM helix interactions. Evidence of dimerization at TM4 led us to investigate this site in SMRs, and we were successful in determining that SMRs dimerize via a highly specific and highly conserved GLxLIxxGV sequence. This dimerization was clearly determined to be exceptionally sensitive to minor modifications, and TM4 was proposed to have a more dynamic role in the efflux mechanism of SMRs: a fact that strengthened our motivation to design a peptide to target this helix-helix interaction.

Our initial peptide inhibitor design mimics the exact sequence of TM4 in the SMR homolog Hsmr. This peptide resulted in approximately a 60% decrease in the efflux rate constant of a common SMR substrate. This is a promising result for the future design of peptides targeting this site. Since our initial peptide design (the exact sequence of Hsmr TM4 comprised of L-amino acids and polar residue tags) is not resistant to protease-degradation over extended periods of time, clearly further development is required of the peptide for the long-term goal of therapeutic applications. Design to resist protease degradation, perhaps by introducing unnatural, D-amino acids and/or peptoids is a must for this application. An improved efficacy, perhaps by shortening the segment and improving membrane insertion to allow for optimal interaction with the native TM4-TM4 contact is also a crucial step to achieve these long-term goals.

This approach, albeit at very early development, can conceivably be used to target other TM-TM interactions, not just those of SMR proteins. Other bacterial multidrug efflux transporters, such as the Resistance Nodulation Division, oligomerize as trimers (Murakami, Nakashima et al., 2006). Targeting these larger oligomeric proteins may be very useful since the RND family extrudes compounds that are tremendously significant from a clinical perspective (Table 1.1). Although an X-ray crystal structure of this important drug efflux family has been determined, the key TM-TM interactions for oligomerization have yet to be established (Murakami, Nakashima et al., 2002; Murakami, Nakashima et al., 2006). Furthermore, monomeric efflux proteins can also be targeted, given they contain key TM-TM contacts whose interactions are required for function and that such disruption will be conveyed by a loss of efflux function. Plausibly, a refined peptide with high specificity and bioavailability may become the newest addition to combinational antibiotic treatments in an effort to re-sensitize drug resistant bacteria to existing small molecule antibiotics.
References


### Appendix 1: Oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Hsmr Mutant</th>
<th>Direction</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>V89A</td>
<td>Forward</td>
<td>GAC GTG GCC GGT GTC GCC GGG CTT GCG CTC ATT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAT GAG CCG AAG CCC GGC GAC ACC GGC CAC GTC</td>
</tr>
<tr>
<td>G90V</td>
<td>Forward</td>
<td>GTG GCC GGT GTC GTC GTT GCG CTC ATT GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC AAT GAG CGC AAG CAC GAC GAC ACC GGC CAC</td>
</tr>
<tr>
<td>L91A</td>
<td>Forward</td>
<td>GCC GGT GTC GTC GGG GCT GCC ATT GTC GCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGC GAC AAT GAG CGC AGC CCC GAC GAC ACC GGC</td>
</tr>
<tr>
<td>L91F</td>
<td>Forward</td>
<td>GCC GGT GTC GTC GGG TTT GCG CTC ATT GTC GCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGC GAC AAT GAG CGC AAA CCC GAC GAC ACC GGC</td>
</tr>
<tr>
<td>L91I</td>
<td>Forward</td>
<td>GCC GGT GTC GTC GGG ATT GCG CTC ATT GTC GCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGC GAC AAT GAG CGC TAA CCC GAC GAC ACC GGC</td>
</tr>
<tr>
<td>L91M</td>
<td>Forward</td>
<td>GCC GGT GTC GTC GGG GTG GTC GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGC GAC AAT GAG CGC CAT CCC GAC GAC ACC GGC</td>
</tr>
<tr>
<td>L91V</td>
<td>Forward</td>
<td>GCC GGT GTC GTC GGG GTT GCG CTC ATT GTC GCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGC GAC AAT GAG CGC AAG CCC GAC GAC ACC GGC</td>
</tr>
<tr>
<td>A92M</td>
<td>Forward</td>
<td>GGT GTC GTC GGG CTG ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCC CGC GAC AAT GAG CAT AAG CCC GAC GAC</td>
</tr>
<tr>
<td>L93A</td>
<td>Forward</td>
<td>GTC GTC GGG CTT GCG GCC ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>L93F</td>
<td>Forward</td>
<td>GTC GTC GGG CTT GCG GCC ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>L93I</td>
<td>Forward</td>
<td>GTC GTC GGG CTT GCG ATC ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAT CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>L93M</td>
<td>Forward</td>
<td>GTC GTC GGG CTT GCG ATG ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT CAT CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>L93V</td>
<td>Forward</td>
<td>GTC GTC GGG CTT GCG ATG ATT GTC GCG GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT CAT CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>I94A</td>
<td>Forward</td>
<td>GTC GGG CTT GCG GCT CTG GCT GCG GCC GGC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>I94F</td>
<td>Forward</td>
<td>GTC GGG CTT GCG GCT TTG GCT GCG GCC GTC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>I94L</td>
<td>Forward</td>
<td>GTC GGG CTT GCG GCT TTG GCT GCG GCC GTC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>I94M</td>
<td>Forward</td>
<td>GTC GGG CTT GCG GCT ATG GTC GCG GGC GTC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>I94V</td>
<td>Forward</td>
<td>GTC GGG CTT GCG GCT GTG GTC GCG GCC GTC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GCC CGC GAC AAT GAG CGC AAG CCC GAC GAC</td>
</tr>
<tr>
<td>V95A</td>
<td>Forward</td>
<td>GGG CTT GCG CTC ATT GCC GCG GCC GTC GTC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAC GAC GAC GCC GCC GCG AAT GAG CGC AGC</td>
</tr>
<tr>
<td>G97V</td>
<td>Forward</td>
<td>GCG CTC ATT GTC GCG GTC GTC GTC TTG TTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTT CAA CAC GAC GAC GAC GCG GAC AAT GAG</td>
</tr>
<tr>
<td>V98A</td>
<td>Forward</td>
<td>CTC ATT GTC GCG GGC GCC GTC GTC TTG TTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GAC GCC GAC GAC GCA GCC GCC GAC AAT GAG</td>
</tr>
<tr>
<td>V98F</td>
<td>Forward</td>
<td>CTC ATT CAC GAC GAC GAC TTG TTG AAC GTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC GAC GCC GAC GAC GCA GCC GCC GAC AAT GAG</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>V98I</td>
<td>CTC ATT GTC GCG GGC ATC GTC GTG TTG AAC GTC</td>
<td>GAC GTT CAA CAC GAC GAT GCC CGC GAC AAT GAG</td>
</tr>
<tr>
<td>V98L</td>
<td>CTC ATT GTC GCG GGC CTC GTC GTG TTG AAC GTC</td>
<td>GAC GTT CAA CAC GAC GAG GCC CGC GAC AAT GAG</td>
</tr>
<tr>
<td>V98M</td>
<td>CTC ATT GTC GCG GGC ATG GTC GTG TTG AAC GTC</td>
<td>GAC GTT CAA CAC GAC CAT GCC CGC GAC AAT GAG</td>
</tr>
<tr>
<td>V100A</td>
<td>GTC GCG GGC GTC GTC GCG TTG AAC GTC GCC TCG</td>
<td>CGA GGC GAC GTT CAA CGC GAC GAC GCC CGC GAC</td>
</tr>
<tr>
<td>L101A</td>
<td>GCG GGC GTC GTC GTG GCG AAC GTC GCC TCG GAC</td>
<td>GTC CGA GGC GAC GTT CGC CAC GAC GAC GCC CGC</td>
</tr>
<tr>
<td>N102A</td>
<td>GGC GTC GTC GTG TTG GCC GTC GCC TCG GAC GCC</td>
<td>GGC GTC CGA GGC GAC GGC CAA CAC GAC GAC GCC</td>
</tr>
<tr>
<td>A104V</td>
<td>GTC GTG TTG AAC GTC GTC TCG GCC GCC TAC ACG</td>
<td>CGT GTA GGC GTC CGA GAC GAC GTT CAA CAC GAC</td>
</tr>
<tr>
<td>S105A</td>
<td>GTG TTG AAC GTC GCC GCG GAC GCC TAC ACG CGG</td>
<td>CGG CGT GTA GGC GTC CGC GGC GAC GTT CAA CAC</td>
</tr>
</tbody>
</table>
Figure A2.1. Mass spectrometry analysis of purified Hsmr. Two main peaks are seen with masses of 14527 and 14551 Da, respectively. The difference can be accounted for a sodium and hydrogen ion (23 and 1 Da, respectively). The theoretical molecular weight of Hsmr is 14526.6 Da.
Figure A2.2. Antiparallel TM4 dimer models. Potential dimerization sites for the TM4 sequence were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS). Helices are coloured green with the Gly90 and Gly97 positions coloured red. The N-termini are coloured black for clarity. The asterix (*) denotes the model compatible with the mutagenesis data as presented in Figure 2.4.
Figure A2.3. Parallel TM4 dimer models. Potential dimerization sites for the TM4 sequence were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS). Helices are coloured green with the Gly90 and Gly97 positions coloured red. The N-termini are coloured black for clarity. The asterix (*) denotes the model compatible with the mutagenesis data as presented in Figure 2.4.
Appendix 3: Supplementary data for Chapter 3

Table A3.1. ClustalW multiple sequence alignment of SMR family members.

<table>
<thead>
<tr>
<th>gi</th>
<th>ref</th>
<th>accession</th>
<th>MHP</th>
<th>Y</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>16554504</td>
<td>ref</td>
<td>NP_444228.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>185372677</td>
<td>gb</td>
<td>ABC70131.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>55377154</td>
<td>ref</td>
<td>YP_135004.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>227390541</td>
<td>ref</td>
<td>ZP_03873872.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>227883243</td>
<td>ref</td>
<td>ZP_04001072.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>109896676</td>
<td>ref</td>
<td>YP_660021.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>11994522</td>
<td>ref</td>
<td>YP_943202.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>167993516</td>
<td>ref</td>
<td>YP_001713109.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>193077844</td>
<td>gb</td>
<td>ABC12721.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>189706324</td>
<td>ref</td>
<td>YP_001424190.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>148555916</td>
<td>ref</td>
<td>YP_001263498.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>33593459</td>
<td>ref</td>
<td>NP_881103.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>33597982</td>
<td>ref</td>
<td>NP_885625.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>163855881</td>
<td>ref</td>
<td>YP_001630179.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>187479171</td>
<td>ref</td>
<td>YP_787196.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>149744744</td>
<td>gb</td>
<td>ABR28416.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>89094495</td>
<td>ref</td>
<td>ZP_01167434.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>218778505</td>
<td>ref</td>
<td>YP_002429823.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>25598833</td>
<td>ref</td>
<td>ZP_0531891.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>145376729</td>
<td>ref</td>
<td>ZP_0389497.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>17987328</td>
<td>ref</td>
<td>NP_539962.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>256061029</td>
<td>ref</td>
<td>ZP_05451185.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>254719026</td>
<td>ref</td>
<td>ZP_05180837.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>153095789</td>
<td>ref</td>
<td>YP_001340708.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>239831802</td>
<td>ref</td>
<td>ZP_04680131.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>39995814</td>
<td>ref</td>
<td>NP_951765.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>88800946</td>
<td>ref</td>
<td>ZP_01116498.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>126667317</td>
<td>ref</td>
<td>ZP_01738290.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>120553989</td>
<td>ref</td>
<td>YP_958430.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>10955231</td>
<td>ref</td>
<td>NP_044260.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>223886350</td>
<td>gb</td>
<td>ACN22632.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>223886403</td>
<td>gb</td>
<td>ACN22662.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>88857055</td>
<td>ref</td>
<td>ZP_01131698.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>192361037</td>
<td>ref</td>
<td>YP_001983646.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>226952641</td>
<td>ref</td>
<td>ZP_03823105.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>114561260</td>
<td>ref</td>
<td>YP_748773.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>91775236</td>
<td>ref</td>
<td>YP_544992.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>16783675</td>
<td>ref</td>
<td>ZP_02463637.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>83719484</td>
<td>ref</td>
<td>YP_443025.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>167562937</td>
<td>ref</td>
<td>ZP_02355289.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>53719474</td>
<td>ref</td>
<td>YP_783460.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>238027528</td>
<td>ref</td>
<td>YP_002911759.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>115351513</td>
<td>ref</td>
<td>YP_773352.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>113429560</td>
<td>ref</td>
<td>YP_001119365.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>171317507</td>
<td>ref</td>
<td>ZP_02906697.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>254252487</td>
<td>ref</td>
<td>ZP_04945805.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>167587283</td>
<td>ref</td>
<td>ZP_02379671.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>79066117</td>
<td>ref</td>
<td>ZP_368940.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>107022632</td>
<td>ref</td>
<td>YP_620959.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>206559980</td>
<td>ref</td>
<td>YP_00230744.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>254245529</td>
<td>ref</td>
<td>ZP_04938850.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>187923725</td>
<td>ref</td>
<td>YP_001895367.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>91783317</td>
<td>ref</td>
<td>YP_558523.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>170962924</td>
<td>ref</td>
<td>ZP_02883141.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>209515712</td>
<td>ref</td>
<td>ZP_03264576.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>186476417</td>
<td>ref</td>
<td>YP_001857887.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>194290259</td>
<td>ref</td>
<td>YP_00206166.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>113866633</td>
<td>ref</td>
<td>YP_727122.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>17906090</td>
<td>ref</td>
<td>YP_283677.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>158421950</td>
<td>ref</td>
<td>YP_001523024.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
<tr>
<td>gi</td>
<td>22125892</td>
<td>ref</td>
<td>NP_669315.1</td>
<td>-------------------</td>
<td>MHP</td>
</tr>
</tbody>
</table>

111
uncharged polar (green), positively charged (pink), and negatively charged (blue).

Hsmr is shown in bold. "*" means that the residues in that column are identical in all sequences in the alignment. "." means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed. Colouring of residues is as follows: hydrophobic (red), uncharged polar (green), positively charged (pink), and negatively charged (blue).
Figure A3.1. **Wild type and representative mutant TM4 dimer models.** Potential dimerization sites for the functionally disruptive TM4 mutants were identified using the CNS searching of helix interactions (CHI) software suite of the crystallography and NMR system (CNS) and were fit with WT (grey helices). The model for each mutant with the smallest RMSD to wildtype is shown as helices coloured red (L91V), blue (L93V), green (I94V), and yellow (V98L). Gly90 and Gly97 positions are shown in as spheres, and sticks represent the substituted side chain relative to WT at each position. Of all mutants, the largest RMSD from WT is the V98L mutant at 1.909 Å.
Figure A4.1. Purification and MS of the synthetic TM4 peptide. (Top) Peptides were purified by reverse phase high performance liquid chromatography on a C4 preparative column (Phenomenex) with a water/acetonitrile gradient (20-100% acetonitrile over 45 minutes) in the presence of 0.01% trifluoroacetic acid. The largest peak (circled) was collected and the TM4 molecular weights were confirmed by mass spectrometry (Bottom). The theoretical and experimental molecular weights of TM4 are 2631.2 and 2631.17 Da, respectively.
Figure A4.2. MS results of the synthetic TM4 peptide variants. Peptides were purified as in Fig. A4.1 and confirmed by mass spectrometry. The theoretical molecular weight of D-TM4, GVSCR, and SCR is 2631.2 Da, and their experimental molecular weights as shown above are 2631.7, 2629.6, and 2629.7 Da, respectively. The theoretical and experimental molecular weights of K-TM4 are 2689.4 and 2689.7 Da, respectively.
Copyright Acknowledgements