11-6-2015

Novel Host Defence Peptide Prodrugs for use in Cystic Fibrosis

Éanna Forde
Royal College of Surgeons in Ireland, eannaforde@rcsi.ie

Citation

This Thesis is brought to you for free and open access by the Theses and Dissertations at e-publications@RCSI. It has been accepted for inclusion in PhD theses by an authorized administrator of e-publications@RCSI. For more information, please contact epubs@rcsi.ie.
Novel Host Defence Peptide Prodrugs for use in Cystic Fibrosis

Éanna Breandán John Forde, BSc (Pharm), MPharm

Departments of Pharmaceutical and Medicinal Chemistry, and Clinical Microbiology, Royal College of Surgeons in Ireland, Dublin

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Philosophy

Under the supervision of Professor Marc Devocelle and
Dr Deirdre Fitzgerald-Hughes

October 2015
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed_____________________________________________ 
RCSI Student Number________________________________ ______ 
Date_______________________________________________ _____
Table of contents

OUTPUTS AND PUBLICATIONS FROM THIS PROJECT .......... 9

Posters .................................................................................................................. 9

Presentations ........................................................................................................ 9

Publications .......................................................................................................... 10

LIST OF ABBREVIATIONS ............................................................................... 11

LIST OF FIGURES ............................................................................................. 16

LIST OF TABLES .................................................................................................. 19

ABSTRACT ........................................................................................................... 20

ACKNOWLEDGMENTS ....................................................................................... 22

1. INTRODUCTION ............................................................................................ 24

1.1 Cystic fibrosis ................................................................................................ 24
   1.1.1 Aetiology of lung infection in CF ......................................................... 25
   1.1.2 P. aeruginosa infection in CF ............................................................... 26
   1.1.3 S. aureus infections in CF ................................................................. 27
   1.1.4 H. influenzae infection in CF ............................................................... 28
   1.1.5 The B. cepacia complex ................................................................. 28
   1.1.6 Fungal infections in CF ................................................................. 29

1.2 Cystic fibrosis and the immune response to infection ........ 29
   1.2.1 Sequelae of neutrophil dysfunction in CF ........................................ 32

1.3 Proteases in cystic fibrosis ........................................................................... 34
   1.3.1 Neutrophil elastase ............................................................................ 34
   1.3.2 Proteinase 3 and cathepsin G ............................................................ 35
1.3.3 Pseudomonas proteases ........................................... 36
1.3.4 Host metalloproteases ........................................... 37
1.3.5 Summary ............................................................. 38

1.4 Management of cystic fibrosis ...................................... 39
1.4.1 CFTR modifier use in CF ........................................... 41

1.5 Host defence peptides ................................................. 43
1.5.1 Classes of HDPs ....................................................... 44
1.5.2 Mechanisms of action of HDPs .................................... 45
1.5.3 Models for membrane disruption by HDPs .................... 46
1.5.4 Immunomodulation of HDPs ....................................... 49
1.5.5 Shortcomings for HDP therapeutics and potential solutions 50
1.5.6 Potential use of HDPs in CF ........................................ 50

1.6 Host defence peptide prodrugs ....................................... 52
1.6.1 Net charge reduction as a prodrug strategy .................... 53
1.6.2 PEG as a pro-moiety ............................................... 56
1.6.3 Antibodies as pro-moieties ........................................ 60
1.6.4 Antibiotics as pro-moieties ....................................... 61
1.6.5 Summary of HDP prodrugs ........................................ 62

AIMS AND SUMMARY OF CHAPTERS .................................. 65

2. MATERIALS AND METHODS .......................................... 66

2.1 Peptide synthesis and in vitro testing .............................. 66
2.1.1 Strains and Clinical isolates ..................................... 66
2.1.2 CF BAL fluid collection .......................................... 66
2.1.3 Blood collection ..................................................... 66
2.1.4 Peptide synthesis ..................................................... 67
2.1.5 TFA removal of HDPs .............................................. 69
2.1.6 Enzymatic cleavage of pro-HDPs ............................... 69
2.1.7 Neutrophil elastase determination .............................. 69
2.1.8 Susceptibility testing .............................................. 70
2.1.9 Bactericidal killing activity .................................................. 70
2.1.10 Static Biofilm studies ......................................................... 71
2.1.11 Flow Biofilm studies .......................................................... 71
2.1.12 Cell culture ........................................................................ 72
2.1.13 Neutrophil isolation ............................................................ 72
2.1.14 Cytotoxicity assays ............................................................. 73
2.1.15 Cytokine release assays ....................................................... 73
2.1.16 Haemolysis assays ............................................................. 74
2.1.17 Statistical analyses .............................................................. 74

2.2 Inhalation studies .................................................................... 75
   2.2.1 Light-scattering particle size analysis ................................. 75
   2.2.2 Impaction particle size analysis .......................................... 75
   2.2.3 Breathing apparatus .......................................................... 76

2.3 In vivo studies .......................................................................... 76
   2.3.1 Transgenic mice ............................................................... 76
   2.3.2 Bacterial challenge ........................................................... 76
   2.3.3 Lung lavage ...................................................................... 77
   2.3.4 Toxicity studies ............................................................... 77
   2.3.5 BAL cell count, total and differential ................................ 78
   2.3.6 BAL cytokine release assay .............................................. 78

3. RESULTS CHAPTER ONE: DEVELOPING HOST DEFENCE
PEPTIDE PRODRUGS FOR CYSTIC FIBROSIS ......................... 79

3.1 Introduction ............................................................................ 79
   3.1.1 Rationale ....................................................................... 79
   3.1.2 Peptide synthesis ........................................................... 79
   3.1.3 Choice of linker .............................................................. 84
   3.1.4 Choice of active peptides ................................................ 85
   3.1.5 Considerations for CF ...................................................... 86

3.2 Results .................................................................................. 87
   3.2.1 Pro-HDPs are cleaved by purified NE ............................... 87
3.2.2 Pro-HDPs have greater MIC values than parent and fragment HDPs ................................................................. 88
3.2.3 Pro-HDPs are bactericidal towards PAO1 in the presence of NE .............................................................................. 90
3.2.4 In the presence of NaCl, pro-HDPs are activated by CF BAL fluids ........................................................................ 92
3.2.5 The pro-HDPs potentially inhibit biofilm formation .......... 98
3.2.6 Pro-HDPs displayed lower cytotoxicity against CFBE41o- cells than fragment HDPs ......................................................... 105

3.3 Discussion ................................................................................. 105

4. RESULTS CHAPTER TWO: OPTIMISING AND EVALUATING HOST DEFENCE PEPTIDE PRODRUGS IN CYSTIC FIBROSIS 111

4.1 Introduction .............................................................................. 111
4.1.1 Rationale .............................................................................. 111
4.1.2 Further elongation of oligoglutamic acid ......................... 111
4.1.3 PEG as a pro-moiety ......................................................... 113
4.1.4 Replacement of TFA as a counter-ion .............................. 114
4.1.5 Investigating alternative HDP sequences ......................... 116

4.2 Results .................................................................................... 119
4.2.1 pH does not decrease the MIC of the pro-HDPs................. 119
4.2.2 Further elongation of oligoglutamic acid yielded no improvements ........................................................................ 119
4.2.3 PEGylation produced an inferior pro-moiety to oligoglutamic acid ............................................................................ 122
4.2.4 Replacing TFA as a counter-ion yielded no improvements in in vitro specificity .............................................................. 122
4.2.5 New HDP sequences yielded improved specificity ........... 123
4.2.6 Pro-HDPs do not stimulate IL-6/IL-8 release .................... 127
4.2.7 Pro-WMR demonstrates low human cell cytotoxicity ......... 128
4.2.8 AAG-WMR kills pre-formed biofilms ............................... 130
4.3 Discussion .................................................................................. 131

5. RESULTS CHAPTER THREE: EVALUATING THE NEBULISATION OF PRO-WMR ............................................. 140

5.1 Introduction .................................................................................. 140
   5.1.1 Rationale ............................................................................. 140
   5.1.2 Inhaled delivery ................................................................. 140
   5.1.3 Metered dose inhalers ....................................................... 142
   5.1.4 Nebulisation ....................................................................... 143
   5.1.5 Inhaled powder ................................................................. 144
   5.1.6 Inhaled delivery of HDPs - options and challenges .......... 145

5.2 Results ....................................................................................... 147
   5.2.1 Pro- and AAG-WMR are unchanged after nebulisation .... 147
   5.2.2 The particle size distribution of the peptides is favourable for lung delivery ................................................. 151
   5.2.3 The peptides are deliverable to a model lung ................. 153

5.3 Discussion .................................................................................. 155

6. RESULTS CHAPTER FOUR: AN IN VIVO STUDY INTO THE ANTI-INFECTIVE AND TOXIC EFFECTS OF PRO-WMR ......... 161

6.1 Introduction .................................................................................. 161
   6.1.1 Rationale ............................................................................. 161
   6.1.2 In vivo studies with HDPs .................................................... 161
   6.1.3 CF mouse model ............................................................... 162

6.2 Results ....................................................................................... 167
   6.2.1 Murine NE cleaves pro-WMR but is less active than the human enzyme ................................................................. 167
   6.2.2 Murine NE is produced in response to P. aeruginosa infection but it is insufficient for cleavage ................................................. 170
   6.2.3 Pro-WMR does not reduce the bacterial load of acute P. aeruginosa lung infection ......................................................... 171
6.2.4 Prodrug modification reduces the toxicity of active peptides 173
6.2.5 Prodrug modification reduces the immunogenicity of AAG-WMR ................................................................. 174
6.2.6 Cytokine release is induced by PAO1 but not aggravated further by active peptides ........................................... 176

6.3 Discussion .................................................................................................................................................. 176

7. CONCLUSION AND FUTURE PLANS ......................................................... 182

7.1 Conclusion .......................................................................................................................................... 182

7.2 Future Plans ....................................................................................................................................... 184
  7.2.1 Expanded clinical isolate groups ..................................................................................................... 184
  7.2.2 Quorum sensing effects ................................................................................................................... 185

8. REFERENCES ........................................................................................................................................ 187

9. APPENDICES AND PAPERS ................................................................. 218
Outputs and publications from this project

Posters

“Investigating the antimicrobial activity of a series of novel neutrophil elastase targeted host defence peptide prodrugs” - Sheppard Prize 2013, Beaumont Hospital, Dublin


“Novel Host Defence Peptide Prodrugs as Potential Anti-Infectives for Cystic Fibrosis” - BioAT Research Day 2013, NUIM, Maynooth

“Novel Host Defence Peptide Prodrugs as Candidate Anti-Infectives for Cystic Fibrosis” - ICAAC 2013, Denver, Colorado

“Novel Host Defence Peptide Prodrugs: Evaluating their activity for use in Cystic Fibrosis” - Sheppard Prize 2014, Beaumont Hospital, Dublin


“Host defence peptide prodrugs are respirable when delivered by vibrating mesh nebuliser” - ERS International Congress 2015, Amsterdam

Presentations

“Novel Host Defence Peptide Prodrugs for use in Cystic Fibrosis” - All Ireland Chemistry Colloquium 2013, TCD, Dublin (Awarded second place in organic chemistry)

“Evaluating Host Defence Peptide prodrugs targeted for use in the cystic fibrosis lung” - UKICRS symposium 2014, UCC, Cork
“Neutrophil elastase-activated host defence peptide prodrugs kill *Pseudomonas aeruginosa* in a cystic fibrosis lung milieu” - ERS International Congress 2014, Munich

“Host Defence Peptide prodrugs for use in cystic fibrosis” - RCSI Research Day 2015, Dublin

**Publications**

“Potential of host defence peptide prodrugs as neutrophil elastase dependent anti-infective agents for cystic fibrosis”

Éanna Forde, Hilary Humphreys, Catherine M. Greene, Deirdre Fitzgerald-Hughes, Marc Devocelle

Published February 2014 in Antimicrobial Agents and Chemotherapy

“Pro-Moieties of Antimicrobial Peptide Prodrugs”

Éanna Forde and Marc Devocelle

Published January 2015 in Molecules

(Papers are included at the end of the appendices)
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1AT</td>
<td>α1 antitrypsin</td>
</tr>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
</tr>
<tr>
<td>Bcc</td>
<td><em>Burkholderia cepacia</em> complex</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFB BE cells</td>
<td>Cystic fibrosis bronchial epithelial cells</td>
</tr>
<tr>
<td>CFTE cells</td>
<td>Cystic fibrosis tracheal epithelial cells</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CG</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>Chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell-penetrating peptide</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal violet</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>$N,N$-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPI</td>
<td>Dry powder inhaler</td>
</tr>
<tr>
<td>eDNA</td>
<td>Extra-cellular deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDT</td>
<td>1,2-Ethanedithiol</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray mass spectrometry</td>
</tr>
<tr>
<td>FEV$_1$</td>
<td>Forced expiration volume in the first second</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>FPF</td>
<td>Fine-particle fraction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FPRL1</td>
<td>Formyl peptide receptor-like 1</td>
</tr>
<tr>
<td>GSD</td>
<td>Geometric standard deviation</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene] 1H-1,2,3- triazolo [4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HDP</td>
<td>Host defence peptide</td>
</tr>
<tr>
<td>HD5</td>
<td>Human defensin 5</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophil peptide</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDR</td>
<td>Innate defence regulator</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL-1β, -2, -4, -5, -6, -8, -10, -12p70 -13</td>
<td>Interleukin 1β, 2, 4, 5, 6, 8, 10, 12 active heterodimer, 13</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix assisted laser desorption ionisation – time of flight mass</td>
</tr>
<tr>
<td>MBHA</td>
<td>(4-methyl)benzhydrylamine</td>
</tr>
<tr>
<td>MCC</td>
<td>Mucociliary clearance</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MH</td>
<td>Mueller Hinton</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MMAD</td>
<td>Mass median aerodynamic diameter</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>mNE</td>
<td>Murine neutrophil elastase</td>
</tr>
<tr>
<td>MOC</td>
<td>Micro-orifice collector</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>Nal</td>
<td>β-napthylalanine</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NMP</td>
<td><em>N</em>-Methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>PE</td>
<td>Pseudomonas elastase (or Pseudolysin)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PR-3</td>
<td>Proteinase 3</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-specific membrane antigen</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute 1640 medium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Term</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>VMD</td>
<td>Volume mean diameter</td>
</tr>
<tr>
<td>VMN</td>
<td>Vibrating mesh nebuliser</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
</tbody>
</table>
List of Figures

**Figure 1.1**: The cycle of infection and inflammation in CF................................. 39

**Figure 1.2**: Three models of HDP disruption of bacterial membranes.............. 48

**Figure 1.3**: Overview of the HDP prodrug model.............................................. 53

**Figure 1.4**: Poly(ethylene glycol) structure ..................................................... 57

**Figure 1.5**: An overview of the effects of the different pro-moieties on the affinity of HDPs for mammalian and bacterial cell membranes............... 63

**Figure 3.1**: Fmoc-D-Arg(Pbf)-OH..................................................................... 80

**Figure 3.3**: Summary of SPPS......................................................................... 82

**Figure 3.4**: Side-chain protecting groups for each amino acid (A) with their corresponding scavenging agents (B)......................................................... 83

**Figure 3.5**: The effect of NaCl on the bactericidal activity of Bac8c and HB43 (12.5μg/ml) against P. aeruginosa PAO1................................................................. 86

**Figure 3.6**: Bactericidal activity of parent and fragment HDPs against P. aeruginosa PAO1.................................................................................................................. 91

**Figure 3.7**: Effect of 5-20μg/ml purified NE on the bactericidal activity of pro-HDPs against P. aeruginosa PAO1 ................................................................. 92

**Figure 3.8**: Effect of 25% v/v BAL fluid on the bactericidal activity of pro-Bac8c (A), pro-HB43 (B), and pro-P18 (C) against P. aeruginosa PAO1............. 94

**Figure 3.9**: Effect of 300mM NaCl and 25% v/v CF BAL fluids on the bactericidal activity of pro-HDPs (25μg/ml) against P. aeruginosa PAO1...... 95

**Figure 3.10**: Effect of 300mM NaCl on the bactericidal activity of CF BAL fluid (25% v/v) against P. aeruginosa PAO1 ................................................................. 96

**Figure 3.11**: Effect of 300-600mM NaCl and 25% v/v CF004 BAL fluid on the bactericidal activity of pro-HB43 (25μg/ml) against P. aeruginosa PAO1 .... 97

**Figure 3.12**: Effect of 300mM NaCl and 25% v/v CF004 BAL fluid on the bactericidal activity of pro-HB43 (25μg/ml) against P. aeruginosa clinical isolates from CF patients................................................................. 98

**Figure 3.13**: Antibiofilm activity (metabolism) of 2-256μg/ml fragment peptides against 48h established biofilms of three P. aeruginosa isolates/strains; PABH02, PABH03, PAO1 ................................................................. 100
**Figure 3.14:** Antibiofilm activity (mass) of 2-256μg/ml fragment peptides against 48h established biofilms of three P. aeruginosa isolates/strains; PABH02, PABH03, PAO1

101

**Figure 3.15:** Biofilm inhibition (metabolism) of 2-64μg/ml fragment peptides against biofilms of three P. aeruginosa isolates/strains; PABH02, PABH03, PAO1 grown in the presence of the peptides

103

**Figure 3.16:** Biofilm inhibition (mass) of 2-64μg/ml fragment peptides against biofilms of three P. aeruginosa isolates/strains; PABH02, PABH03, PAO1 grown in the presence of the peptides

104

**Figure 4.1:** The different conjugate base and acid forms (γ-carboxyl group) of Glutamic acid at pH 7.4 and 4.1

113

**Figure 4.2:** PEG-AAAG-P18

114

**Figure 4.3:** HB43 with five TFA counter ions for the positively charged lysine residues and N-terminal NH₃

116

**Figure 4.5:** The effect of NaCl on the bactericidal activity of WMR, AAG-WMR, and AAG-WR12 (12.5μg/ml) against P. aeruginosa PAO1

124

**Figure 4.6:** Effect of 20μg/ml NE on the bactericidal activity of pro-WMR and pro-WR12 against P. aeruginosa PAO1

125

**Figure 4.7:** Effect of 25% (v/v) CF BAL fluids on the bactericidal activity of pro-WMR (25μg/ml) against P. aeruginosa PAO1 in the presence of 300mM NaCl

126

**Figure 4.9:** Haemolytic activity in response to HDPs

129

**Figure 4.10:** Viability of a 48h PAO1 flow biofilm exposed statically to AAG-WMR in PBS for 6h

131

**Figure 4.11:** 214nm RP-HPLC of crude pro-WR12

135

**Figure 5.1:** Aerogen Aeroneb® Solo vibrating mesh nebuliser

144

**Figure 5.2:** Next generation impactor

147

**Figure 5.3:** HPLC and ESI-MS of AAG-WMR before (A) and after (B) nebulisation

149

**Figure 5.4:** HPLC and ESI-MS of pro-WMR before (A) and after (B) nebulisation

150
Figure 5.5: Mass balance of each stage of the next generation impactor for pro-WMR ................................................................. 152

Figure 5.6: Mass balance of each stage of the next generation impactor for AAG-WMR .............................................................. 153

Figure 5.7: Image of the breathing apparatus ........................................... 154

Figure 6.1: A demonstration of the effects of CFTR and ENaC on mucociliary clearance ................................................................. 164

Figure 6.2: A comparison of the retention times of pro-WMR (A), AAG-WMR (B), and pro-WMR after overnight incubation with 5μg/ml of mNE (C) ...... 168

Figure 6.3: A comparison of the % cleavage of 500μg/ml pro-WMR by 5μg/ml of human and murine NE ...................................................... 169

Figure 6.4: A comparison of the cleavage of the substrate .................... 170

Figure 6.5: The relationship between CFU/ml and mNE levels in mouse BAL fluid ............................................................................. 171

Figure 6.6: The CFU/ml of P. aeruginosa PAO1 recovered from BAL fluids after treatment with pro- and active HDPs ........................................ 172

Figure 6.7: The % weight loss of wildtype mice treated twice intratracheally with 50μg/ml of peptide (A) and the total BAL mouse cell counts after treatment (B) ........................................................................... 174

Figure 6.8: Wildtype mouse BAL fluid levels of IL-5 (A), IL-6 (B), KC/GRO (C), and TNF-α (D) ......................................................................................... 175

Figure 6.9: β-ENaC mouse BAL fluid levels of KC/GRO (A) and IL-5 (B) ...... 176
## List of Tables

**Table 3.1:** MIC values for parent HDPs, fragment HDPs, and pro-HDPs vs. *P. aeruginosa* PAO1 and clinical isolates ........................................................... 89

**Table 3.2:** Comparison of the neutrophil elastase concentration and bactericidal activity of different CF BAL fluid samples. Patient details are included .......................................................................................................... 93

**Table 3.3:** Comparison of the IC$_{50}$ values of pro- and fragment HDP against CFBE41o- cells .............................................................................................. 105

**Table 4.1:** MIC values for pro-HDPs vs. *P. aeruginosa* PAO1 in different conditions of pH ........................................................................................................... 119

**Table 4.2:** MIC values for parent HDPs, fragment HDPs, and pro-HDPs vs. *P. aeruginosa* PAO1 and clinical isolates PABH01-04 ...................................... 120

**Table 4.3:** IC$_{50}$ values for parent HDPs, cleaved HDPs, and pro-HDPs versus CF bronchial (CFBE) and tracheal (CFTE) epithelial cells lines, and healthy neutrophils ........................................................................................................... 121

**Table 4.4:** MIC values for HB43 chloride and TFA salts vs. *P. aeruginosa* PAO1 and clinical isolates PABH01-04. IC$_{50}$ values against CFBE41o- cells........ 123

**Table 5.1:** MIC values for pre- and post-nebulisation HDPs vs. *P. aeruginosa* PAO1 and clinical isolates ............................................................................ 148

**Table 5.2:** VMD, MMAD, GSD and %FPF values for pro- and AAG-WMR, determined using both laser diffraction and cascade impaction...................... 151

**Table 6.1:** Mouse survival after two intratracheal treatments with 50μg of peptide ........................................................................................................... 173
Abstract

Host defence peptides (HDPs) are short antimicrobial peptides that form a crucial component of the innate immune system. Deficiencies in HDP activity contribute to enhanced susceptibility to infections; this is the case in cystic fibrosis (CF). Exogenously-applied HDPs can compensate for these deficiencies and are attractive as new anti-infectives, but their development as antimicrobials is limited by cytotoxicity. This thesis describes the development of a series of HDP prodrugs whose cytotoxicity is reduced by net-charge decrease. As high levels of the host protease neutrophil elastase (NE) are co-localised in the lung with *Pseudomonas aeruginosa* infection, the enzyme is the target for prodrug activation. This system limits antimicrobial activity and cytotoxicity to areas infected with the bacterium.

It is demonstrated here using *in vitro* assays that the prodrug modification reduces the antimicrobial activity against CF *P. aeruginosa* clinical isolates and reference strains. This activity is specifically restored with NE, both purified and in CF bronchoalveolar lavage (BAL) fluid. *In vitro* assays against epithelial cells, erythrocytes, and neutrophils demonstrate how the modification reduces cytotoxicity. Refinement of the model has produced pro-HDPs, with very low cytotoxicity, which are active in BAL fluid, can kill bacterial biofilms, and display low immunogenicity. The requirements for CF lung activity, such as the need for D-amino acids are also demonstrated. We show that the pro-HDPs are deliverable by nebulisation in a model of the human lung using a vibrating mesh nebuliser. *In vivo* results in mice show the toxicity and inflammatory effects of the pro-HDPs are reduced compared to the active peptides, in agreement with *in vitro* results.

This thesis describes the design, synthesis, *in vitro/in vivo* evaluation, and drug delivery of pro-HDPs. It describes how the large and growing library of active HDPs may be harnessed for use in CF. The final
candidate pro-WMR has the potential to be used in CF to treat infection and warrants further investigation.
Acknowledgments

I would first like to thank my supervisors Professor Marc Devocelle and Dr Deirdre Fitzgerald-Hughes for their inexhaustible support and mentorship throughout my PhD. It has been a pleasure to work with such fantastic scientists and I have enjoyed every minute of the project. Special thanks must also go to Professor Catherine Greene, who has always given generously of her time and expertise and without whom much of the project would have been impossible.

I am extremely grateful to Professor Clifford Taggart of Queen’s University, Belfast for agreeing to examine my thesis and act as external examiner. I would also thank Dr Emer Reeves for acting as internal examiner. I appreciate the significant time and effort they have given to make the examination possible.

I would like to thank my collaborators and friends in Heidelberg, especially Prof Dr Marcus Mall and Dr rer. nat. André Schütte, without whom the *in vivo* study would have not have been possible. Their kindness and hospitality is something I will never forget.

I would like to thank the head of Clinical Microbiology, Professor Hilary Humphreys, who has always been approachable and gave freely of his time, and the head of Pharmaceutical and Medicinal Chemistry, Professor Donal O'Shea, who has always ensured that my research was facilitated in the chemistry lab. Professor Kevin Nolan and Dr Helen McVeigh gave me the opportunity to take part in the BioAT program and for that I am very grateful.

I would like to acknowledge my funding body, the Higher Education Authority of Ireland. Funding for my PhD under the BioAT program was obtained from Cycle 5 of the Programme for Research in Third-Level Institutions. Science Foundation Ireland, under Equipment Grant no. 06/RFP/CHO024/602 EC07, provided for the peptide synthesizer.

I would like to thank my many colleagues and friends in RCSI, who have made the last four years some of the most enjoyable of my life:
Dr Alan Hibbitts, Dr Graeme Kelly, Emmet Campion, Suzanne Donnelly, Aoife McKeon, Donal Keogan, Harrison Daly, Eolann Kitteringham, Reece Kenny, Dr Colm Duffy, Dr James Reck, Dr Lorraine Blackmore, Dr Aoife O’Connor, Dr James Parker, Dr. Łukasz Frankiewicz, Dr Darren Griffith, Tadhg McGivern, Ziga Ude, Dr Michael Thai, Dr Marta Zapotoczna, Siobhan Hogan, Dr Niall Stevens, Dr Paul McKiernan, Dr Bojana Mirković, Gillian Lavelle, Dr Jonathan Cowman, William Whyte, Paul Dillon, and many others. Special mention must go to my fellow BioAT scholars: Hugh, Ross, Sinead, Cian, Claire, Valerio, Irene, and Cormac.

I would also like to thank my collaborators in Aerogen, Galway, in particular Dr. Ronan MacLoughlin.

My friends from TCD Pharmacy and at home in Ballina have always supported me in everything I have done, there are too many to name but I’d like to thank especially Gerard, Alan, Stephen, Cormac, Lorcán, Ronan Grimes, Ronan Scanlan, and Colm.

My parents, Catherine and Brendan, and sisters, Ailbhe and Niamh, have provided me with nothing but love and encouragement. My girlfriend Claire has undoubtedly suffered the most out of all the people mentioned and has been my rock. I dedicate this thesis to her and my family in love.

I would like to finish with a quote from one of my heroes, Sir Ernest Shackleton. It would never compare the undertaking of a PhD to the extreme rigours of Antarctic exploration but a recruitment advert he purportedly placed in a London paper for one of his expeditions would not seem out of place on a research recruitment page:

"Men wanted for hazardous journey. Low wages, bitter cold, long months of complete darkness, constant danger, safe return doubtful. Honour and recognition in case of success."
1. Introduction

1.1 Cystic fibrosis

Mutations in the gene coding for the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) cause the autosomal recessive disorder cystic fibrosis (CF) (1). CFTR is a cAMP-regulated chloride (Cl\textsuperscript{-}) channel found in epithelial cell membranes, the dysfunction of which leads to a multisystem disorder that is the most common lethal genetic disease in Caucasians (2). Over 1000 genetic mutations can lead to CFTR dysfunction and CF, including those that cause defects in function (\textit{i.e.} a channel gating defect), prevent the channel reaching its membrane target (\textit{i.e.} a trafficking dysfunction), or stop its initial production. Some mutations, such as ΔF508, cause both a trafficking and gating defect (3). This is the most common mutation, accounting for 70% of cases, and involves the deletion of a phenylalanine residue at position 508. The faulty CFTR will not pass the cell’s quality control mechanisms, which will prevent the proper trafficking of the CFTR from the endoplasmic reticulum and leads to an absence of activity (2). As CFTR is expressed in many organs, multiple systems are affected, although most common manifestations of the disease are found in the respiratory, gastrointestinal and reproductive tracts (4-6).

The result of CFTR mutation is the dysregulation of ion and water transport across epithelial membranes. This has a profound effect on numerous biological systems \textit{e.g.} male reproduction is impaired by the obstruction of the vas deferens by dehydrated secretions, rendering patients infertile (7). Digestive issues associated with CFTR dysfunction meant that in the past many patients died in early life, owing mainly to pancreatic insufficiency (6). Mucous plugs block the pancreatic canaliculi, preventing the release of digestive enzymes. This, in conjunction with a more acidic pH in the duodenum, results in poor digestion and malabsorption of nutrients. With improved
understanding of how CF interferes with these processes, nutritional problems are now managed with digestive enzyme treatment. Damage to the pancreas in CF can also lead to glucose intolerance (8), diabetes being a co-morbidity in 8-15% of patients. However, since the improvement in nutritional regimens, life expectancy has greatly increased, with chronic lung disease becoming the main cause of death (6, 9, 10). Over 80% of CF patients die directly or indirectly from lung disease (11). In the respiratory tract CFTR dysfunction leads to hypossecretion of chloride, hyperabsorption of sodium, and a dehydrated and volume-depleted airway surface liquid (ASL) (12). This 20µm fluid layer is a crucial component of the pulmonary innate defence and mediates mucus clearance (2). The dysfunction critically impairs the host defensive response and leads to severe infections and progressive pulmonary damage (13).

1.1.1 Aetiology of lung infection in CF

CFTR dysfunction leads to the chronic lung disease that characterises CF. It impairs the innate immune system of the host in a variety of ways. Amongst the most profound alterations is to the ASL which provides one of the first barriers to bacterial pulmonary invasion. As stated above, CFTR dysfunction results in ASL dehydration through Cl⁻ and fluid hypossecretion, which in turn leads to the impairment of mucociliary clearance and the promotion of mucus stasis in the lung (13). The result of this is increased contact time between bacteria and the lung epithelium, and the facilitation of bacterial adhesion that would otherwise not occur in healthy lungs (4, 12, 14). CF infections are typically caused by *Staphylococcus aureus* (*S. aureus*) and *Haemophilus influenzae* (*H. influenzae*) in younger children and *Pseudomonas aeruginosa* (*P. aeruginosa*) in adults. Other pathogens include *Burkholderia cepacia* (*B. cepacia*), *Aspergillus fumigatus* (*A. fumigatus*) and *Candida albicans* (*C. albicans*) (15). It has been demonstrated that the airways are not sterile in healthy patients and the nasal cavity may act as a reservoir for potentially harmful lung
pathogens. An investigation with paediatric patients demonstrated that the nose contains a diverse microbiome in which competition between bacteria is normally low or stable. In contrast, in exacerbations in CF patients it can become over-colonised by pathogenic bacteria like *P. aeruginosa*. The abnormal airway microbiome that characterises CF is established in patients well before adulthood (16).

1.1.2 *P. aeruginosa* infection in CF

*P. aeruginosa* is the most important pathogen in CF (4, 15, 17). It is generally acquired from the environment and not from other patients, and in a normal clinical situation would be rapidly cleared from the lung by mucociliary clearance and other components of the innate immune system. However, in CF these systems are compromised as an indirect result of CFTR dysfunction (4, 14). CFTR dysfunction also has direct innate immune effects, with CFTR normally acting as a receptor for lipopolysaccharide (LPS) on the bacterium, internalising it and eliciting an inflammatory response that ultimately aids in eradication (18). Other changes to the lung epithelium in CF, in addition to impaired clearance, aid the adherence of the bacterium. For example, *P. aeruginosa* uses highly inflammatory fimbral adhesins, such as with type 4 pili and flagellae, to initially adhere to the epithelium. The receptor for these is contained on asialylated glycolipids, such as asioloGM1 (14), the levels of which are relatively high in CF epithelial cells (4). Altered fucosyl residues on glycopeptides, increased heparan sulphate levels, and the accumulation of ceramide also contribute to adhesion (19).

As stated above, since the introduction of CF nutritional regimens, chronic *P. aeruginosa* infection has become the primary cause of mortality (6). Early treatment of acute infection in CF is crucial as, once it becomes established the bacterium can alter its phenotype to greatly increase its ability to evade eradication. The resulting chronic infection, localised to the endobronchial space, is very difficult to remove (20). As infection progresses, the initially non-mucoid
bacterium produces the polysaccharide alginate (6). This can protect
the bacteria from phagocytosis by host defences and from the effects
of exogenously applied antibiotics (4, 12, 19). In addition, further
phenotypic changes occur; flagellae and pili are lost (14), biofilms are
established (4, 19), and the bacterium becomes non-motile (19). The
rate of infection with multidrug-resistant *P. aeruginosa* has increased
substantially in older patients in recent years, perhaps resulting from
the exposure to multiple antibiotics over a lifetime. This imposes
selective pressure on the infecting population where resistant
phenotypes are favoured (21).

1.1.3 *S. aureus* infections in CF

*S. aureus* is the most commonly isolated CF pathogen in infants and
young children and was the first pathogen linked to lung disease in
CF. *P. aeruginosa* was not recognised as a major pathogen until the
1950s. Previously, when average life expectancy was below ten years,
most CF bacterial cultures were collected from the age groups where
*S. aureus* is most prevalent (11). The bacterium is found in 80% of
patients aged 6-10, being replaced by *P. aeruginosa* as the main
pathogen by the age of 18 (21). CFTR dysfunction may contribute
directly to *S. aureus* infection. For example, it has been shown that the
infection of Calu-3 cells by *S. aureus* increases when CFTR activity is
removed (22). Despite its prevalence in younger patients and historical
pathogenicity, whether *S. aureus* contributes to decreased survival in
modern times is controversial (11). While it has been shown that
infection with *P. aeruginosa* is often preceded by *S. aureus* infection in
infancy (23), it has been asserted that the latter does not have a major
impact on the status of patients (for example, it is cultured less as lung
function declines) (24). Of greater clinical concern is the increasing
proportion of methicillin-resistant *S. aureus* (MRSA) among *S. aureus*,
which is being increasingly isolated from clinical samples, currently
found in 25% of CF patients (21). Anecdotal evidence suggests that
more virulent strains of MRSA may potentially cause severe lung damage (11).

1.1.4 *H. influenzae* infection in CF

*H. influenzae* is another common pathogen in CF, present in approximately 16% of patients (21). Like *S. aureus* it most regularly infects patients in childhood, being one of the first organisms to infect the lung, and again like *S. aureus* its pathogenicity is controversial (11). The isolation of *H. influenzae* from CF lungs has been observed to increase during acute exacerbations (25), but the presence of *H. influenzae* (and *S. aureus*) have been found to not be associated with lower-than-predicted lung function (26). Nevertheless, the expert opinion is that this bacterium has the potential to cause exacerbations, and when these occur they should be treated (11).

1.1.5 The *B. cepacia* complex

The *B. cepacia* complex (Bcc) is a group of 17 related Gram-negative bacterial species that have become notorious for their ability to spread rapidly between CF patients and cause great morbidity, even beyond that of *P. aeruginosa*. The phylogenetic similarities of the species make them difficult to separate, but it has been determined that *Burkholderia cenocepacia* and *Burkholderia multivorans* account for the majority of Bcc CF isolates (11). Unlike *S. aureus* and *H. influenzae*, it generally infects patients later in life with a relatively low prevalence of approximately 3% (21). The low rate of infection compared to other bacteria discussed here is fortunate when one considers that it has been shown to have a significant adverse effect on the survival of CF patients (27). The prognosis after *B. cenocepacia* infection is poor, with the estimated 5-year survival rate after acquisition of one strain being 66.6%, in comparison to 85.3% with *P. aeruginosa*. It must be noted, however, that virulence is strain-dependent. Of great concern is the fact that it is intrinsically resistant to polymyxins, aminoglycosides, and most β-lactam antibiotics (28).
1.1.6 Fungal infections in CF

The presence of the fungi *A. fumigatus* and *C. albicans* is also commonly reported in CF lung samples, with recent average prevalence across a series of US centres reported at 11.9% and 7.8% respectively (21). The lungs by their nature are continuously exposed to the atmosphere which is a rich source of fungal spores, and as with bacteria the CF lung has limited capacity to clear them (29). A large proportion of CF patients infected with *A. fumigatus* may exhibit allergic bronchopulmonary aspergillosis (ABPA), where the infection induces an allergic response to the fungus. Episodic wheezing ensues that is unresponsive to bronchodilators but may be treated with corticosteroids. However, the degree to which infection can lead to long-term lung function decline is controversial. There is a correlation between non-ABPA *A. fumigatus* infection and poorer lung function but whether the relationship is causal has yet to be satisfactorily determined (11). The effectiveness of treatment with antifungals such as itraconazole has also yet to be demonstrated beyond doubt in both ABPA and non-ABPA *Aspergillus* infection (29). *C. albicans* can cause oral and genital candidiasis in CF patients, as well as infecting the lung. Life-long treatment with inhaled steroids and antibiotics puts CF patients at increased risk of acquiring it. However, the clinical significance of *C. albicans* infection is unclear and there is no evidence supporting treatment (29).

1.2 Cystic fibrosis and the immune response to infection

Bacterial infections must be recognised by the lung epithelium before the immune system can launch an appropriate response. This is achieved using a broad range of Toll-like receptors (TLRs) to sense pathogenic molecules associated with bacteria. For example, LPS is detected by TLR4, and flagellin by TLR5. In CF, dysfunction in these systems contributes to airway pathology via the combination of TLR hyper-responsiveness and an abundance of TLR agonists that are not
competently removed by mucociliary clearance (15). The manner in which airway dysfunction leads to TLR overstimulation can be illustrated by the case of the protease neutrophil elastase (NE). This enzyme is released in large quantities by CF neutrophils (30), activates TLR4 and causes exaggerated release of the cytokine interleukin-8 (IL-8) (31). IL-8 itself is a potent chemoattractant for NE-producing neutrophils, leading to an amplified immune response and positive feedback loop of immune system stimulation (13). Other components of the CF lung milieu have similar effects, for example haem, released into the lung from microbleeds resulting from tissue damage, also activates the TLR system in a similar manner (32). Sex also has a role in the immune response to infection, the prognoses for female CF patients are worse than that in males. The cause for the discrepancy is thought to involve the female hormone 17β-estradiol. Although its role in vivo is complex, this hormone may have a net pro-inflammatory effect in CF. Exogenous application of the hormone in male CFTR−/− mice has been shown to exacerbate inflammation in P. aeruginosa infection and lead to increased lung bacterial burden (33). The hormone also contributes to the sexual dichotomy of the disease by promoting the early conversion of P. aeruginosa to a mucoid phenotype. It has been observed that the median age of chronic infection with this phenotype is 1.7 years earlier in females and that the frequency of infective exacerbations is increased (34).

The constant activation of innate immune receptors by pathogens (and host molecules) leads to a dysregulated overproduction of cytokines such as IL-6 and tumour necrosis factor-α (TNF-α). The resulting pro-inflammatory cascade then recruits neutrophils from the bloodstream to combat the inflammatory insult. In the healthy lung these immune cells are vital for killing bacteria, using three mechanisms: phagocytosis, granule release of agents such as NE, and neutrophil extra-cellular trap formation (13). This component of the immune response is also dysregulated in CF. Numerous studies have demonstrated how CFTR dysfunction can lead to differences between
normal and CF neutrophils that significantly contribute to lung damage rather than ameliorate it. For example, phagocytosis by neutrophils has been shown to be impaired in paediatric CF patients compared to controls (35). CFTR is present in phagolysosomes of neutrophils, and its defective activity leads to lower Cl⁻ in the compartments, which is required for their activity against *P. aeruginosa* (36). In a neutrophil of a healthy patient, a phagocytosed foreign particle triggers the formation of O₂⁻ by the enzyme NADPH oxidase, which quickly forms H₂O₂. Myeloperoxidase, another enzyme, can then catalyse halogenation (and death) of the foreign particle by using H₂O₂, Cl⁻ and the correct pH conditions to form hypochlorite. This is called the respiratory burst and can cause oxygen consumption by neutrophils to increase 100-fold (37). Inappropriate O₂⁻ production by CF neutrophils has also been linked to tissue damage. One study observed that after an exacerbation, CF neutrophils release larger quantities of O₂⁻ compared to healthy controls. The spontaneous release of NE was also elevated (38). In a similar manner, and despite the fact that the NE content of the granules of CF neutrophils is not increased, an elevated elastase response to bacterial stimulus compared to normal neutrophils has been noted with CF neutrophils after pre-incubation with bronchoalveolar lavage (BAL) fluid. This indicates that CF neutrophils intrinsically release more NE in response to bacterial stimulus (30).

Neutrophil chemotaxis (movement in response to a chemical stimulus) in CF infection has been demonstrated to differ in comparison to healthy controls, displaying a degree of hypo-responsiveness to IL-8. This may illustrate receptor desensitisation as a result of persistently high levels of IL-8 present in the lung. However, as noted in a review of CF neutrophil dysfunction, the high levels of IL-8 in CF ensures that recruitment is not reduced *in vivo* and in the absence of infection, CF neutrophils can out-migrate their non-CF counterparts (37).
1.2.1 Sequelae of neutrophil dysfunction in CF

While they are essential to clearing infection, the dysfunctional action of neutrophils in CF leads to tissue damage and remodelling (13). The neutrophil-dominated response to *P. aeruginosa* leads to the release of large quantities of NE which, apart from its ability to stimulate IL-8 release as detailed above, causes extensive tissue damage (39, 40). Thus, the exaggerated inflammatory response leads to long-term reduction in lung function and is associated with premature death (17, 19, 20). Neutrophils represent approximately 70% of the airway inflammatory cell population in CF, in contrast to 1% in healthy patients (41). This is mostly related to elevated neutrophil chemokine levels in the lung as a result of the ineffective clearance of *P. aeruginosa* (39). The lungs of the majority of CF patients appear normal at birth, however chronic neutrophilic inflammation begins early, with one study finding that neutrophils represented on average 31.5% of immune cells for a group of 1 to 5 year old children (42).

High NE levels overwhelm epithelial antiprotease defences, which normally protect against proteolytic damage, and can inactivate other components of the immune response, such as complement and immunoglobulins (41). The enzyme may further contribute to impaired bacterial clearance by inhibiting the receptors that mediate pathogen recognition such as CD16 (37). It can also cleave flagellin, compromising the host’s ability to recognise and react to the molecule (43).

Dysfunctional neutrophil death is a major contributor to the lung disease of CF. The necrosis of the large number of neutrophils results in the release of extra-cellular DNA (eDNA), further increasing mucous viscosity (19). When neutrophils, which normally have a short lifespan, are inhibited in the normal process of controlled apoptosis, tissue damage can be increased by the uncontrolled release of pro-inflammatory substances. Host and bacterial mediators such as LL-37 (44) and LPS (45) have been shown to delay apoptosis, an effect which may help clear infection in healthy lungs but has the potential to
exacerbate lung disease in CF. To resolve inflammation properly, neutrophils must first apoptose and then be cleared to prevent the above effects. Many of the conditions of the CF lung confound this process. For example, pyocyanin, a metabolite produced by \textit{P. aeruginosa}, has been shown to impair the engulfment of apoptotic neutrophils by macrophages, leading to a large increase in necrotic neutrophils in an \textit{in vivo} mouse model (46).

In a healthy lung, a balance exists between proteases and anti-proteases, such as secretory leukoprotease inhibitor (SLPI), α1-antitrypsin (A1AT), and elafin. This facilitates tight regulation of protease activities and is required to avoid the lung damage and immune dysfunction detailed above (42). Despite their potential for damage in CF, proteases have an important protective role in a healthy lung and this can be illustrated by the effects of their absence. \textit{S. aureus} and \textit{C. albicans} have both been shown to be markedly more virulent in mice lacking neutrophil-granule proteases (47). In another study, an NE-knockout mouse model was found to be more susceptible to \textit{Klebsiella pneumoniae} and \textit{Escherichia coli} \textit{(E. coli)} infection than wildtype mice, confirming that NE is crucial for the antibacterial action of neutrophils (48).

In CF the protease-antiprotease balance is dysregulated and proteases produced from the neutrophil-dominated epithelial inflammation can overwhelm the inhibitory defences (42). Unlike in many other lung diseases, the issue is not one of antiprotease deficiency but more of enzyme excess. High neutrophils numbers mean that large quantities of unopposed proteases are released into the ASL (41). This leads to lung matrix destruction and inflammation (49). There are several proteases from different sources, both host and bacterial, including serine, aspartyl, and metallo-proteases that are relevant to CF (32).
1.3 Proteases in cystic fibrosis

Neutrophils are the source (although not exclusively) of many of the proteases associated with CF. The azurophilic (or primary) granules of neutrophils are the source of the serine proteases NE, cathepsin G (CG), and proteinase 3 (PR-3). These enzymes share significant sequence homology and play an important role in both infectious and non-infectious inflammation e.g. by digesting phagocytised microorganisms and other ingested material intra-cellularly (43, 50). In response to cytokines such as IL-8 and bacterial LPS, large quantities of proteases can be released extra-cellularly (49). Like all serine proteases they are characterised by the presence of serine in their catalytic centre, and all three belong to the chymotrypsin superfamily. However they differ in their substrate specificity, with NE and PR-3 preferentially accommodating hydrophobic residues such as alanine and isoleucine and CG preferring phenylalanine and methionine and positively charged residues such as arginine (49).

In normal protease notation, substrates are cleaved between amino acids termed P1 and P1’ (amino- and carboxy-terminal residues respectively). The adjacent residues on each side are named P2, P2’, P3, P3’ and so on (51).

1.3.1 Neutrophil elastase

NE is the most important protease in CF, with a number of proteolytic and immunomodulatory properties. Its name would suggest activity against elastin (the first substrate used to characterise its activity (49)) but NE can cleave a wide variety of matrix and plasma proteins ranging from collagen and fibronectin, to complement and protease inhibitors (41). It contributes to the killing of bacteria, both extra- and intra-cellularly, allowing the clearance of foreign material after phagocytosis by the neutrophils. It is also involved in the regulation of the innate immune response (39). NE itself can stimulate the release of other proteases such as cathepsin B and matrix metalloprotease-2
(MMP-2) and may stand at the peak of a hierarchy of protease regulation (52). The abundance of NE in CF is the source of much morbidity for patients, it is the primary source of haemoglobin degradation in CF BAL fluid, producing haem, which can then stimulate the production of IL-8 (32). It can also directly stimulate the release of IL-8 from bronchial epithelial cells, leading to enhanced neutrophil accumulation and a cycle of inflammation (53). The observation that the application of SLPI can remove 90% of the elastinolytic activity of CF sputum is indicative that NE represents the main source of protease activity in the CF lung (SLPI is an inhibitor for NE but not PR-3) (32, 54).

1.3.2 Proteinase 3 and cathepsin G

PR-3 is thought to account for approximately 7% of the remaining protease activity of the CF lung (32) although levels higher than NE have been reported in CF sputum in one study (55). This serine protease has an important role in the regulation of the precursor Host Defence Peptide (HDP) hCAP-18. PR-3 and hCAP-18 are both located in different compartments of neutrophils, but upon exocytosis the protease cleaves the HDP, releasing the active peptide LL-37. Both CG and NE also cleave hCAP-18 but at different sites and do not generate the active LL-37. The activation cleavage site on hCAP-18 is an Ala-Leu bond, a bond preferred by PR-3. The enzyme has a preference for cleaving between two small aliphatic amino acids, in contrast to NE which prefers to cleave at a valine (56). This illustrates that, despite the sequence homology between the three serine proteases and many shared substrates, they can still display different cleavage patterns to each other. For example, PR-3 is unable to cleave the NE substrate Suc-AAA-Nitroanilide. PR-3 is also 40 times less catalytic than NE against the substrate MeO-Suc-AAPV-Nitroanilide, a common substrate for many NE-quantification assays (cleavage of the amide bond releasing a chromophore) (50) while CG does not cleave it at all (57). CG has a significant preference for
substrates with a phenylalanine at P1 (58). The differences extend to inhibitors, PR-3 is not inhibited by SLPI, but is inhibited by A1AT for example (50).

CG is generally less active against most substrates than the other serine proteases, having a lower intrinsic catalytic activity (59) that has been estimated to be two orders of magnitude lower than NE (58). However, it has still been shown to cleave components of the extracellular matrix (but not to the same extent as NE) (60). Like NE, it has also been shown to be crucial for killing of bacteria by neutrophils (47). However in one study the clearance of *P. aeruginosa* from mouse lungs was improved in CG-deficient mice, illustrating the potentially harmful effects of the unopposed protease on the lung immune response (61).

### 1.3.3 Pseudomonas proteases

The contribution of proteases from *Pseudomonas* in CF is often overlooked. The bacterium produces two metalloproteases relevant to CF, pseudomonas elastase (PE) and alkaline protease that have a number of important biological properties. Like NE and PR-3, both metalloproteases cleave haemoglobin, allowing the bacterium to use microbleeds in the CF lung as a potential iron source (32). They also degrade many components of the host immune system and enhance survival of the bacteria. For example, PE can cleave and inactivate LL-37, reducing the bactericidal activity of the HDP *in vitro*, an effect abolished by the addition of metalloprotease inhibitors (62). Like NE, PE can degrade elastin and a number of other substrates, hydrolysing a number of tetrapeptide sequences such as tetra-alanine (63). *Pseudomonas* metalloproteases have also been shown to degrade the protease inhibitors SLPI, A1AT, and elafin. Purified PE was shown to reduce the NE-inhibitory effects of elafin, disturbing the lung’s anti-elastase defences (64). While PE is a major virulence factor for *Pseudomonas* and can cause extensive damage to host tissue, it has
a biological function in the bacterium beyond virulence, acting as an important mediator in the production of alginate (65).

1.3.4 Host metalloproteases

MMPs are a large group of zinc metalloproteases that have a number of functions in the healthy lung. These include degradation of membrane components and their repair, cytokine release, and modulation of cell mobility. They are characterised by an active catalytic domain with a zinc-binding region. When they are dysregulated, their ability to degrade the membrane can lead to pathogenic destruction of the lung epithelium and the impairment of repair. Some have been the subject of much interest in CF, e.g. MMP-9. Others, such as MMP-2, while known to contribute to the pathogenesis of diseases such as cancer, have been studied less in the context of CF (66).

MMP-9 is one of the best characterised proteases and is released from the tertiary granules of neutrophils. Enzyme levels have been found elevated in the sputum of CF patients compared to healthy controls (with a mean of 1.21 µg/ml). In the same study MMP-8, on the other hand, was found to be decreased in CF patients. While MMP-9 is normally held as an inactive pro-form in healthy lungs, it is found constitutively active in CF. In addition, the high levels of NE in CF are able to both activate MMP-9 and inactivate its inhibitor (67). Substrate studies indicate that, because of the structure of its active site, MMP-9 prefers to cleave near a large hydrophobic residue, showing marked preference for leucine over alanine. An arginine residue at P2’ and a proline at P3 have both been shown to highly favourable for proteolysis (68).

MMP-7, the smallest known MMP, is thought to have a major role in repair of the epithelium and is found in the conducting airways of healthy patients. Its expression has been found to be upregulated in CF, especially at sites of epithelial damage. It is normally not found in
the lower bronchioles and alveoli but expression has been observed in CF alveolar cells (69).

MMP-12 is secreted by macrophages and may play a role in the killing of Gram-negative and -positive bacteria (66). It is also detectable in the sputum samples of CF patients (67). In a CF mouse model, it has been demonstrated that upregulation of the genes expressing MMP-12 is linked with worsening emphysema and that MMP-12 activity is increased on the surface of mouse and human macrophages in CF, but not found free in mouse BAL fluid. MMP inhibition and genetic deletion have both been shown to reduce airway disease in these mice (70). It shares a number of substrates with MMP-3, -7, -13, all characterised by the presence of proline (71). This seems to be a common requirement for MMP substrates, for example PLGLWAR is cleaved very efficiently by a number of MMPs at the Gly-Leu bond and forms part of a very sensitive reporter for MMP activity (72).

1.3.5 Summary

Both host and bacterial enzymes contribute to the cycle of infection and inflammation that characterises the lung disease of CF. Figure 1.1 illustrates how *P. aeruginosa* can directly and indirectly cause tissue damage, with proteases being central to the perpetuation of the cycle.
Figure 1.1: The cycle of infection and inflammation in CF. *Pseudomonas* can directly damage host tissue but also induce the release of pro-inflammatory cytokines. These recruit neutrophils which can also damage host tissue by releasing proteases. The proteases degrade antimicrobial peptides as well as other components of host defence. This facilitates further infection, which continues the cycle. This continues with progressive tissue damage that eventually leads to fatal pulmonary disease. Taken from (11).

1.4 Management of cystic fibrosis

In addition to nutritional and digestive enzyme therapy, the main focus of CF treatment is the eradication of pulmonary infection with antibiotics. As stated previously, the primary source of morbidity and mortality in CF is persistent lower respiratory tract infection. The most common pathogens are *S. aureus* and *H. influenzae* in young children, and *P. aeruginosa* in older children and adults (10). Rigorous antibiotic therapy with drugs such as inhaled tobramycin is recommended;
improving lung function and reducing the number of pulmonary exacerbations. Other antibiotics used include inhaled colistin and ciprofloxacin (15). While systemic antibiotics also form an important part of the treatment strategy, inhaled antibiotics have several advantages, including allowing higher local drug concentrations while limiting systemic toxicity (as infection is restricted to the endobronchial space, systemic delivery is not an absolute requirement) (20).

Antibiotics delivered systemically operate mainly in the alveolar sacs while those that are inhaled have their effects from the trachea to the bronchioles. A combination of both routes would theoretically provide maximum coverage, although the use of inhaled antibiotics alone has had considerable success (10).

Other components of CF treatment include hypertonic saline, delivered at concentrations of 3-7% w/v, which improves lung function through a variety of means, including restoring the height of the ASL and increasing mucociliary clearance (73). Similarly, dornase alfa (recombinant human deoxyribonuclease) is used as a mucolytic. It is an enzyme that selectively cleaves eDNA and was the first drug developed specifically for CF (74). It non-specifically hydrolyses double stranded DNA into low molecular weight fragments, relying on Mg$^{2+}$ for catalysis (75). Cleaving the high levels of eDNA can improve the access of antibiotics to bacteria and co-administration has been demonstrated to improve the ability of both endogenous and exogenous HDPs to kill *P. aeruginosa*; complexation of LL-37 to eDNA has been shown to be detrimental to activity (76, 77). Functionalisation of tobramycin/alginate/chitosan nanoparticles with dornase alfa has been shown to increase the ability of the antibiotic to penetrate CF sputum (78). NE is also bound to eDNA, and treatment of patients with dornase alfa has been demonstrated to increase the levels of free NE in sputum, releasing the enzyme from eDNA-complexation. However, this effect is transient and long-term therapy modestly reduces the protease burden in the CF lung (79).
Despite improvements in quality of life and longevity, CF and the treatments involved place a significant burden on patients. Daily exercises and treatments can take up to 2h each day (15, 20). The importance of this disease in an Irish context is clear from the high incidence rate relative to other countries, 1 in 1461 births compared to 1 in ~2000 in Caucasians in general (80). The combined prevalence of ∆F508 homozygous and heterozygous mutations is 90.18% and 13.9% for the G511D mutation (the next most common). 65.4% of adult Irish CF patients are chronically infected with P. aeruginosa (81).

1.4.1 CFTR modifier use in CF

The identification of the mutations that cause CF has led to intensive efforts to discover small molecules that can directly correct dysfunctional CFTR activity. The resulting compounds have begun to reach clinics and may be divided into “correctors”, which promote the maturation of CFTR, and “potentiator”, which increase the open time of existing CFTR channels (82). Treating a CF patient with a modifier requires one to consider the consequences of the specific mutation (of which there are >1000) on the CFTR. The many mutations are divided into five classes. Class I comprises synthesis defects, Class II protein processing defects, Class III protein regulation defects, Class IV protein conductance defects, and Class V reduced functioning protein. Class I and II have the highest mortality rates (37). G551D is a class III defect and results in impaired gating of the channel, and ∆F508, while primarily a class II defect where the CFTR is degraded before reaching the membrane, encompasses both a trafficking and gating defect (3).

Ivacaftor is a CFTR potentiator, which can restore CFTR function in patients with the G551D mutation and was the first approved treatment that addresses the underlying cause of CF. However, it has only been found to be effective in patients with the specific mutation, who represent less than 5% of all CF patients worldwide (83). There may also be some benefit in patients with similar defects to G551D but it is
estimated that in total this only represents 10% of the CF population at most (3). The increase in the chloride transport afforded by the drug is only effective if the CFTR receptor reaches the cell surface, which is not the case in ΔF508, and a clinical trial investigating the use of ivacaftor in ΔF508 found no benefit over placebo (84).

The combination of trafficking and gating dysfunction in ΔF508 requires the co-administration of both a corrector to allow delivery of the protein to the cell surface, and a potentiator to allow proper gating and ion flow. Corrector molecules have been developed, and one, lumacaftor was the subject of a clinical trial with homozygous ΔF508 patients. In vitro data had previously demonstrated significant correction of the defect, but this was not replicated in vivo, where no improvements in lung function were observed. The implication is that more than one corrector is needed to overcome the defect in patients (3). The combination of ivacaftor and lumacaftor has also been investigated, hoping to combine the effects of both to overcome the dual defect in ΔF508. A phase II trial demonstrated that the combination could provide a significant improvement in lung function (85). Both studies were carried out over a relatively short time period, and the effects of long-term therapy are not yet fully understood. One in vitro study has demonstrated that chronic ivacaftor treatment may increase the turnover of lumacaftor-corrected CFTR channels, possibly reversing the effects long-term and requiring longer-term in vivo studies (82). However, on the basis of two 24-week phase III trials, TRANSPORT and TRAFFIC, a new drug application was submitted by the manufacturers for the use of the combination of lumacaftor and ivacaftor in ΔF508 CF patients (86). The results indicate an improvement in lung function compared to placebo, with an absolute improvement of forced expiration volume (FEV₁) of 2.6 to 4.0%, a reduction in lung exacerbations, and increased weight gain (possibly due to decreased energy expenditure) (87). The use of the combination (brand name Orkambi®) in ΔF508 patients over 12 years old was approved by the US Food and Drug Administration in July.
2015 (88). However, it must be noted that the improvements were not as profound as with the treatment of G551D patients with ivacaftor, where an absolute FEV\textsubscript{1} improvement of over 10% was observed, as was an even greater reduction in exacerbations. The authors of that study noted that ivacaftor compares well with FEV\textsubscript{1} improvements seen with other treatments, e.g. 12%, 5.8%, and 3.2% for tobramycin, dornase alfa, and hypertonic saline respectively (89).

The cost of therapy with these small molecules remains a major issue, with the initial estimated annual cost of ivacaftor treatment per patient being €243,804 in Ireland. The National Centre for Pharmacoeconomics advised against the reimbursement of the drug as a result (90). However, political pressure and renegotiation of the price with the manufacturer led the government of Ireland to approve its provision to patients (91). The financial burden of providing the ivacaftor, in conjunction with the reduced efficacy of the combination therapy in ΔF508 patients, may potentially prevent the use of these drugs in the wider CF population.

1.5 Host defence peptides

HDPs are short, cationic, amphipathic, peptides that play a crucial role in the innate immune system of all multi-cellular organisms (92). They are generally 12-50 amino acids in length with a net positive charge due to an excess of basic residues such as lysine and arginine. In addition, they contain over 30% hydrophobic residues (92, 93). Some HDPs with a net negative charge have also been described such as SAAP, but they are the exception (94, 95). Many of these highly-conserved peptides possess substantial antimicrobial activity (96), which is initiated through electrostatic interactions with the anionic phospholipid head-groups of the bacterial cell envelope. This can lead to either membrane perturbations or translocation across the membrane and interaction with various intracellular targets (97-99).
Nowhere is the importance of HDPs to innate defence more apparent than in those animals that have no adaptive immunity, such as invertebrates. Insects, for example, are entirely reliant on the production of a number of highly-active HDPs, reaching µM concentrations in the insect haemolymph a few hours after initial infection. This is a process that is 100 times more energy efficient than immunoglobulin production, and over 100 times faster. The highly active peptides of insects have been the subject of much interest for exogenous application in humans but are not without their drawbacks: many are toxic to eukaryotic cells and, while they are relatively stable in insect haemolymph, are highly susceptible to mammalian proteases such as trypsin (100). They can, however, be modified to produce desirable characteristics while maintaining their activity. For example, the peptide Cecropin A from the silk moth larvae, *Hyalophara cecropia*, has been combined with the peptide Magainin 2 from the clawed frog, *Xenopus laevis*, to produce the hybrid peptide P18, which displays both high activity against bacterial cells and low haemolytic activity against mammalian erythrocytes (101).

### 1.5.1 Classes of HDPs

There are a wide range of HDPs described in the literature with diverse characteristics and, at times, little sequence homology, which complicates their classification. However, there are several structural characteristics, crucial for activity, shared between HDPs that go beyond the simple presence of hydrophobic and cationic residues that may be used to group them.

Many HDPs share three-dimensional topology and this can be used to classify them. Alternative methods of classification are by source, precursor, or intramolecular bonds. The two largest structural groups are the α-helical and β-sheet peptides. This method of classification may be broad at times, with many of the former only becoming helical upon interaction with amphipathic membranes and the latter representing a highly diverse group in structure. Despite this, both
groups can be characterised by structurally distinct hydrophobic and hydrophilic regions which mediate membrane interaction and are crucial for the pore-formation and cell disruption (102).

The main mammalian families of HDPs include the defensins, cathelicidins, and histatins. Defensins consists of parallel $\beta$-sheets linked by disulphide bonds (classed by structure). Cathelicidins consist of a highly conserved precursor domain (classed by precursor, but including among them $\alpha$-helices or disulphide-bonded peptides) (103). The diversity of sequences is enormous, with one species potentially possessing dozens of different HDPs, and with no similar sequences found in related organisms. This may be due to rapid evolution of sequences, with modification occurring as each species faces very different microbial challenges (92). The result of this rapid evolution is a rich library of potential sequences, of varying characteristics and activities that may be harnessed for exogenous use against infection (104).

### 1.5.2 Mechanisms of action of HDPs

Several models exist for HDP membrane disruption and each depends on the characteristics of the HDP (Figure 1.2). However, all rely on the exploitation of the fundamental differences between mammalian and bacterial cell membranes, including composition, transmembrane potential, and structural features. A key feature for a HDP is the degree in which it can differentiate between membranes. While all biological membranes are essentially composed of amphipathic phospholipid bilayer, beyond this, prokaryotic and eukaryotic membranes differ greatly in composition. Human cell membranes are predominantly composed of the lipids phosphatidylethanolamine, phosphatidylcholine, and sphingomyeline, all of which are without net negative charge. This is an important characteristic for the initial electrostatic interaction with the cationic peptides. In contrast, bacterial membranes, composed predominantly of phosphatidylglycerol, cardiolipin, and phosphatidylserine, are generally negatively charged
(102). In cell membrane-mimicking liposomes, the increased net negative charge of lipid membranes has been shown to increase HDP adsorption and HDP-induced leaking of liposomal contents compared to zwitterionic membranes, demonstrating the higher affinity of the HDPs for anionic lipid components (105). Bacteria are completely lacking membrane sterols such as cholesterol or the ergosterol found in fungal membranes. Cholesterol condenses the lipid bilayer and renders the adsorption of bulky amino acids such as tryptophan, found commonly in HDPs, energetically unfavourable (105). Mammalian membranes also have a lower transmembrane potential than bacterial membranes, $\Delta \Psi$ of -90 to -100mV versus -130 to -150mV respectively. This difference may also act to concentrate the positively charged HDPs on the bacterial cell surface. In general, the membranolytic action of HDPs is non-stereospecific, a fact that is demonstrated by the comparable antimicrobial activity of both natural L-amino acid HDPs and their D-amino acid isoforms (102).

There are many structural determinants of HDP activity, including conformation, net charge, amphipathicity, hydrophobicity, and polar angle. They confer specificity for bacterial membranes to HDPs in different ways. A high cationic net charge allows strong initial electrostatic interaction and is correlated with activity. However, mammalian cytotoxicity can ensue when the net charge is too high. Similarly, high amphipathicity and hydrophobicity is linked to high activity (linked to portioning the lipid bilayer) but increasing it beyond a certain threshold leads to the loss of antimicrobial specificity. The polar angle, i.e. the relative proportion of polar and non-polar facets in a peptide helix, must also be balanced: too acute and membrane pore formation rate is weakened, too obtuse and pore stability is compromised (102).

1.5.3 Models for membrane disruption by HDPs

Several models exist for membrane disruption. The barrel-stave model concerns the aggregation of HDPs in a barrel-like ring on the bacterial
membrane, forming an aqueous pore and the leaking of cytoplasmic components (106). The peptides initially bind to the membrane as monomers and self-aggregate after reaching a threshold concentration. The hydrophilic surfaces of the peptides then face inward, forming pores of differing diameters spanning the membrane. The threshold concentration for this model, and indeed all pore forming models, is dictated by not just the peptide’s propensity to self-assemble (with distinct hydrophilic and hydrophobic domains being crucial) but also membrane phospholipid composition, fluidity, and transmembrane potential. Relatively few peptides have been demonstrated to display this activity, and a requirement is that the peptide must be long enough to span the membrane (102).

In the toroidal pore model another channel is formed, but lipids are intercalated with the adsorbed HDPs and a fold in the membrane is induced. This model is more common than the barrel-stave (106). The curvature strain that is induced by the lipid-peptide complex (relative ratio of 1:30 with Magainin for example) causes a transient pore to form. The eventual disintegration of the pore may also be the mechanism by which many peptides enter into the cytoplasm. Membrane-spanning length is not a requirement for peptides in this model (102).

The carpet model involves a detergent-like effect induced by high concentrations of HDPs accumulated at the membrane surface. This is not, however, as indiscriminate a process as it sounds and is a common mechanism for many HDPs (106). The adsorption of the peptides causes the displacement of phospholipids, changes in membrane fluidity, and the reduction in barrier properties. When a high concentration is reached, rather than forming a pore as above, the peptides cause unfavourable membrane energetics, with the loss of membrane integrity ensuing. Cecropin is an example of a peptide that uses this mechanism (102).
Other mechanisms have been described and one possible target for HDPs are intra-cellular components, with the possibility existing for a peptide to kill bacteria without the involvement of membrane disruption. HDPs could, for example, bind to negatively-charged DNA fragments. Buforin II has been shown to kill bacteria by disrupting intra-cellular functions (102).

Because HDPs have multiple modes of action, including membrane depolarisation, pore formation, induction of degradative enzymes and disruption of intracellular targets, bacteria may have a lower propensity for the development of resistance to HDPs compared to conventional antibiotics. This would potentially increase the lifetime of any exogenous HDP in clinical use (107) and provide another option for antimicrobial therapy. In addition, their ability to permeabilise bacterial membranes may facilitate the activity of conventional antibiotics (102, 108).

Figure 1.2: Three models of HDP disruption of bacterial membranes. Barrel-stave (A), Toroidal pore (B), and Carpet (C). Taken from (106).
1.5.4 Immunomodulation of HDPs

In addition to antibacterial activity, many HDPs possess immunomodulatory effects, involving cellular recruitment and stimulation of components of innate immunity (93). This has been extensively investigated with human cathelicidin, LL-37, found in high concentrations at sites of inflammation. LL-37 has been shown to have a chemotactic effect on immune cells such as neutrophils and monocytes at high concentrations (109), attracting them to the site of infection and helping clear the inflammatory insult (110). LL-37 can modulate the release of pro-inflammatory cytokines such as IL-8 (and has been found to directly stimulate IL-8 release itself) in response to stimuli (111). It can also both reduce and increase cytokine levels in response to LPS in different cell types (112, 113). Although these differing effects may appear to be paradoxical, its role in vivo may involve maintaining a balance between pro- and anti-inflammatory responses (110). The mechanism of action is still not fully elucidated and while the “formyl peptide receptor-like” receptor (FPRL1) has been found to play a role (44, 109, 114), the action of LL-37 may not be entirely receptor-mediated. For example d-amino acid LL-37 can induce a greater IL-8 response than the natural l-amino acid sequence. This indicates that the response may be related to membrane effects and, like with antimicrobial activity, not stereospecific (115). LL-37 is not alone amongst HDPs in immunomodulation, e.g. Human β-defensin 2 has been shown to induce some neutrophil chemotaxis (116), and human defensin 5 stimulates IL-8 release in epithelial cells (117). This is not surprising when one considers that defensins in general, with their β-sheets and disulphide bonds share many structural features with chemokines. Similarities between both are further demonstrated by the fact that many chemokines have direct antimicrobial activity themselves (103). Immunomodulatory HDPs have also been rationally-designed peptides. The peptide Innate Defence Regulator-1 (IDR-1), for example, has been shown to be protective against infection in vivo,
with an accompanying modulation of cytokines such as IL-6 and IL-10, but without direct \textit{in vitro} antimicrobial activity (118).

\subsection*{1.5.5 Shortcomings for HDP therapeutics and potential solutions}

Despite the wealth in number and characteristics, the development of HDPs as antimicrobial agents has been hampered so far by a series of shortcomings. The main issues include the cost of production, lability to proteases and potential for toxicity. Shorter peptide sequences can address some of the cost issues and the use of non-natural amino acids (such as \(\text{D}\)-amino acids) can prevent protease degradation (as the mechanism of action is not stereospecific, as stated above, it will not compromise activity). Toxicology can only be dealt with via careful selection of sequence and proper screening (104).

\subsection*{1.5.6 Potential use of HDPs in CF}

The rationale exists for using HDPs as exogenous therapeutics for CF as the activity of endogenous HDPs can be compromised. In addition to other immune components, pulmonary proteases in CF cleave endogenous HDPs. This can take the form of the proteinase 3-regulated activation of LL-37 from hCAP-18 (39), but also degradation and inactivation by NE. The effect of the dysregulation of protease expression in CF has been demonstrated by comparing BAL fluids of healthy controls and CF patients. The former will process hCAP-18 to LL-37, while the latter will further degrade LL-37 (119). Similarly, the \(\beta\)-defensins have been shown to be cleaved and inactivated by cathepsins B, L, and S. Again, these enzymes are present in CF BAL fluid (120). In addition to high protease activity, it has been suggested that CFTR dysfunction leads to high pulmonary salt levels, which can deactivate HDPs (121). However, whether the ionic composition of the ASL is abnormal in CF is a subject of debate (5, 122-124).

Compounding the impaired host HDP function in CF, fears over bacterial resistance in CF provides further impetus for developing
HDPs as therapeutics. The growth of resistance has led to previously-restricted therapeutics, such as the use of colistin, being re-examined and re-entering the clinic (125). Concern is growing in particular over the development of multi-drug resistant *P. aeruginosa* and the need for new drugs to treat it (10, 15, 20).

However, the issues of protease degradation and salt-inactivation are not the only barriers to HDP use in CF. Significant research has been undertaken to develop HDPs as viable therapeutics for a host of other infectious diseases, but progress has been limited by a variety of factors. While a number of HDPs are the subject of clinical trials, none have yet received approval, and the majority of these trials focus on topical applications (107, 126); rapid metabolism and a lack of affinity for their target means that HDPs often have very narrow therapeutic indices (107, 127). An issue with rational design, as seen above, is that increased activity, *e.g.* due to increased hydrophobicity, is often at the expense of selectivity between eukaryotic and prokaryotic membranes and results in host toxicity (103). Even endogenous HDPs, such as LL-37, can be cytotoxic at high concentrations (128).

Some of the most potent natural HDPs are the β-hairpin peptides, such as polyphemusin 1 derived from the horseshoe crab. It has a very low MIC against *P. aeruginosa* of 0.25µg/ml (129). Though highly active, it exhibits haemolytic activity at higher concentrations and a derivative, iseganan, failed at phase III trials against oral mucositis (104). This represents a major obstacle to the use of HDPs in CF; the primary aim of chronic *P. aeruginosa* infection therapy is the preservation of lung function (10, 15). Non-selective HDPs will bind to both bacterial and human cell membranes, and can form transmembrane pores, such as via the barrel-stave mechanism (130). Additionally, when internalised in eukaryotic cells, HDPs can interact with mitochondrial membranes, which, like bacterial membranes, are negatively charged, and induce their permeation, the release of cytochrome c, and induction of apoptotic pathways (130). The potential for mammalian cell toxicity has been exploited in the area of
For example, when hydroxyalkanoic acid is coupled to P18 it increases its cancer specificity, killing the cancer cell primarily via induction of apoptosis (131). In addition, many α-helical HDPs kill cancer cells by damaging cell membranes and causing necrosis, with HDP classes including cathelicidins, cecropins, and magainins all displaying activity against human cancer cells (132).

1.6 Host defence peptide prodrugs

Designing a peptide rationally with the correct combination of high antimicrobial activity, low cytotoxicity, and protease-resistance that is short in length and has favourable immunomodulatory effects for a specific disease model has not been achieved to date. Many peptides have been described that cover many, but not all of these requirements. As a result there are currently no HDPs in clinical use for internal infections like those in CF. An alternative approach is to convert favourable HDPs into prodrugs. Prodrugs are chemically-modified inactive derivatives of active agents that are transformed in vivo to produce the active drug (133). This can allow the activity of the HDP to be targeted to a specific bacterium or biological system and reduce cytotoxicity distal from the site of activation (Figure 1.3). This approach has parallels in nature with activation of hCAP-18 to LL-37 by PR-3. In this manner, the activity, and potential cytotoxicity, of the endogenous HDP can be limited until required to fight infection (56). Colistin, which is widely used as an antimicrobial agent against *P. aeruginosa* in CF, is delivered as a prodrug to the lung for reasons of toxicity (20, 134).

---

1 Please note that some of this section has been adapted from a review written on the topic by the author of the thesis (134). This review is available in the appendices.
Figure 1.3: Overview of the HDP prodrug model. The pro-moiety prevents the active HDP from interacting with cell membranes, both bacterial and mammalian, reducing both activity and cytotoxicity. The targeting mechanism, e.g. bacterial or host enzymes, releases the active HDP, restoring activity and limiting cytotoxic effects distal from the site of activation. Adapted from (135).

1.6.1 Net charge reduction as a prodrug strategy

There are a variety of ways in which peptides can be modified to convert them to prodrugs. Numerous approaches and accompanying pro-moieties have been investigated (135). One of the most effective methods is the reduction of net charge. As discussed above, the different electrostatic interactions between HDPs and membranes grant them a degree of selectivity (96) and allows them to disrupt bacterial cytoplasmic membranes, both Gram-positive and -negative (93). The importance of this interaction is underlined by the fact that nearly all HDPs have a net charge in the range of +2 to +9 (136). Peptides derive their net positive charge from their N-terminus amine and basic residues such as arginine and lysine. The prodrug of the peptide drug colistin is an example of net charge reduction. Its use was limited for many years over fears of nephrotoxicity but was
reconsidered with the emergence of bacterial resistance (125). Electrostatic interaction with LPS in the outer Gram-negative membrane is the basis of its activity and is not unlike that of HDPs (137). Currently it is delivered as its prodrug colistimethate via inhalation (20, 134). Colistimethate is inactive and less toxic due to the addition of sulfomethyl groups to the five primary amines of colistin (125). This changes the net charge from +5 to -5, reducing both antimicrobial activity and host toxicity (138). The sulfomethyl groups are hydrolysed in vivo, releasing active colistin and restoring the net positive charge (139). However, sulfomethyl is not an attractive pro-moiety for HDPs. The release of the active colistin relies on spontaneous hydrolysis, an inefficient and non-selective process, releasing a mixture of partially sulfomethylated derivatives alongside free colistin, with the net charges of the derivatives ranging from -3 (four attached sulfomethyl groups) to +3 (one attached group). In addition, factors such as pH and temperature have a major effect on release. For example, an in vitro study reported that only 31.2% of colistimethate was hydrolysed to colistin after 4h in aqueous solution at 37°C (125). Commercially-available preparations of colistimethate may also differ between suppliers, with spontaneous hydrolysis resulting in undefined mixtures of substituted compounds with different activities (140).

The reduction of net charge has been used to reduce the uptake of cell-penetrating peptides (CPP) that, like HDPs, rely on high net positive charge as a mechanism of action. The addition of anionic glutamic or aspartic acid residues has been shown to inhibit their cellular uptake. In one study, the insertion of an MMP-targeted PLGLAG linker in between the CPP and anionic groups allowed MMP-2 and -9-dependent increases in CPP uptake to occur, the enzyme cleaving the anionic group and restoring the net positive charge (141). It is of note that this linker is consistent with the characteristics of an ideal substrate discussed in section 1.3.4 for MMP-9 (68).

Independently, a similar approach to that of the CPPs was
successfully employed by our group using the relatively facile addition of an oligoglutamic acid pro-moiety to reduce the net charge of P18 from +8 to +3, reducing its antimicrobial activity against *P. aeruginosa* and *S. aureus*, and lowering its haemolytic activity. Included in the design was a tri-alanine linker designed for cleavage by NE, allowing NE-dependent restoration of antimicrobial activity. In order to prevent the cleavage of the active sequence by NE, P18 was synthesised from D-amino acids, leaving the linker and pro-moiety as enzyme-labile L-amino acids. However, the peptide resulting from NE activation, AA-P18, had inferior antibacterial activity compared to P18 (142).

The combination of an anionic pro-moiety and an enzyme-targeted linker sequence has been applied to a series of disease models, with the potential to target both host and bacterial enzymes for cleavage and active HDP release. Some HDP prodrugs, for example, are being examined for anticancer applications. MMP-2 is overexpressed in cancer cells and has been targeted with a peptide linker consisting of the sequence GPLGIAGQ. The pro-moiety, based on a sequence from magainin, DAEAVGPEAADEEKDED, reduces the net charge of the HDP by 8 units and has been used with buforin, which has a net charge of +7. MMP-producing cancer cells were found to be susceptible *in vitro* to the pro-peptide. In cells without the enzyme, no toxicity was demonstrated (143). Similarly, the amoebapore lytic peptide H-3 has been modified with C-terminal γ-linked glutamic acids which prevent membrane interaction. The prodrug is targeted at the carboxypeptidase enzyme prostate-specific membrane antigen (PSMA), which can remove terminally-linked γ-glutamic acids and is found in malignant prostate tissue. Anticancer activity was demonstrated in cells overexpressing PSMA, with little lytic effects on PSMA-negative cell lines (144). *In vivo* studies using a mouse tumour xenograft model demonstrated reduced tumour size in treated mice compared to control and, in a separate experiment, no significant toxicity was observed in healthy mice after IV administration of a 30mg/kg dose (145).
In targeting cancer, one of the issues associated with intravenous delivery is stability. A very recent paper (citing work carried out as part of this thesis) addresses this issue. The peptide Melittin is inactivated by the blocking segment PEPAPePAGADAEADPEA, reducing the charge from +3 to -5, and targets MMP-9 with the linker GPQGIAGQ, cleavage occurring at Gly-Ile bond. It reduced the cytotoxicity against human 2F2B cells (increasing IC\textsubscript{50} from 2.4\(\mu\)M to >100\(\mu\)M).

Incorporating the pro-HDP into perfluorocarbon nanoparticles increases its circulating half-life and slows cleavage. This minimises non-specific cleavage in the bloodstream but still allows cancer-targeting. In a mouse model of cancer, treatment for 14 days with the pro-HDP nanoparticles reduced tumour size by 88% (146).

The major advantage of the net charge reduction approach is that, even with the incorporation of an enzyme-targeted linker, the final peptide remains relatively short. This is important when the cost of production is taken into account but also in terms of the ease in which the sequence can be produced and modified in a research setting. Solid phase synthesis (see section 3.1.2) and RP-HPLC can be used to produce highly-pure well-characterised final products, allowing for the generation of \textit{in vitro} data quickly. One can then return and optimise the pro-HDP based on the \textit{in vitro} results. Industrial scale-up, and therefore economic viability and clinical approval, are also likely to be facilitated, as compared to a multi-component system.

1.6.2 PEG as a pro-moiety

Poly(Ethylene Glycol) (PEG) is one of the most widely used polymers for improving the pharmacokinetic and pharmacodynamic properties of low molecular weight drugs, peptides, proteins and oligonucleotides (Figure 1.4). It is synthesized via the nucleophilic attack of a hydroxide ion on the epoxide ring of ethylene oxide, leading to anionic polymerization. It can be covalently attached without crosslinking to drugs with a reactive functional group such as \textendash\textendash\textendash\textendash\textendash\textendash\textendashCOOH, \textendash\textendash\textendash\textendash\textendash\textendash\textendashSH, \textendash\textendash\textendash\textendash\textendash\textendash\textendashOH, or \textendash\textendash\textendash\textendash\textendash\textendash\textendashNH\textsubscript{2} (147), making the attachment to a resin-bound, protected HDP
relatively straightforward. PEG is currently used as a conjugate for many protein drugs, conferring increased aqueous solubility, reduced immunogenicity, prolonged half-life and higher specificity than the active agent alone (148). PEG, when bound to water, has a hydrodynamic radius 5-10 times that of a globular protein of similar molecular mass. The water-associated PEG protects the attached active constituent from enzymatic degradation, protein interaction and fluctuations of temperature and pH. PEGylation can also prevent the attached drug from interacting with its target, but the resulting extensions in half-life can offset the effect of reduced activity (149).

Many PEGylated anticancer agents, once localized in the tumour, are designed with a release mechanism that exploits lysosomal enzymes such as cathepsin B with a peptide linker of sequence GFLG (148, 150, 151), or lower pH levels of tumour cells with a hydrazone linker (152).

![Figure 1.4: Poly(ethylene glycol) structure.](image)

PEG may be easily incorporated into resin-bound peptides, using supra-stoichiometric concentrations of the desired PEG in conjunction with a solid support that allows the diffusion of the polymer in its matrix. However, while widespread in anticancer therapies, PEG has not been used as extensively with HDPs. As with other molecules, PEGylation can decrease the binding of peptides to their biological targets *i.e.* cell membranes, and while this will reduce antimicrobial activity and potentially interaction with a targeted activating enzyme, it will also lower cytotoxicity and haemolysis (153).

The reduction in both host- and bacterial-targeting effects has been exploited to improve the selectivity of HDPs, with mixed results. While
not incorporating a prodrug release mechanism, a conjugate of Magainin and 5kDa PEG had far lower cytotoxicity against CHO-K1 cells but only a four-fold reduction in MIC against *E. coli* and *Staphylococcus epidermidis* (154). A conjugate of tachyplesin and 5kDa PEG displayed similar reductions, again with no release mechanism incorporated into the design. However, while reducing the cytotoxicity of tachyplesin, PEG had a major deleterious effect on antimicrobial activity, increasing the MIC against *E. coli* from 0.5µg/ml to 32µg/ml (153). Reductions in haemolysis compared to control were noted in a PEGylated HDP, KYE28, and was inversely related to length of the attached PEG. Improved selectivity was demonstrated in a blood mixture supplemented with a bacterial inoculum, where PEGylation reduced haemolysis while maintaining antimicrobial effect. This ran in parallel with reductions seen in interaction with model lipid membranes (both anionic and zwitterionic) and antimicrobial effect with increasing PEG length. Also noted was a modest reduction in LPS binding with PEGylation, which was interesting from an immunomodulatory aspect. Furthermore, while the parent peptide reduced LPS-induced NF-κB activation of macrophages, the PEGylated peptide was moderately less effective, indicating that the pro-moiety was interfering with immunomodulatory effects (155).

Using a short PEG, another group was able to demonstrate modest reductions in cytotoxicity with small decreases in antimicrobial activity. They proposed that PEGylation reduced the association with zwitterionic membranes, such as those in mammals, while leaving the interaction with anionic bacterial membranes relatively unaltered. They demonstrated that the PEGylated HDP, compared to the unaltered HDP, had an increased preference for binding with anionic model membranes than their zwitterionic counterparts. Interestingly they demonstrated that the PEG used was unable to prevent HDP degradation by rat lung proteases and that the PEGylated peptide was more labile than the free HDP. They proposed that in this case PEGylation may interfere with the HDP’s ability to self-associate and
reduce its stability (156). Conversely, the use of short PEGs has been shown to protect against degradation by chymotrypsin and serum proteases, which is important when one considers the impediment to HDP development that protease-lability represents. A comparison between an active HDP and its PEGylated derivative showed that, while the latter had reduced antimicrobial activity, it maintained this activity in serum, while serum had a major deleterious effect on the activity of the parent HDP. The authors of the study noted that the PEG length was below that which is usually required to extend peptide half-life (2kDa and higher) but that the short PEG may still shield the peptide core from degradation (157). The ability of PEG to protect from proteolytic degradation may depend on the enzyme in question and the length of PEG used. Relevant degradative enzymes would have to be taken into account when considering the length of PEG for use in a prodrug system.

While PEGylation alone may be used to improve the selectivity of HDPs, the inclusion of targeted release mechanisms can take advantage of the reductions in cytotoxicity while circumventing the issue of reduced antimicrobial activity, creating a true prodrug. The covalent linkage of PEG or other polymers to the HDP can be designed for cleavage under certain physiological and enzymatic conditions to ensure controlled release of the active peptide and limitation of host toxic effects, producing a prodrug (152). Nollman et al produced a series of PEG-HDP conjugates targeted for release by trypsin-like proteases found in serum using a GARSG linker sequence. The active sequences had previously been optimised for stability against mouse serum proteases. This provides a controlled systemic release of active HDP. It was again found that PEGylation reduced antimicrobial activity, in one case with MIC increasing from 0.8µM to 9.3µM. Addition of mouse serum (containing the target proteases) restored activity and reduced the MIC back to 0.6µM. Release kinetics varied between pro-HDPs and depended on PEG length and linker, e.g. a longer PEG was found to slow the release rate. This is
consistent with the PEG shielding the linker from enzymatic degradation (158).

PEGylation has produced mixed results in HDP prodrugs models. Improved specificity has been reported in some cases while major deleterious effects on activity were found in others. Its ability to shield peptides from protease degradation is also mixed, and the use of too large a PEG may preclude efficient release of the active HDP in vivo. However, taken together, the results of previous studies indicate that it may have a useful role in future HDP prodrugs, warranting further study with a suitable enzyme-linker system and an optimised PEG and linker length. Like oligoglutamic acid, it can be produced using solid-phase synthesis, allowing highly-pure products to be obtained, although, unlike oligoglutamic acid, a polymeric prodrug would likely be relatively large, requiring careful economic consideration.

1.6.3 Antibodies as pro-moieties

In certain clinical scenarios it may be highly desirable to target the activity of HDPs at a pathogenic bacterium with minimal effect on normal commensal flora, such as in the mouth or the colon. Using antibodies as conjugates has been attempted to achieve this. In one study, the HDP SMAP was linked to immunoglobulin G antibodies specific for the bacterium Porphyromonas gingivalis. Preferential killing against P. gingivalis was demonstrated in a simulated microbial community at 20µg/ml but specificity was lost at higher concentrations (159). Antibody conjugates, however, have a major drawback in that modest improvement in specificity comes with a greatly increased product size and cost of production. The final therapeutic would likely have a molecular mass in the region of 150kDa and cannot be synthesised by solid-phase chemistry (135). For example, the HDP dhvar5 was conjugated to an antibody against Streptococcus mutans with a linker sequence targeted at the endoproteinase factor Xa, a true prodrug. While it was demonstrated that the linker allowed factor Xa-dependent restoration of antimicrobial activity, purification and testing
of the larger antibody conjugate was not possible due to low yield, product degradation, and poor bacterial vector growth (160).

1.6.4 Antibiotics as pro-moieties

Conjugating HDPs to conventional antibiotics can use characteristics of both components to overcome issues of toxicity and selectivity. For example, vancomycin has been combined with Magainin. Vancomycin targets Lipid II which is essential for peptidoglycan synthesis and has a much higher affinity for Gram-positive membranes compared to the non-selective electrostatic attraction of Magainin (and other HDPs). Like all HDPs, Magainin can take advantage of the lower propensity for resistance to develop in bacteria. The resulting conjugate had an MIC of 16µg/ml against vancomycin-resistant Enterococci compared to 128µg/ml for vancomycin alone (161). However, activity would potentially be limited to Lipid II-containing bacteria.

Additional design characteristics can incorporate the antibiotic as a targeted pro-moiety. Conjugating antibiotics can provide a reduction in HDP net charge by masking the N-terminus amine and via the negative charge of antibiotic functional groups such as –COOH, inactivating the HDP in much the same way as oligoglutamic acid. A conjugate of the HDP Bac8c and the cephalothin was designed where the antibiotic acts solely as a pro-moiety as opposed to a co-drug. The net charge of the HDP is reduced from +4 to +2. The prodrug, targeted at the bacterial enzyme β-lactamase, has lower antimicrobial activity than both separate constituents in the absence of the target enzyme, with β-lactam resistant E. coli the target. The enzyme cleaves the β-lactam bond of the antibiotic and releases the active HDP. Cleavage of the prodrug has been demonstrated with purified β-lactamase, but the prodrug showed reduced activity compared to Bac8c alone. However, the comparison of the activity of an uncleavable control confirms that a degree of enzyme-dependent activity was present but with the uncleaved prodrug still somewhat active (162).
1.6.5 Summary of HDP prodrugs

The prodrug approach has the potential to greatly expand the number of HDPs suitable for anti-infective therapy. Highly-active HDPs that would otherwise be too cytotoxic under normal circumstances may be reconsidered for use as a prodrug that targets activity to a particular biological system such as the CF lung. Several options for pro-moieties, with varying characteristics, have been investigated. Figure 1.5 illustrates how these different conjugates may be used to decrease the absolute affinity of HDPs for cell membranes, reducing host cytotoxicity and, in many cases, antibacterial activity. While many of the modifications such as PEGylation will increase the selectivity of HDPs by increasing the relative affinity for bacterial cell membranes, the accompanying loss of absolute bactericidal activity necessitates the incorporation of a release mechanism for the active HDP *i.e.* the synthesis of HDP prodrugs. The various enzymatic targets used by the different prodrug models underlines how HDP activity can be tailored to a specific bacterium or region of the body, limiting cytotoxic effects of the already selective AMPs.
Figure 1.5: An overview of the effects of the different pro-moieties on the affinity of HDPs for mammalian and bacterial cell membranes, the size of the arrows representing the higher intrinsic affinity for HDPs towards bacterial cells. Lipid composition means that bacterial membranes are of net negative charge (-), while mammalian ones are neutral (+/-) with cholesterol as a major component (its hydroxyl group aligning with the phosphate heads of the outer surface of the membrane). These characteristics direct HDP affinity. The pro-moieties also modulate this affinity to different degrees, with net charge reduction having the most impact. Adapted from (135).
Considering the complexity of the targeted biological systems, an important future milestone in the development of pro-HDPs would be an in-depth *in vivo* study of not just the antimicrobial activity of the pro-HDP but also the activation by the target biological system, including analysis of the released active peptide in biological samples such as CF BAL fluid or serum. The reduction in toxic effects, if any, afforded by the modification must also be investigated. In addition, something that has not yet been studied in detail is the potential effects of the prodrug approach on the other effects of HDPs *i.e.* immunomodulatory activity, which may be a potential source of host toxicity with exogenous application. When one considers that membrane association and translocation may play a role in the immunomodulatory mechanism, it is possible that the addition of a pro-moiety may reduce these effects. Furthermore, where the immunomodulatory effect is the result of binding to immunogenic molecules such as LPS, the possibility that the pro-moiety will reduce this effect must be investigated (155). Another important design consideration is whether the active sequence will be resistant to degradation by both target and off-target enzymes. HDP prodrug systems may rely on high concentrations of host or bacterial enzyme for activation, such as NE in CF, which could have the potential to degrade the active sequence. Strategies to avoid this must be incorporated into design, whether it be via PEGylation (157), *D*-amino acids (142), a protease-resistant sequence (158), or otherwise. These approaches, particularly *D*-amino acids, may also increase the future cost of production of pro-HDPs and alter immunomodulatory activity and must be carefully considered whether they are necessary.
Aims and summary of chapters

This study aims to develop HDP prodrugs for use against bacterial infection in CF. HDPs form an important part of the innate immune system but their endogenous activity may be compromised in CF, contributing to chronic infection and lung disease. Developing HDPs for exogenous delivery is hampered by a series of factors including host toxicity. This project develops HDP prodrugs that will be active against CF lung infection but with reduced host toxicity.

The overall aim was achieved through a series of related studies described in four chapters which are summarised as follows:

- Chapter 1 describes the development of a new prodrug approach and its application with three candidate HDPs, P18, Bac8c and HB43.
- Chapter 2 describes the approaches taken to achieve further improvements in activity and reduced cytotoxicity with CF conditions in mind. A more detailed in vitro evaluation of the previous series of pro-HDPs and further candidates is described and a final candidate peptide, pro-WMR was selected for further studies.
- Chapter 3 describes the evaluation of pro-WMR in a nebulisation drug-delivery system.
- Chapter 4 describes an in vivo CF mouse study of the toxicity and effectiveness of pro-WMR against P. aeruginosa lung infection.
2. Materials and methods

2.1 Peptide synthesis and *in vitro* testing

2.1.1 Strains and Clinical isolates

The laboratory strain PAO1 (American Type Culture Collection, Rockville, MD, USA) was used as a reference. *P. aeruginosa* clinical isolates from CF patients were obtained from the Microbiological Diagnostic Laboratory of Beaumont Hospital, Dublin, Ireland. Isolate identity was confirmed by a combination of the BBL™ DrySlide™ oxidase test (*P. aeruginosa* is oxidase-positive) (BD, USA), the C-390 Diatab™ disk test (where growing *P. aeruginosa* in the presence of the antibiotic disk produces no zone of inhibition) (Rosco Diagnostics, Germany), and Matrix Assisted Laser Desorption Ionisation – Time of Flight (MALDI-TOF) mass spectrometry (Bruker, Germany).

2.1.2 CF BAL fluid collection

Samples of CF BAL fluid were collected from consenting CF patients. Non-CF BAL was collected from patients with stage I or II sarcoidosis from a previous study. Both protocols for collection were approved by the Beaumont Hospital Ethics Research Committee (references 10/43 and 99/94 respectively). Briefly, 30ml of sterile 0.9% NaCl was instilled into the right or left sub-segmental bronchus, collected immediately and stored at 4°C. After centrifugation at 500xg for 10min at 4°C, the supernatants were removed and stored in aliquots at -80°C until required.

2.1.3 Blood collection

Blood samples were collected from consenting healthy individuals. The protocol was approved by the RCSI Research Ethics Committee (reference REC951). For neutrophil isolation, blood was collected in
7.5ml tubes containing heparin lithium-coated beads (Sarstedt, Ireland). Blood collection tubes (2.7 ml) containing EDTA were used for erythrocyte collection (Sarstedt, Ireland).

### 2.1.4 Peptide synthesis

Five peptide amide sequences were investigated; d-Bac8c<sup>2,5 Leu</sup> (rlwvlwrr), d-HB43 (fakkllaklkll), d-P18<sup>8 Leu</sup> (kwkflkklpfhlahkkf), d-WMR<sup>3,6 Leu</sup> (wglrlkkygkrs), and d-WR12 (rwwrwwrrwrr). The isoleucine residues normally found in Bac8c, P18, and WMR were replaced by leucine. This has previously been shown not to negatively impact on activity in Bac8c and P18 while decreasing the cost associated with d-isoleucine synthesis (131, 162). This modification has not previously been made to WMR and was investigated with comparison to L-WMR (WGIRRILKYGKRS) (see Results Chapter Two). Henceforth, the d-HDPs will be referred to as Bac8c, HB43, P18, WMR and WR12.

The parent sequences were assembled by automated solid phase peptide synthesis on a 433A synthesiser (Applied Biosystems, UK) from 9-Fluorenylmethyloxycarbonyl (Fmoc)-protected d-amino acids (Merck Chemical, UK) according to the Fmoc-tBu strategy with 1-[Bis(dimethylamino)methylene] 1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)/N,N-Diisopropylethylamine (DIEA) coupling chemistry from a Rink Amide MBHA resin (Sigma, Ireland). Single coupling cycles using a 10-fold excess of Fmoc-amino acids were employed, except for the leucine at position 8 in P18<sup>8 Leu</sup>, where double coupling was required. The excess ensures complete coupling during each synthetic step. Removal of Fmoc was carried out using 20% v/v piperidine in N-Methyl-2-pyrrolidone (NMP) and the reaction was monitored by the UV absorbance at 301nm of the Fmoc-dibenzylfulyene adduct in the run-off. For the pro-HDPs, the addition of the AAAG linker, glutamic acids and N-terminal acetylation were carried out manually with L-amino acids in a syringe fitted with a Teflon frit. Coupling and deprotection, were monitored using the Kaiser test.
Addition of more than four glutamic acids was attempted using a combination of microwave-assisted synthesis and repeated couplings.

The PEGylated pro-peptide was generated by first synthesising P18 on PEGA resin (EMD Millipore, UK) using automated synthesis on a 0.1mM scale, AAAG was then added manually with Kaiser test monitoring. PEGylation was carried out using 2 equivalents of 2000Da MeO-PEG-COOH (Iris Biotech, Germany) and HATU/DIEA coupling chemistry.

Peptides were deprotected and cleaved from the synthesis resin using a mixture of 80% trifluoroacetic acid, 5% water, 5% triisopropylsilane, 5% thioanisole and 5% 1, 2-ethanedithiol, at RT for either 2h (pro-HB43 and pro-P18) or 4h (pro-Bac8c, pro-WMR, and pro-WR12), with the number of arginine residues present dictating the cleavage time. They were then precipitated by addition of diethyl ether, washed three times with 10 ml portions of diethyl ether, dried, dissolved in distilled water and lyophilised.

Chromatographic analysis and purification were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (PerSeptive Biosystems, UK) using Gemini columns (Phenomenex, 110Å, 5m, C18, 4.6mmd/250mmL or 100mmd/250mmL, for the analytical or semi-preparative columns, respectively). Buffers used were mobile phase A: 0.1% trifluoroacetic acid (TFA) in water; mobile phase B: 0.1% TFA in acetonitrile with a gradient of 5 to 65% B in 18 column volume (analytical) or 5 column volume (semi-preparative) with a flow rate of 1ml/min (analytical) or 5ml/min (semi-preparative) and single wavelength detection at 214nm. A secondary wavelength at 280nm was used for Bac8c, P18, WMR, and WR12, and at 254nm for HB43. The PEGylated pro-peptide was purified as above but with a C5 Jupiter column (Phenomenex, 300 Å). Purified peptides were finally characterised by MALDI-TOF MS using an α-cyano-4-hydroxy-cinnamic acid matrix.
2.1.5 TFA removal of HDPs

In order to exchange the TFA counter-ion with a Cl\(^-\), 20mg of HB43 was dissolved in 15ml of deionised water. 100mg of Dowex® 1X8 chloride form ion exchange resin (Sigma, Ireland) was added to the solution, which was then stirred at RT for 1h. The solution was filtered to remove the resin and lyophilised. The resulting peptide was analysed in D\(_2\)O by \(^{19}\text{F}\) NMR and compared to unexchanged peptide to investigate TFA removal. The ion exchange procedure was repeated until the \(^{19}\text{F}\) peak was completely removed to ensure that TFA had been completely exchanged with Cl\(^-\).

2.1.6 Enzymatic cleavage of pro-HDPs

Each pro-HDP (250µg/ml) was incubated with 5µg/ml purified NE, 5 or 10µg/ml PE (Elastin Products Company, USA) or 5µg/ml CG (Sigma, Ireland), at 37°C, in phosphate buffered saline (PBS), pH 7.4. At various time-points, samples were removed from the incubation mixture and analysed by HPLC and MALDI-TOF MS. To confirm the protease-resistance of the D-amino acid sequences, Bac8c. HB43 and P18 were incubated at 37°C with 100µg/ml NE for 24h and analysed by HPLC.

2.1.7 Neutrophil elastase determination

Neutrophil elastase activity of CF BAL fluids was estimated by measuring the cleavage of the NE substrate, \(N\)-methoxysuccinyl-Ala-Ala-Pro-Val-p-Nitroanilide (AAPV-pNA) (Sigma, Ireland). Briefly, 1mM AAPV-pNA was added to BAL fluids (diluted as appropriate) in buffer containing 0.05M sodium acetate and 0.1M sodium chloride, pH 7.5 in 96 well plates. The rate of AAPV-pNA cleavage was measured as the increase in absorbance at 405 nm over 5 min at 37°C using a 2030 Multilable Reader Victor X3 (Perkin Elmer, USA). Assays containing 0.25 – 5µg/ml purified NE (Elastin Products, USA) were used to construct a standard curve on Graphpad Prism software. The standard
curve was used to calculate the NE concentration of the CF BAL fluids.

2.1.8 Susceptibility testing

Minimum inhibitory concentrations (MIC) were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (163), with modifications for cationic peptides as described by Wu & Hancock (164). Briefly, serial doubling dilutions of peptide were made in a sterile solution containing 0.2% w/v bovine serum albumin (BSA) and 0.01% v/v acetic acid with a concentration range of 0.5 - 64µg/ml (units chosen for consistency with guidelines). These were added to a 96-well microtitre plate with a 1.5 x 10^5 CFU/ml inoculum of *P. aeruginosa* reference strain PAO1 or clinical isolates in Mueller-Hinton broth (non-cation adjusted, Oxoid, UK). The lowest peptide concentration showing no visible growth was recorded as the MIC. The result was confirmed by measuring absorbance at 620nm and determining the concentration at which it was reduced by 90% compared to control.

2.1.9 Bactericidal killing activity

*P. aeruginosa* strain PAO1 and clinical isolates were grown overnight at 37°C on Mueller-Hinton (MH) agar. Suspensions were prepared in 0.9% w/v NaCl from isolated colonies to the density of a 1.0 McFarland standard using a Densichek meter (Biomerieux, Ireland) and were further diluted 1/100 in potassium phosphate buffer, pH 7.4 containing 0.2% BSA. Assays were carried out in microcentrifuge tubes and contained 0.39 - 25µg/ml peptides, 10% v/v *P. aeruginosa* suspension (approximately 1.5 x 10^6 CFU/ml) and 10mM potassium phosphate buffer, pH 7.4 containing 0.2% BSA. Assays were incubated at 37°C, 200rpm in a shaking incubator for 1h (Gallenkamp, UK) and then diluted 1/10 with 0.95% w/v NaCl. After vortex mixing for 30s, a 100µl aliquot was spread onto MH agar and incubated overnight at 37°C. Killing activity (%) was calculated from viable
counts (CFU/ml) from assays containing peptides compared to control assays not containing peptides. The effects of the addition of purified NE (5-20µg/ml), CF BAL fluid (25% v/v) and NaCl (300-600mM) on killing activity were determined by the addition of these to the assays and the inclusion of appropriate controls.

2.1.10 Static Biofilm studies

The reference strain, PAO1 and *P. aeruginosa* clinical isolates with strong biofilm-forming ability based on the criteria of Stephanovic *et al* (2000) (165) were used. Overnight cultures of the isolates were diluted in 5ml of tryptic soy broth (TSB) (Oxoid, UK) supplemented with 0.5% w/v NaCl and incubated with shaking at 37°C to an optical density of 0.8 at 600nm. The cultures were diluted 1:40 with fresh TSB/NaCl and 150µl was added to each well of a 96 well microtitre plate (HDPs were present from seeding in some experiments). The plates were sealed with parafilm and incubated for 48h at 37°C. Treatment was with active HDPs in TSB for 24h or dH₂O as a positive control. Biofilm staining was with crystal violet (CV). Briefly, after washing three times with dH₂O, CV was applied to the biofilms for 15min and removed, biofilms were washed three times with dH₂O and the remaining CV solubilised with 30% v/v acetic acid. The absorbance was measured at 595nm. In addition, metabolic activity in biofilms was investigated by incubating the biofilms with 440mM Resazurin sodium (Sigma, Ireland) in NaCl/Pₐ for 3h, and measuring fluorescence at 590nm after excitation at 544nm. The activity of the peptides both in preventing biofilm formation and against pre-formed biofilms was tested.

2.1.11 Flow Biofilm studies

An overnight culture of *P. aeruginosa* PAO1 was diluted and grown to a concentration of $1 \times 10^8$ CFU/ml and seeded in the eight flow chambers of a Vena8 Fluoro+ biochip (Cellix, Ireland) and incubated at 37°C for 3h to allow the bacteria to adhere. The chambers were subjected to flow using a Kima pump (Cellix, Ireland) at a rate of
30µl/min using 25% v/v tryptic soy broth (TSB) for 48h. After incubation, the biofilms were treated statically with peptide in PBS for 6h. Live/dead staining was then carried out by incubating with 0.027mM SYTO®9 green and 0.16mM propidium iodide (Molecular probes, USA) for 1h. Green and red fluorescence (live and dead) was detected on an inverted Zeiss LSM 510 META confocal microscope. At least three representative images were obtained per chamber per experiment. Image J 1 software was used to quantify fluorescence intensity. Each experiment was performed three times.

2.1.12 Cell culture

The human ΔF508 homozygous CFBE41o- bronchial and CFTE29o-tracheal epithelial cell lines were obtained as a gift from D. Gruenert (California Pacific Medical Centre Research Institute, San Francisco, CA) (166, 167). Cells were cultured in minimal essential medium (MEM) supplemented with 10% v/v foetal calf serum (FCS), 100U/ml penicillin, and 100µg/ml streptomycin, at 37°C in a humidified atmosphere with 5% CO₂. Cells were used or detached with Trypsin/EDTA and re-seeded at lower cell densities when they reached 90% confluency in flasks pre-coated with fibronectin.

2.1.13 Neutrophil isolation

4ml of a 10% w/v solution of Mr 500000 Dextran (Sigma, Ireland) in purified water was added to 40ml of freshly-collected blood, gently mixed, and allowed to rest until settled. To 15ml of the resulting supernatant, 5ml of Lymphoprep™ (Axis-shield, UK) was underlayed, and it was centrifuged at 800xg for 10min. Remaining erythrocytes were lysed from the resulting pellet by exposure to 25ml of purified water for 10s, with the isolated neutrophils recovered by the immediate addition of an equal volume of 1.8% w/v NaCl. After further centrifugation at 500xg, the remaining pelleted neutrophils were resuspended in 1ml PBS/0.09% w/v glucose. Neutrophils were then counted using Trypan Blue staining (VWR, Ireland) and a
haemocytometer under a microscope, and were routinely recovered at a purity of at least 95%.

2.1.14 Cytotoxicity assays

CFBE41o- and CFTE29o- cells were seeded on 96 well plates at a density of $3 \times 10^5$ cells/ml, and neutrophils were seeded at a density of $5 \times 10^6$ cells/ml. The epithelial cells were incubated for 24h at 37°C. The cells were then treated in triplicate with a concentration range of $0.2 – 300/600\mu M$ of the peptides and their prodrugs in serum-free media (units chosen for consistency with literature and for accurate comparison of pro- and active HDPs). MEM media was used for the epithelial cells and Roswell Park Memorial Institute (RPMI) 1640 was used for neutrophils. Incubation was for 24h for epithelial cells and 3h for neutrophils. After incubation, media was removed and the cells were incubated with 500µg/ml of thiazolyl blue tetrazolium bromide (MTT) (Sigma, Ireland) in serum-free media. Incubation time was 4h for epithelial cells and 2h for neutrophils. The MTT solution was removed and 100µl of dimethylsulphoxide (DMSO) was added to each well, mixed by shaking, and the absorbance at 560nm was recorded. The IC$_{50}$ values, defined as the peptide concentration that resulted in 50% cell death, were calculated using Graphpad Prism software from the resulting sigmoidal dose-response curve.

2.1.15 Cytokine release assays

CFBE41o- cells were seeded on 24 well plates at a density of $3 \times 10^5$ cells/ml and incubated for 24h at 37°C. The cells were treated in triplicate with sub-IC$_{50}$ concentrations of the peptides and their prodrugs in MEM media containing 1% FCS. The plates were centrifuged at 700xg for 5min and the cell supernatant was removed. The cytokine concentration of each supernatant was measured using a Human Pro-inflammatory Panel V-PLEX Plus Kit (MSD, Ireland) analysing levels of IL-1β, IL-6, IL-8, and TNF-α. Briefly, BAL samples were diluted and incubated on the plate for 2h at RT. Wells were
washed three times with PBS/0.05% TWEEN-20 and then incubated for 2h at RT with the secondary antibodies. Plates were washed again and then read by chemiluminescence on a MESO QuickPlex SQ 120 (MSD, Ireland). LPS stimulation for 24h at a concentration of 50µg/ml was used as a positive control.

2.1.16 Haemolysis assays

Freshly-collected human blood was centrifuged at 100xg for 5min. The supernatant was removed and the erythrocyte-rich pellet resuspended in sterile PBS and centrifuged again. The blood was washed twice in this manner and resuspended to twice its original volume in PBS. In a 96 well plate, 50µl of the erythrocyte suspension was mixed with 50µl of a peptide solution in PBS, to give a final assay concentration range of 0.195-500µM. The assay was performed in triplicate. Positive controls containing 0.1% Triton-X and negative controls containing no peptide were also included. After 24h incubation at 37°C, the % haemolysis relative to control was calculated based on the absorbance at 450nm.

2.1.17 Statistical analyses

Statistical analyses of the data were carried out using Graphpad Prism software and the two-tailed unpaired t-test. P values are given throughout the results and in figures with * denoting P<0.05, ** P<0.01, and *** P<0.001. In most cases analyses were carried out in relation to controls. Where the relationship between other data groups are investigated, the significance of this relationship is provided graphically in the figures. This statistical analysis was used in all experiments where p values are given.
2.2 Inhalation studies

2.2.1 Light-scattering particle size analysis

Laser diffractometry was carried out on a 1mg/ml aqueous peptide solution using a Spraytech Laser Light Scattering System (Malvern, UK). Briefly, 250µl of the 1mg/ml solution was nebulised into the apparatus using an Aeroneb® Solo vibrating mesh nebuliser (Aerogen, Ireland). An extraction flow of 15l/min was applied to prevent aerosol re-entry into the measurement zone. Particle sizing of the aerosol spray was carried out measuring the intensity of the light scattered from the laser beam as it passes through the spray. This allowed the calculation of the volume median diameter (VMD), where half the volume of the spray contains particles of larger and smaller diameter, and was carried out four separate times.

2.2.2 Impaction particle size analysis

A next generation impactor was also used to assess the droplet diameter of the nebulised spray. The nebuliser (Aeroneb® Solo) was attached to the apparatus via a plastic connector and metal inlet (throat). The intake was 15l/min and 1ml of the 1mg/ml solution in dH₂O was nebulised. After nebulisation was complete, the apparatus was dismantled and the eight plates, connector, throat, nebuliser, and end filter (Respigard 303, Baxter, Ireland) were all thoroughly rinsed with dH₂O to collect the impacted peptide. The washings of each stage were analysed by HPLC and compared to a standard line of peptide concentration versus peak area. The % deposition in each stage was used to calculate the mass median aerodynamic diameter (MMAD) of the spray and the mass balance of each stage. This was carried out three times for each peptide. Samples of low concentration were lyophilised and reconstituted in 1/10th the original volume to increase the HPLC signal and improve accuracy.
2.2.3 Breathing apparatus

The Aeroneb® Solo was used to nebulise 1ml of a 1mg/ml solution in dH$_2$O into a breathing apparatus. The conditions of a healthy human breathing pattern was simulated using a Salter valved facemask 81070-0 (Salter, USA) and an ASL 5000 active servo lung (IngMar Medical, USA). The parameters were 15 breathes/min, inhalation:expiration ratio of 1:1, tidal volume of 500ml, and 2l of supplementary gas flow. The nebulised peptides were collected below the model on a Respigard 303 filter (Baxter, Ireland) which was washed afterwards with 10ml dH$_2$O. The nebuliser was also washed. The washings were analysed by HPLC and the % of the original dose delivered to the filter was calculated, giving the % deliverable to the lung. This was carried out three times for each peptide.

2.3 In vivo studies

2.3.1 Transgenic mice

All in vivo studies were granted ethical approval by the Regierungspräsidium (Regional Council) of Karlsruhe, Baden-Württemberg, Germany (reference G-284/14) and were carried out in collaboration with the laboratory of Prof Dr. Marcus Mall, University of Heidelberg, Germany. The animal handling licence was obtained from Laboratory Animal Science and Training, Ireland. The mouse strains used were $Scnn1b$-Tg (β-ENaC), and $Scnn1b$-Tg NE$^{-}$ (β-ENaC NE-knockout) on a C57BL/6 background. All mice used in infection studies were 8-10 weeks old. For toxicity studies wildtype and NE$^{-}$ C57BL/6 mice aged 11-15 weeks were used.

2.3.2 Bacterial challenge

Mice were sedated using 3% v/v isoflurane in O$_2$ at a flow rate of 2l/min and then suspended vertically by their incisors on a wire
attached to an upright examination board. 50µl of a \textit{P. aeruginosa} PAO1 inoculum (1 - 2.5 \times 10^7 CFU/mouse) was pipetted into their throat and their nose was covered until the mice fully inhaled the bacterial suspension. After 6h the mice were then treated in a similar manner with either 50µl of PBS or a 1mg/ml peptide solution. Mice were sacrificed 24h after initial infection.

### 2.3.3 Lung lavage

Mice were euthanized by intraperitoneal injection of 120mg/kg ketamine and 16mg/kg xylazine and exsanguination. The midline anterior was incised and the trachea isolated, cannulated with a 22G needle, and tied. 0.035ml cold PBS/g (bodyweight) was used to lavage the lungs. Bacterial counts were carried out on each BAL fluid using the plate count method, after which the BAL fluids were centrifuged at 600xg for 5min and the supernatant removed. Murine NE was quantified in each BAL supernatant using MeOSuc-AAPV-AMC, measuring the change in fluorescence at 460nm after excitation at 380nm. Recombinant murine NE was used as a standard (R & D systems, Germany), this was pre-activated by incubating for 4h at 37°C with murine cathepsin C (R & D systems, Germany). This step is required to cleave the activation dipeptide from the amino terminal of the proenzyme (168). Please note that this is a different protocol to that used above for the determination of human NE levels in CF BAL fluid.

### 2.3.4 Toxicity studies

Wildtype mice were sedated and suspended by their incisors as with the infection studies. The mice were treated intratracheally with 50µg of peptide in the morning and evening and sacrificed 24h after the first dose. Any mice that were found to have died were not processed further. In living mice, lung lavage was carried out on half lungs while the other half was stored in 4% formaldehyde solution overnight,
washed three times in dH$_2$O, and stored in 70% ethanol at -20°C for histological processing and analysis.

2.3.5 BAL cell count, total and differential

BAL fluids were centrifuged at 600xg at 4°C for 5min. The cell pellet was resuspended in 50µl of cold PBS. The BAL fluid cell count was carried out microscopically using Trypan blue staining and a haemocytometer. The cell suspension was then spread onto a slide by centrifugation using a Cytospin (3 x 10$^4$ cells per slide). After air-drying overnight the slides were stained using May-Grünwald-Giemsa staining to count the relative proportion of each cell type. Briefly, slides were placed in May-Grünwald solution for 3min, rinsed in dH$_2$O, placed in Giemsa solution for 7min and rinsed in dH$_2$O. After drying the slides were placed in xylene for 5s and then mounting media and a coverslip were added. The proportion of each cell type was quantified using oil-immersion microscopy at 100x magnification.

2.3.6 BAL cytokine release assay

BAL supernatants were analysed for cytokine levels using a V-PLEX Plus Proinflammatory Panel 1 (mouse) Kit (MSD, Ireland), analysing levels of interferon γ (IFN-γ), IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, keratinocyte chemoattractant (KC), and TNF-α. Briefly, BAL samples were diluted and incubated on the plate for 2h at RT. Wells were washed three times with PBS/0.05% TWEEN-20 and then incubated for 2h at RT with the secondary antibodies. Plates were washed again and then read by chemiluminescence on a MESO QuickPlex SQ 120 (MSD, Ireland). Analysis was carried out on BAL fluids from mice in the toxicity study and from mice infected with 2.5 x 10$^7$ PAO1 and then treated with PBS/AAG-WMR/AAG-P18.
3. Results Chapter One: Developing Host Defence

Peptide Prodrugs for Cystic Fibrosis

3.1 Introduction

3.1.1 Rationale

The treatment of the most important pathogen in CF, *P. aeruginosa*, with a pro-HDP targeted at the most common enzyme, NE, forms the basis of the prodrug model. The large quantities of the enzyme found in the CF lung would predict that the active sequence must be synthesised from d-amino acids to ensure that it is not degraded. Maintaining an L-amino acid linker will ensure that it is cleaved upon application of the pro-HDP to the endobronchial space with the release of the active peptide. Based on our own review of the literature, oligoglutamic acid is the most promising pro-moiety (135).

3.1.2 Peptide synthesis

Synthesis of the peptides in this project was carried by standard Solid Phase Peptide Synthesis (SPPS), using the Fmoc/t-Bu protection scheme. It involves incrementally synthesising the peptide from the C- to the N-terminus, in a manner that minimises racemisation and is easily automated, allowing large peptides to be synthesised quite rapidly. All amino acids used in the synthesis are temporarily protected with Fmoc, a base-sensitive $N^\alpha$-amino protecting group. The C-terminal carboxylic acid is left free, while any side-chain functional groups of the amino acid are also semi-permanently protected, but with an acid-sensitive group instead of Fmoc. An example of the protection scheme is given in Figure 3.1, with Fmoc-$d$-Arg(Pbf)-OH.
Figure 3.1: Fmoc-D-Arg(Pbf)-OH. The amine is protected by the base-labile Fmoc, while the side-chain is protected by the acid-labile Pbf group. The C-terminal carboxylic acid is left free.

The process of coupling multiple amino acids together to form a peptide begins with the attachment of the first amino acid onto an insoluble resin, a technique pioneered by Bruce Merrifield (169). The resin, which is retained by a filter in the reaction chamber, allows the application of multiple phases of excess reagents relative to the immobilised peptide. The excesses of reagents at each stage of the synthesis allow a very high level of conversion to be maintained. This is crucial when one considers that even a level of 99% coupling efficiency will lead to an overall synthetic yield of 82% for a 20 amino acid peptide. The coupling agent used, HATU, is one of the most efficient available and allows fast coupling with little loss of chiral integrity. This is important for the pro-HDPs for the maintenance of distinct D- and L-amino acid motifs. The reaction mechanism is illustrated in Figure 3.2 where the first amino acid has already been coupled to the resin and a second is being added. First, HATU reacts with the -COOH group of the second Fmoc-protected amino acid (AA2) (which is deprotonated by the base DIEA) [1]. This releases a side product, OAt, which reacts with the concomitantly-formed isouronium ester to form another active ester [2], eliminating a substituted urea at the same time [3]. Both intermediate esters can next form an amide bond with resin-bound amino acid 1 (AA1) whose amine is not protected [4]. The coupling efficiency of HATU is superior as at this point compared to other agents as OAt forms an N-H
hydrogen bond with the reacting amide [5] in a phenomenon known as
the “neighbouring effect” (170). As the amide bond is formed, OAt is
eliminated, leaving a dipeptide coupled to the resin, but with the amino
group protected by Fmoc [6].

Figure 3.2: Reaction scheme for the addition of amino acid 2 (AA2) to
resin-bound amino acid 1 (AA1), using HATU/DIEA coupling
chemistry.

As stated above, Fmoc is base-sensitive (171), and can be removed
with the application of 20% v/v piperidine. This allows intermittent
steps of deprotection and coupling to be carried out (Figure 3.3A). To
cleave the finished peptide, of n amino acids in length, and remove
side-chain protecting groups, acidic conditions are used (Figure 3.3B).
The conditions required to remove both sets of protecting groups
(temporary Fmoc of the N\textsuperscript{\alpha}-amino and semi-permanent t-butyl-derived
of the side-chain functions) are mutually exclusive (described as orthogonal), which ensures that side-chain branches cannot form and that only the amine of the N-terminal amino acid is free to react.

![Peptide Synthesis Cycle Diagram](image)

**Figure 3.3**: Summary of SPPS. The peptide synthesis cycle involves incremental steps of coupling with HATU/DIEA onto an insoluble resin support followed by deprotection with piperidine. $R$ = amino acid side-chain, $PG$ = protecting group, and $n$ = number of amino acids (A). The complete resin-bound peptide is cleaved from the resin in acidic conditions which also remove side-chain protecting groups (B).

Electrophilic and reactive entities are released from the protecting groups during the acidolytic deprotection. To prevent them from recombining with the nucleophilic liberated side-chain functional groups, the addition of nucleophilic scavenging agents to the cleavage
mixture is required. Different protecting groups are required for each amino acid, and in the same way, different scavenging agents are required for each protecting group (Figure 3.4).

Figure 3.4: Side-chain protecting groups for each amino acid (A) with their corresponding scavenging agents (B). In addition Pbf is the protecting group for arginine. 1, 2 ethane-dithiol is required for cleavage reactions containing tryptophan (to protect its indole ring), and methionine and cysteine (to protect their sulphur-containing side-chains).

Synthesis of the entire D-amino acid active sequence is generally possible using automated peptide synthesis. However, further elongation with L-amino acid linker group and oligoglutamic acid pro-moiety requires careful monitoring of coupling and deprotection by manual synthesis due to the potential aggregation of relatively long and hydrophobic (protected) peptide chains with the polymer matrix of the synthetic resin. Monitoring in this case is carried out by the Kaiser test, which measures the presence of a free N\textsuperscript{ε}-amino group. A positive result indicates deprotection, while a negative indicates coupling. With this monitoring it is possible to determine if repeated
coupling steps are required to complete the addition of an amino acid *i.e.* a double- or triple-coupling step. As the oligoglutamic acid sequence is elongated, multiple coupling steps are often required for each additional residue, as aggregation of the peptide chain can be promoted by the tri-alanine motif.

### 3.1.3 Choice of linker

The linker motif, first used with HDPs in (142) was slightly modified to AAAG. Glycine was added between the heterochiral sequences (L-amino acid pro-moiety and D-amino acid active HDP) as it is achiral and can therefore act as a short spacer; the goal being a cleavage product with comparable bactericidal activity. The choice of linker was determined by the balance between enzyme-specificity and the difficulty in synthesis. The sequence AAPV is commonly used as a specific substrate in NE assays and would seem the logical choice, however, valine favours the aggregation of peptide chains and proline is the least reactive of the amino acid residues (as amino partner in the coupling reaction). Both amino acids would therefore compound the difficulties in the addition of the subsequent glutamic acids. In addition, PR-3 cleaves this substrate, albeit at a much reduced rate compared to NE, removing a degree of specificity. Conversely, NE cleaves AAA, while PR-3 does not (50). Considering that PR-3 constitutes approximately 7% of CF lung protease activity (32), this would mean that although other enzymes may cleave AAA, they would likely be at very low concentrations relative to NE. This linker also does not contain a proline or large hydrophobic groups which should render it unfavourable for MMP-cleavage (68). One enzyme of interest is PE, which has been shown to cleave AAAA sequences (63), and this was also investigated in this chapter. The lability of AAA to CG has not been determined and is investigated in Results Chapter Two.
3.1.4 Choice of active peptides

Three peptides were chosen to generate the first prodrug candidates. This allows the choice of a range of different net charges and peptide lengths in order to determine the optimum peptide for the CF model. Bac8c, HB43, and P18 were modified to contain the linker motif and the anionic oligoglutamic acid pro-moiety.

The three peptides were initially selected based on the requirement for relatively short HDPs, that can be readily synthesised, and that are active against *P. aeruginosa*. Bac8c is an eight amino acid peptide derived from Bactenecin, a product of bovine neutrophils (172). It has relatively high bactericidal activity against Gram-positive and Gram-negative bacteria (173). Its mechanism of action is complex, targeting multiple membrane-associated and intra-cellular processes. Studies into moderate resistance of bacteria against Bac8c, have demonstrated that energy metabolism and membrane disruption may play crucial roles in its mechanism of action (174). Preliminary studies indicated that its activity is salt-sensitive (Figure 3.5) but, despite this, its short length and reported MIC against *P. aeruginosa* of 8µg/ml makes it an attractive candidate for modification (172). HB43 is a thirteen residue HDP. It was previously shown to maintain high activity against *P. aeruginosa* in conditions representative of the CF lung, *e.g.* high levels of mucins, eDNA and NaCl. Its relatively short length and MIC of 8µg/ml against PAO1 are desirable (121). As stated in the introduction, P18 is an eighteen residue, hybrid sequence derived from Cecropin A and Magainin 2 (175). It has demonstrated high activity against Gram-negative bacteria in the presence of high NaCl concentrations *e.g.* an MIC of 2µg/ml against *P. aeruginosa* in 200mM NaCl (176). These characteristics are highly desirable, despite the relatively long sequence of P18.
Figure 3.5: The effect of NaCl on the bactericidal activity of Bac8c and HB43 (12.5µg/ml) against *P. aeruginosa* PAO1. The ability of P18 to maintain activity in high salt concentrations (up to 200mM) has already been established in the literature (176). Values shown are the means ± SEM for three independent assays carried out in duplicate. Statistical analyses were carried out using an unpaired two-tailed t-test * denotes p<0.05, ** p<0.01, and *** p<0.001 compared to the peptide in 0mM NaCl.

3.1.5 Considerations for CF

The pro-HDPs are being developed for local delivery to the CF lung, similarly to that described for tobramycin therapy (134). This will potentially limit cleavage of the pro-HDPs to the region of the bronchioles, where mucus and NE are localised (10). To simulate the physiological conditions of the ASL, with which the pro-HDPs will interact, CF BAL fluid was added to bactericidal assays. Incubation of the pro-HDPs with BAL fluid provides endogenous NE for pro-HDP cleavage. As components of BAL fluid have previously been shown to inhibit the activity of some HDPs (77, 119, 121, 177-179), it also acts as a robust test of the feasibility of local lung delivery. Failure of the pro-HDPs to be activated in BAL fluid would mean that their inhaled delivery to the lung would likely fail to treat infection.
The purpose of this chapter was to demonstrate that the reversible reduction of a peptide’s net charge may be used to reduce the epithelial cell cytotoxicity. It is also the goal to determine whether the inhibition of HDP activity in CF can be circumvented by the rational design of HDP prodrugs and whether an environment similar to that found in the CF lung may be effectively exploited in the targeted application of pro-HDPs. This chapter illustrates the potential of pro-HDPs as a delivery strategy to the CF lung on the basis of reduced cytotoxicity and increased specificity.

3.2 Results

3.2.1 Pro-HDPs are cleaved by purified NE

Cleavage of pro-HDPs by 5µg/ml NE was shown after 1h incubation. Cleavage mainly occurred between the 1st and 2nd alanine of the linker group and to a lesser extent, between the 2nd and 3rd alanine (numbered from the N-terminus). This cleavage pattern (i.e. generation of AAG-HDP and AG-HDP) was found for pro-Bac8c and pro-P18. Pro-HB43 yielded predominantly AG-HB43. These cleavage products were synthesised as controls and are called thereafter fragment peptides to differentiate them from the parent sequences. The former peptides differ therefore from the latter ones by the presence of 2 to 3 residual amino acids from the linker. The HPLC chromatograms for the pure cleaved peptides were consistent with the products produced by the original cleavage experiments (see appendices for further information). The AAAG linker was not labile to PE, as indicated by the detection of only pro-HDPs after incubation with this enzyme for 24h. Incubation of Bac8c, HB43, and P18 with 100µg/ml NE for 24h resulted in no cleavage (data not shown).
3.2.2 Pro-HDPs have greater MIC values than parent and fragment HDPs

The MIC values for both parent and fragment HDPs were comparable (Table 3.1). The additional glycine and alanine residues increased the MIC from 4 to 8µg/ml for the HB43 series and there was no increase for the Bac8c or P18 series. In all cases, the pro-HDPs had MIC values greater than or equal to the highest concentration tested (64µg/ml). The MICs for both pro- and active HDPs towards PAO1 were comparable to those found for the four clinical isolates tested (Table 3.1).
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>MIC vs. P. aeruginosa strains (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PAO1</td>
</tr>
<tr>
<td>Bac8c</td>
<td>rlwvlwrr-NH₂</td>
<td>4</td>
</tr>
<tr>
<td>AAG-Bac8c</td>
<td>AAGrlwvlwrr-NH₂</td>
<td>4</td>
</tr>
<tr>
<td>Pro-Bac8c</td>
<td>Ac-EEEEAAAGrlwvlwrr-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>P18</td>
<td>kwlkfklkpkfhhlaklf-NH₂</td>
<td>2</td>
</tr>
<tr>
<td>AAG-P18</td>
<td>AAGkwlkfklkpkfhhlaklf-NH₂</td>
<td>2</td>
</tr>
<tr>
<td>Pro-P18</td>
<td>Ac-EEEEAAAGkwlkfklkpkfhhlaklf-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>HB43</td>
<td>fakkllakllkll-NH₂</td>
<td>4</td>
</tr>
<tr>
<td>AG-HB43</td>
<td>AGfakkllakllkll-NH₂</td>
<td>8</td>
</tr>
<tr>
<td>Pro-HB43</td>
<td>Ac-EEEEAAAGfakkllakllkll-NH₂</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

* Upper case = L-amino acids, lower case = D-amino acids. NT = Not Tested
3.2.3 Pro-HDPs are bactericidal towards PAO1 in the presence of NE

The bactericidal activity of parent and cleaved active HDPs were comparable (Figure 3.6) over the concentration range tested. As 100% killing of PAO1 was achieved at 6.25µg/ml for parent and fragment HDPs, this concentration was selected for bactericidal testing of pro-HDPs in the presence of NE. Bactericidal activity was negligible for pro-Bac8c and pro-HB43 in the absence of NE, whereas pro-P18 was bactericidal (66.8 ± 11.7% killing activity) under these conditions (Figure 3.7). Bactericidal activity increased in the presence of NE for all pro-HDPs. The bactericidal activity, with 5µg/ml NE, increased to 73.3 ± 0.2% for pro-Bac8c, and 90.1 ± 5.8% for pro-HB43 (p<0.0001 for both). The activity also increased for pro-P18, to 93.5 ± 4.7%, but this was not statistically significant (p = 0.102). The level of bactericidal activity appeared to be independent of the NE-concentration tested. No bactericidal effect was observed for NE alone in phosphate buffer pH 7.4, at any of the concentrations investigated (data not shown).
Figure 3.6: Bactericidal activity of parent and fragment HDPs against *P. aeruginosa* PAO1. Data shown are the mean ± SEM for three independent assays carried out in duplicate.
Figure 3.7: Effect of 5-20μg/ml purified NE on the bactericidal activity of pro-HDPs against *P. aeruginosa* PAO1. NE was added to assays containing 6.25μg/ml pro-HDPs. Killing activities shown are the mean ± SEM from three independent assays carried out in duplicate. Where no killing activity was observed, no bar is shown. Statistical analyses were carried out using an unpaired two-tailed t-test, ** denotes p<0.01, and *** p<0.001 compared to the control of pro-HDP with 0μg/ml NE.

### 3.2.4 In the presence of NaCl, pro-HDPs are activated by CF BAL fluids

Six CF BAL fluids were used to investigate pro-HDP activation. The NE concentration in CF BAL fluids varied (range 0 - 193.3μg/ml, Table 3.2). With the exception of CF005, which had significant bactericidal activity (at 25% v/v final assay concentration) against PAO1 (74.5%), the killing activity of the CF BAL fluids against PAO1 was <20%.
Table 3.2: Comparison of the neutrophil elastase concentration and bactericidal activity of different CF BAL fluid samples. Patient details are included.

<table>
<thead>
<tr>
<th>CF BAL</th>
<th>Age</th>
<th>Gender</th>
<th>Genotype</th>
<th>NE Concentration (µg/ml)</th>
<th>% killing*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF001</td>
<td>28</td>
<td>M</td>
<td>ΔF508/E60X</td>
<td>35.7</td>
<td>0</td>
</tr>
<tr>
<td>CF002</td>
<td>22</td>
<td>F</td>
<td>ΔF508/ΔF508</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CF003</td>
<td>25</td>
<td>F</td>
<td>ΔF508/G542X</td>
<td>136</td>
<td>7.8 ± 6.8</td>
</tr>
<tr>
<td>CF004</td>
<td>26</td>
<td>M</td>
<td>ΔF508/ΔF508</td>
<td>46.4</td>
<td>10.4 ± 10.6</td>
</tr>
<tr>
<td>CF005</td>
<td>24</td>
<td>M</td>
<td>ΔF508/Unknown</td>
<td>193.3</td>
<td>74.5 ± 4.6</td>
</tr>
<tr>
<td>CF006</td>
<td>29</td>
<td>M</td>
<td>ΔF508/ΔF508</td>
<td>62.8</td>
<td>15.6 ± 3.3</td>
</tr>
</tbody>
</table>

* Mean ± SEM for 3 separate determinations where 25% v/v BAL fluid was added to assays.

The bactericidal activities of the pro-HDPs were not initially restored by any of the CF BAL fluids investigated. Pro-P18 retained bactericidal activity in the absence of CF BAL fluid. However, after accounting for activity due to BAL alone, the bactericidal activity of pro-P18 was reduced by the addition of CF BAL fluid (Figure 3.8C).
Figure 3.8: Effect of 25% v/v BAL fluid on the bactericidal activity of pro-Bac8c (A), pro-HB43 (B), and pro-P18 (C) against *P. aeruginosa* PAO1. BAL fluid (CF001-006) was added to assays containing 6.25µg/ml pro-HDPs. Killing activities shown are the means ± SEM for three independent assays where activity due to CF BAL fluid alone was subtracted. Where no killing activity was observed, no bar is shown. Controls represent the corresponding cleaved HDP *e.g.* AAG-Bac8c at 6.25µg/ml for pro-Bac8c.

A change in assay conditions, *i.e.* the addition of 300mM NaCl (which reduces non-specific electrostatic interaction of cationic entities such as HDPs with anionic components of CF BAL fluid) and an increase of pro-HDP concentration to 25µg/ml in the bactericidal assays, resulted in the restoration of bactericidal activity of pro-HB43 and pro-P18 but not pro-Bac8c. This effect was shown for three selected CF BAL fluids with NE concentrations in the range of 46.4 - 136µg/ml (BAL fluids with significant
antimicrobial activity were excluded) (Figure 3.9). NaCl alone did not increase the bactericidal activity of CF BAL fluid (Figure 3.10).

![Bar chart showing bactericidal activity of pro-HDPs against P. aeruginosa PAO1](image)

**Figure 3.9:** Effect of 300mM NaCl and 25% v/v CF BAL fluids on the bactericidal activity of pro-HDPs (25µg/ml) against *P. aeruginosa* PAO1. Values shown are the means ± SEM for three independent assays where activity due to CF BAL fluid alone was subtracted. Where no killing activity was observed, no bar is shown. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, and ** p<0.01, compared to the control of pro-HDP with 300mM NaCl alone.
Figure 3.10: Effect of 300mM NaCl on the bactericidal activity of CF BAL fluid (25% v/v) against *P. aeruginosa* PAO1. Values shown are the means ± SEM for three independent assays.

Further increasing the NaCl concentration (450mM and 600mM) did not increase the bactericidal activity of pro-HB43 (BAL fluid CF004, Figure 3.11). The killing activity of pro-HB43 was significantly increased with 300mM NaCl and CF004 BAL against the CF clinical isolates (Figure 3.12). In the case of the isolate PABH02, the activity increased from 0% (pro-HB43 with NaCl) to 99.2 ± 0.7% (pro-HB43 with NaCl and CF BAL, p<0.0001).
Figure 3.11: Effect of 300-600mM NaCl and 25% v/v CF004 BAL fluid on the bactericidal activity of pro-HB43 (25µg/ml) against *P. aeruginosa* PAO1. Values shown are the means ± SEM for three independent assays where activity due to CF BAL fluid alone has been subtracted. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, and ** p<0.01, compared to the control of pro-HDP with 300mM NaCl alone.
Figure 3.12: Effect of 300mM NaCl and 25% v/v CF004 BAL fluid on the bactericidal activity of pro-HB43 (25µg/ml) against *P. aeruginosa* clinical isolates from CF patients. Values shown are the means ± SEM for three independent assays where activity due to CF BAL fluid alone has been subtracted. Where no killing activity was observed, no bar is shown. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, ** p<0.01, and *** p<0.01 compared to the control of pro-HDP with 300mM NaCl alone.

3.2.5 The pro-HDPS potentially inhibit biofilm formation

The activity of the fragment HDPs against established 48h biofilms was investigated using three *P. aeruginosa* strains; PAO1 and the clinical isolates PABH02 and PABH03. The peptides were unable to disrupt the biofilms to a significant extent at concentrations ranging from 2-128µg/ml when analysed for biofilm metabolism, using rezasurin sodium, and mass (Figures 3.13 and 3.14). At 256µg/ml both AG-HB43 and AAG-P18 had some activity against the biofilms of PABH03 and PAO1 (Figure 3.13). However this effect was
modest and only statistically significant in the case of PABH03 with AAG-P18 (from 97.7 ± 4.7% for 2µg/ml to 57.5 ± 10% for 256µg/ml, p = 0.022). This did not correlate with a reduction in biofilm mass (Figure 3.14). AAG-Bac8c did not reduce biofilm metabolism or mass at 256µg/ml, the highest concentration investigated.
Figure 3.13: Antibiofilm activity (metabolism) of 2-256µg/ml fragment peptides against 48h established biofilms of three *P. aeruginosa* isolates/strains; PABH02, PABH03, PAO1. Biofilm metabolism, as represented by rezasurin sodium conversion, was expressed as % of the ‘no peptide’ control. Values shown are the means ± SEM for three independent assays. A positive control of dH₂O was included. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, compared to 2µg/ml peptide.
Figure 3.14: Antibiofilm activity (mass) of 2-256µg/ml fragment peptides against 48h established biofilms of three *P. aeruginosa* isolates/strains; PABH02, PABH03, PAO1. Biofilm mass, as represented by crystal violet staining, was expressed as % of the ‘no peptide’ control. Values shown are the means ± SEM for three independent assays. A positive control of dH2O was included.

The fragment peptides showed more activity in the prevention of biofilm formation (Figures 3.15 and 3.16). Metabolic studies demonstrated that AG-
HB43 had the highest activity, \( e.g. \ 47.4 \pm 15.8\% \) and \( 59.9 \pm 12.8\% \), against PABH03 and PAO1 respectively at 16\( \mu \)g/ml. This was also demonstrated with biofilm mass. 64\( \mu \)g/ml of AG-HB43 and AAG-P18 compared to 2\( \mu \)g/ml reduced the mass of PAO1 biofilms to \( 26.9 \pm 9.9\% \) compared to \( 84.7 \pm 3.3\% \) \((p = 0.005)\) and \( 23.4 \pm 14.5\% \) compared to \( 96.1 \pm 2.4\% \) \((p = 0.008)\) respectively (Figure 3.16).

While effective against PABH02 and PABH03 at 64\( \mu \)g/ml, AAG-Bac8c demonstrated the lowest antibiofilm activity of the three peptides. At 32\( \mu \)g/ml against PAO1, biofilm metabolic activity was \( 103.2 \pm 13.9\% \) (Figure 3.15). Similarly, the biofilm mass of PAO1 in the presence of 64\( \mu \)g/ml AAG-Bac8c was \( 68 \pm 27.3\% \).
Figure 3.15: Biofilm inhibition (metabolism) of 2-64µg/ml fragment peptides against biofilms of three *P. aeruginosa* isolates/strains; PABH02, PABH03, PAO1 grown in the presence of the peptides. Biofilm metabolism, as represented by rezasurin sodium conversion, was expressed as % of the ‘no peptide’ control. Values shown are the means ± SEM for three independent assays. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, ** p<0.01, and *** p<0.001 compared to 2µg/ml peptide.
Figure 3.16: Biofilm inhibition (mass) of 2-64µg/ml fragment peptides against biofilms of three *P. aeruginosa* isolates/strains; PABH02, PABH03, PAO1 grown in the presence of the peptides. Biofilm mass, as represented by crystal violet staining, was expressed as % of the ‘no peptide’ control. Values shown are the means ± SEM for three independent assays. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, ** p<0.01, and *** p<0.001 compared to 2µg/ml peptide.
3.2.6 Pro-HDPs displayed lower cytotoxicity against CFBE41o- cells than fragment HDPs

The 24h IC\textsubscript{50} values were higher for the pro-HDPs compared to the fragment HDPs (Table 3.3). The addition of the pro-moiety increased the IC\textsubscript{50} from 38.3\textmu M to \textgreater 300\textmu M for pro-Bac8c, from 3.6\textmu M to 50.8\textmu M for pro-HB43, and from 35.5\textmu M to 77.3\textmu M for pro-P18. Cytotoxicity was reduced in AAG-P18 compared to P18 with IC\textsubscript{50} values of 35.5\textmu M and 8.1\textmu M respectively. IC\textsubscript{50} values were similar for AG-HB43 and HB43, being 3.6\textmu M and 2.8\textmu M respectively.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC\textsubscript{50} (\textmu M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Bac8c</td>
<td>Ac-EEEEAAAGrlwvlwrr-NH\textsubscript{2}</td>
<td>\textgreater 300</td>
</tr>
<tr>
<td>AAG-Bac8c</td>
<td>AAGrlwvlwrr-NH\textsubscript{2}</td>
<td>38.3</td>
</tr>
<tr>
<td>Pro-HB43</td>
<td>Ac-EEEEAAAGfakllaklakkll-NH\textsubscript{2}</td>
<td>50.8</td>
</tr>
<tr>
<td>AG-HB43</td>
<td>Agfakllaklakkll-NH\textsubscript{2}</td>
<td>3.6</td>
</tr>
<tr>
<td>HB43</td>
<td>fakllaklakkll</td>
<td>2.8</td>
</tr>
<tr>
<td>Pro-P18</td>
<td>Ac-EEEEAAAGkwklfkklpakhhlakkf-NH\textsubscript{2}</td>
<td>77.3</td>
</tr>
<tr>
<td>AAG-P18</td>
<td>AAGkwklfkklpakhhlakkf-NH\textsubscript{2}</td>
<td>35.5</td>
</tr>
<tr>
<td>P18</td>
<td>kwklfkklpakhhlakkf</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Mean values for 3 separate determinations

3.3 Discussion

Life expectancy in cystic fibrosis has been greatly increased by the use of antibiotics to treat lower airway disease and delay the progression from acute to chronic infection (10). However, significant challenges remain to be overcome. Once a patient becomes chronically infected by \textit{P. aeruginosa}, the current guidelines recommend long-term antibiotic therapy. This has led to the development of antibiotic resistance due to selective pressure and sub-therapeutic local drug concentrations (17, 20, 134, 180, 181). In addition, the ability of \textit{P. aeruginosa} to grow as a biofilm further reduces its susceptibility to certain antibiotics (17, 19, 20, 182). Currently-used antibiotics cannot eradicate \textit{P. aeruginosa} chronic infection, and the continuing exaggerated inflammatory response leads to progressive loss of.
lung function (15). There is a need for novel alternative therapeutic agents that are highly active against CF pathogens and less vulnerable to development of resistance. Delivery of such agents in high concentrations in the endobronchial space without the accompanying systemic effects and toxicity would be desirable. The requirement for high concentrations can be met by the use of inhaled drug delivery (20, 41, 134), while HDPs have the potential to meet the need for new agents. Without novel antimicrobials there is a risk that many of the gains made in CF may be lost with increasing antimicrobial resistance.

Potential host toxicity is currently a limitation to the development of HDPs as antimicrobial agents. Here we demonstrate that prodrug modification can mask HDP bactericidal activity and may reduce host cytotoxic effects. Furthermore, the reversible conjugation of the pro-moiety can be targeted for cleavage by NE, in a manner similar to the enzymatic activation of LL-37 by PR-3 in vivo (127), allowing for targeted restoration of bactericidal activity and limitation of cytotoxic effects. NE cleavage produces HDPs that have similar antimicrobial activity to the parent HDPs and, in some cases, reduced cytotoxicity, e.g. P18 vs AAG-P18. In addition, the reactivation of two of the pro-HDPs by NE has been shown under in vitro conditions that are representative of in vivo conditions in the CF lung, through the use of CF BAL fluid. The NE concentration required to cleave the pro-HDPs was within the range observed in CF BAL fluids. NE levels varied considerably in CF BAL fluids in this study and the wide variation is consistent with what has been reported by others. For example, Rees et al reported ranges from 0.47 – 18.5µM (13.8 – 545.8µg/ml) in the sputum (54). In addition, NE concentrations have also been reported to vary over time within the same CF patients (41). While BAL fluid can be considered to be a dilute representation of ASL, the degree of dilution cannot be accurately stated. However, using urea as a marker, for example, it has been estimated to be as high as a 100-fold dilution (183). Taking the dilution level to be 25 to 50-fold it has previously been estimated, that the NE concentrations at the respiratory surface (as opposed to CF BAL itself) may be in the range of 92 – 185µM (2.7 – 5.5 mg/ml) (40). These levels, in combination with the fact that the pro-
HDPs were cleaved in 5µg/ml NE, demonstrate that the proteolytic needs will be met by CF.

Although purified NE was shown to re-activate pro-HDPs in the present study, this effect was not initially apparent in CF BAL fluids containing NE, despite confirmation of NE concentrations that were high enough to catalyse the cleavage step. However, increased concentration of HDPs and the addition of NaCl restored the bactericidal activity of pro-HB43 and pro-P18 in CF BAL fluid, overcoming the antagonism of BAL components. It has been previously observed that the bactericidal activity of LL-37 was inhibited by proteolytic degradation by NE and cathepsin D in CF BAL fluid (119) and that cationic HDPs bind to anionic components of BAL and CF sputum, such as F-actin, eDNA (77, 177), mucins (178), and glycosaminoglycans (119, 184). In addition, both eDNA and mucins have been reported to increase the MIC values for synthetic HDPs such as HB43 (121). It was previously shown that 300-450mM NaCl can, through the disruption of electrostatic interactions, liberate LL-37 from BAL fluid components and restore its bactericidal activity (119). The use of all D-amino acid active HDPs in the present study prevents their proteolytic degradation in vivo, as evidenced by their stability in 100µg/ml NE for 24h. In addition, the stringent cleavage and reactivation conditions demonstrated here for activation in the lung environment, such as the addition of NaCl, may be met therapeutically by inhalation of hypertonic saline and by using higher concentrations of pro-HDPs. Hypertonic saline is currently used alone at 1.2 M for the improvement lung function in CF (119, 185). The failure to achieve activation of pro-Bac8c in the presence of CF BAL and NaCl, in contrast to pro-HB43 and pro-P18, may be related to the ability of the latter two HDPs to maintain activity in high NaCl concentrations (Figure 3.5) (121, 176).

As stated above, the susceptibility of *P. aeruginosa* to antibiotic treatment can be greatly decreased in its sessile biofilm form (4, 19, 186). The biofilm inhibition and disruption activity of the novel HDPs was therefore investigated. Biofilms have been found to be particularly resistant to HDPs, with many of their anionic components binding to the peptides and deactivating them (187). However, some HDPs have still been found to have...
significant antibiofilm activity. Human cathelicidin, LL-37, has been shown to inhibit the formation of *P. aeruginosa* biofilms at concentrations 1/16\textsuperscript{th} of its MIC (4\(\mu\)g/ml) and decreased the thickness of 24h biofilms pre-formed in flow cells (182). Similarly, shortened sequences of LL-37 have been shown to prevent biofilm formation and disrupt those already established, with improved cytotoxicity compared to LL-37 (188). A shortened variant of chicken cathelicidin, F\(_{2,5,12}\), has also been used to impair *Staphylococcus epidermidis* biofilms (189).

The HDPs did not demonstrate significant activity against pre-formed biofilms but prevented biofilm formation. However, the concentration required for the latter was in excess of the MIC and the killing of planktonic bacteria may have likely contributed to this result. Other mechanisms of inhibition, such as disruption of quorum sensing (QS) pathways, which have been described for LL-37 biofilm inhibition (182) may also exist but were not investigated in this model. Given the nature of CF *P. aeruginosa* infections, the ideal pro-HDP candidate would have activity against established biofilms. However, it must be noted that the microtitre method of biofilm analysis used here provides at most, a crude estimation of the antibiofilm activity of antimicrobial agents as it does not accurately represent the conditions of the CF lung where *P. aeruginosa* biofilms form. The use of more sophisticated methods such as peg lids (186), the Calgary Biofilm Device (190), flow chamber assays, and confocal microscopy may provide a more accurate representation of the effects of pro-HDPs on biofilms. The latter would also allow more detailed analyses of the biofilms such as thickness measurement, live/dead staining, and 3D structural analysis (182, 188). Ideally, before any HDP can be considered for CF infections it would have to demonstrate some degree of activity against mature biofilms and if this cannot be shown, other parent sequences would need to be considered.

The effectiveness of the NE-targeted approach for two pro-HDPs is demonstrated here, but there is further scope for the application of this approach to other HDPs. Highly-active HDPs, with varying length, charge, activity and cytotoxicity have been described (121, 172, 191-195). However, the appropriate selection of parent HDP requires consideration. In the
present study, since the number of glutamic acids added to the pro-HDPs was limited to four, the final pro-HDPs were of different net charges (-1, 0 and +3, for pro-Bac8c, pro-HB43, and pro-P18 respectively). While in principle the number of glutamic acids in the pro-moiety can be adjusted to match the number of positive charges of the parent HDP, practically, this number is limited by synthetic constraints. Therefore, the bactericidal activity and cytotoxicity of P18, which still retained significant cationic net charge, was not masked as markedly as for the other candidates (Figure 3.8). These results demonstrate that a high starting net charge may be undesirable for the modification described here. The net charge reduction, while being sufficient for masking activity against *S. aureus* (142), may be insufficient against Gram-negative organisms such as *P. aeruginosa*. Alternatively, other synthetic approaches may be used for parent HDPs with high net charge in order to extend the oligoglutamic acid moiety (142).

The linker moiety of pro-HDPs may also be tailored to contain specific sequences cleavable by enzymes appropriate to the application or the disease. In the present study, the AAAG linker was labile to human NE but not PE from *P. aeruginosa*, both of which are relevant to CF. It is possible that AAAG can be cleaved by other host/bacterial enzymes and this may need to be determined using sophisticated NE-knockout models. If other off-target enzymes are identified that could potentially cleave a pro-HDP in a disease model, they would ideally be investigated.

Suitable HDP candidates for pulmonary delivery as pro-HDPs against CF infections would ideally be short, highly active against *P. aeruginosa*, highly-salt resistant, and of low to moderate net charge. The issue of net charge has been observed previously by our group for cephalothin-Bac8c conjugates, where activity was incompletely masked (162). The use of D-amino acids may be necessary to avoid deactivation by the abundant proteases of the CF lung and any potential candidate would have to be compatible with local lung delivery such as is used with tobramycin and colistin in CF (20, 134, 196).
This prodrug approach potentially provides the means to deliver effective bactericidal HDPs to the CF lung in therapeutic concentrations, while limiting cytotoxicity distal to the site of activation and infection (i.e. the endobronchial space). HDP prodrugs may provide novel anti-infective agents against acute bacterial infection in CF and alternative therapeutics in the prevention of chronic pseudomonal infections.
4. Results Chapter Two: Optimising and Evaluating Host Defence Peptide Prodrugs in Cystic Fibrosis

4.1 Introduction

4.1.1 Rationale

The prodrugs described in Results Chapter One illustrate how the simple addition of glutamic acid residues can increase the selectivity and reduce the cytotoxicity of HDPs in a CF environment. However, host toxicity still remained an unresolved issue with these prodrugs, with both pro-HB43 and pro-P18 displaying cytotoxicity at concentrations close to the antibacterial range. In addition, the complex immune and bacterial environment of the CF lung necessitates that the pro-HDPs need to be evaluated in more complex in vitro models, taking immunogenicity, haemolysis, and cytotoxicity against a wider range of cell types, both immune and epithelial, into account. Therefore this chapter describes the refinement of the pro-HDP design by a variety of means and the further evaluation of the new pro-HDPs to select a candidate peptide with characteristics more suitable for development as a therapeutic for CF.

4.1.2 Further elongation of oligoglutamic acid

The addition of four glutamic acid residues, in conjunction with N-terminal acetylation, produced a reduction of net charge of 5; this is sufficient to reduce the net cationic charge of moderately charged HDPs, such as Bac8c and HB43, below or near 0. However, as seen previously, many active peptides have high net charges (+8 for P18) and therefore significant net charge remains after modification. Therefore it is possible that further gains in specificity can be achieved with additional pro-moiety modification. It was shown in Results Chapter One that the IC\textsubscript{50} value of P18 against CFBF cells was only moderately improved (from 35.5µM to 77.3µM) when net charge was reduced to +3. One factor limiting the production of pro-HDPs with more
The unique conditions of the CF lung also raise challenges for the use of prodrugs bearing four glutamic acids. Numerous studies have demonstrated, in both human and animal models of CF, that the CFTR dysfunction can reduce the pH of the ASL. CFTR malfunction leads to dysregulation of $\text{HCO}_3^-$ and increased ASL acidity, which can negatively impact the antimicrobial activity of some endogenous HDPs, such as LL-37, arising from changes in conformation (1). Many other defence components of the CF lung may be affected, including ciliary function and mucus viscosity (197). In a CF porcine model, ASL pH was decreased compared to wild-type (~pH 6.8 compared to 7.2) with accompanying lower ASL antimicrobial activity. It was also found that when CF ASL pH was increased \textit{in vivo} to the level found in the healthy lungs (using aerosolised NaHCO$_3$) that the CF ASL antimicrobial activity increased. Conversely, decreasing pH levels in wild-type pigs to that of CF animals had the opposite effect (198). In a human study, breath condensate was collected in both healthy and CF subjects and analysed. It was found that pH was decreased in all CF patients compared to healthy individuals, with the largest difference noted between healthy controls and CF patients undergoing exacerbations (pH 6.15 vs. 5.32 respectively) (197). The importance of this pH change in a pro-HDP context is that the side-chain carboxylic acid of glutamic acid has a pKa of 4.1. This means that at physiological pH it will remain deprotonated, negatively charged, and thus able to reduce the net charge of the pro-HDPs and deactivate them (see Figure 4.1). However, as pH decreases, the $\gamma$-carboxyl becomes protonated and the net charge of the pro-HDPs increases; the possibility exists then that the charge effects of the pro-moiety may be reduced. Additional glutamic...
acid residues might protect against this charge alteration. In addition to investigating longer glutamic acid pro-moieties, the effect of pH on the existing pro-HDPs was investigated.

Figure 4.1: The different conjugate base and acid forms (γ-carboxyl group) of Glutamic acid at pH 7.4 and 4.1.

4.1.3 PEG as a pro-moiety

An alternative to using oligoglutamic acid as a pro-moiety is PEG (Figure 4.2). As discussed in section 1.6.2, PEG is the most widely used polymer in anticancer therapy, with many PEG-prodrug systems displaying higher water solubility, lower degradative enzyme lability, and higher specificity than the active anticancer agent alone. Many cytotoxic drugs, such as methotrexate and cisplatin have been PEGylated, with site-specific delivery built-in, such as controlled degradation in the target cell or tissue (147).
Figure 4.2: PEG-AAAG-P18, in this pro-HDP the oligoglutamic acid pro-moiety is replaced by a PEG of n subunit length (top left of molecule).

PEGylation, while widespread in anticancer therapies, has not been used extensively with antimicrobial peptides. Studies that have been carried out have shown that PEGylation in general will reduce both cytotoxicity and antimicrobial activity (154, 155). However, a combination of correct linker and PEG size can produce PEGylated HDP prodrugs that are cleavable by target enzymes, for example serum-derived trypsin (158). PEGylation has the potential to reduce the cytotoxicity of P18 and for this reason PEG was investigated as an alternative pro-moiety to oligoglutamic acid.

4.1.4 Replacement of TFA as a counter-ion

The cleavage of the peptide from the insoluble resin requires the use of TFA. In addition, TFA is used to improve HPLC purification by sharpening peaks. Therefore all peptides produced by SPPS are TFA salts (see Figure 4.3). It is common practice to convert the peptides from TFA salts to another counter-ion such as acetate before using peptides in in vivo studies due to concerns
about the cytotoxic effects of the salt. The number of TFA ions associated with each peptide depends on the number of basic residues it carries, in addition to the (free) N-terminus, and it is possible that the high cytotoxicity of HB43 may be in part due to the number of TFA counter-ions associated with it. Counter-ion replacement can be achieved by a variety of means, including the use of an acid, such as HCl, that is stronger than TFA and will replace it as counter-ion. However, this approach requires an excess of a strong acid solution which has the potential to damage the peptide. As a result, an alternative approach using ion exchange resins is often employed successfully to replace TFA (199). HB43 can be expected to have five associated TFA molecules. The effects of salt exchange of HB43 on both \textit{in vitro} cytotoxicity and antimicrobial activity were investigated to determine whether additional improvements in specificity could be achieved with this simple procedure and whether some of the cytotoxic effects observed were due to the counter-ion.
Figure 4.3: HB43 with five TFA counter ions for the positively charged lysine residues and N-terminal NH₃.

4.1.5 Investigating alternative HDP sequences

The ideal basis of any pro-HDP for CF has been described in the conclusions of Results Chapter One. A number of HDPs have been described in the literature that meet some or all of these criteria. There is a large library of short HDPs, many of which are very effective against *P. aeruginosa*, e.g. the 18 amino acid horseshoe crab peptide Polyphemusin 1 has a MIC of 0.25μg/ml against PAO1 (129). However, when salt-resistance and a low net charge are required, the number of available sequences decreases. While a number of salt-resistant peptides have been developed, a large proportion of them are too long for the purposes of this study. Examples are the analogues of Human β-Defensins (hBD) 1 and 3 developed by Scudiero *et al*, which combine domains of both defensins. These are active in high NaCl concentrations, unlike hBD-1 alone, and
demonstrate little cytotoxicity at active concentrations. However, their net charge and length are major disadvantages e.g. the most promising peptide 3N1, is 38 residues long with a net charge of +11 (192, 200).

Chu et al synthesised a series of short peptides modified with a bulky β-naphthylalanine (Nal) group at their termini. These showed greatly increased antimicrobial activity against *P. aeruginosa* in 300mM NaCl compared to the non-modified peptides e.g. MIC of 3.125µg/ml compared to >50µg/ml for the HDP S1. The modification also improved the serum-stability of the peptides. However, in addition to economic considerations associated with using a non-natural amino acid like Nal, this approach demonstrated several shortcomings, with the modified peptides displaying increased haemolytic activity at relatively low concentrations (40% haemolysis for S1-NalNalNal at 12.5µg/ml) which would preclude their use *in vivo* (201).

An alternative approach, given the requirement for activity in an environment of high salinity, is investigating marine sources of HDPs. In studies, a synthetic peptide derived from a precursor found in the haemocytes of the tunicate *Ciona intestinalis* Ci-MAM-A24, had an MIC against *P. aeruginosa* of 0.8µM (2.2µg/ml), retained its activity in 150mM NaCl against *E. coli*, and showed little haemolysis at concentrations up to 10µM (202). A further study into its activity against *P. aeruginosa* found a Minimum Bactericidal Concentration range of 1.56-3.125µg/ml against 10 multiresistant clinical isolates (203). However, like the hBD analogues, its length (24 amino acids) and relatively high net charge of +7 do not make it ideal for the prodrug modification. The HDP myxinidin, derived from the hagfish *Myxine glutinosa*, has been shown to have significant antibacterial activity in 300mM NaCl and no haemolytic activity. Being one of the most primitive vertebrates, hagfish lack many components of adaptive immunity, relying instead on a robust innate immune response. Myxinidin is found in the epidermal mucus, acting as a barrier to aquatic infection (204). Further improvements to its activity were achieved in a subsequent study, where a structure-activity relationship produced the peptide WMR. They found an MIC of 2µM (3.3µg/ml) against *P. aeruginosa* with little haemolysis and cytotoxicity against Vero cells at
200µM. This, combined with its length of 13 residues and net charge of +5 made it a promising candidate for pro-HDP modification (205, 206).

Another potential source of short, salt-resistant HDPs are those rich in tryptophan and arginine. A number of studies have produced relatively-short Trp-Arg peptides that retain their activity in NaCl. As mentioned above, hBD-3 is salt-resistant, with its C-terminal RRKK motif believed to contribute to this. Saravana et al attempted to incorporate this into the design of a 10 residue synthetic analogue with increased hydrophobicity with tryptophan substitution and increased net charge with lysine and arginine substitution. They produced a series of peptides that had good activity against *E. coli*, *S. aureus* and *P. aeruginosa* with low haemolytic potential, but unfortunately the activity was lost against *P. aeruginosa* in 150mM NaCl (207). Another group produced a 13 residue peptide with an MIC of 3.125µg/ml against *P. aeruginosa* in 200mM NaCl. It was designed rationally with a tryptophan substitution at position 9 found to greatly increase activity in high salt concentrations. The authors conclude that this leads to an increase in hydrophobic surface available to insert into target membranes and is the basis of the increased activity in NaCl. This promising peptide was unsuitable for prodrug modification however as it had a net charge of +8 (208).

Deslouches et al synthesised a series of Trp-Arg peptides ranging from 6 – 18 residues in length. This was based on the observation that substituting tryptophan for valine in Trp-Val peptides resulted in increased broad-spectrum antibacterial activity in challenging conditions such as in saline, serum, and whole blood. The most promising of the candidates was WR12. This peptide has net charge +6 and 12 residues in length, had a reported MIC of 11µg/ml against PAO1, retained activity in 150mM NaCl, and had limited haemolysis at active concentrations (209). Against a panel of 100 CF *P. aeruginosa* clinical isolates it demonstrated good activity (210). Its combinations of high activity and short sequence led to its selection as a possible pro-HDP candidate.
4.2 Results

4.2.1. pH does not decrease the MIC of the pro-HDPs

The effects of reducing pH on the MIC of pro-Bac8c, pro-HB43, and pro-P18 was investigated against PAO1. The antimicrobial activity of the pro-HDPs was unaltered when measured in Mueller-Hinton broth adjusted to pH 5 and 6, with MIC values remaining the same (Table 4.1). This was compared to unadjusted MH broth (pH 7.3). In the case of pro-P18, MIC increased from 64µg/ml to >64µg/ml in media adjusted to pH 5 and 6 compared to pH 7.3.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>MIC vs. P. aeruginosa PAO1 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 7.3</td>
</tr>
<tr>
<td>Pro-Bac8c</td>
<td>Ac-EEEEAAAGrlwvlwrr-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Pro-HB43</td>
<td>Ac-EEEEAAAGfakllakakkll-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Pro-P18</td>
<td>Ac-EEEEAAAGkwklfkklpkfhlhlakkf-NH₂</td>
<td>64</td>
</tr>
</tbody>
</table>

* Upper case = L-amino acids, lower case = D-amino acids.

4.2.2 Further elongation of oligoglutamic acid yielded no improvements

While a five glutamic acid pro-moiety was successfully added to P18 (named 5E-P18), it was not possible to add six or more. Lability of 5E-P18 with NE was confirmed by HPLC. As with the original pro-moiety, 5E-P18 had a higher MIC than the AAG-P18 against *P. aeruginosa* (Table 4.2). When tested against PAO1, 20µg/ml NE increased the bactericidal activity of 0.78µg/ml 5E-P18 from 13 ± 1.5% to 89.3 ± 3.3% (p<0.0001) (Figure 4.4). However, the difference in cytotoxicity compared to the original pro-P18 was negligible with IC₅₀ values of 79.4µM and 77.3µM respectively (Table 2.3).
Table 4.2: MIC values for parent HDPs, fragment HDPs, and pro-HDPs vs. *P. aeruginosa* PAO1 and clinical isolates PABH01-04

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>MIC vs. <em>P. aeruginosa</em> strains (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PAO1</td>
</tr>
<tr>
<td>WMR</td>
<td>wglrrllkygkrs-NH₂</td>
<td>64</td>
</tr>
<tr>
<td>L-WMR</td>
<td>WGIRRILKYGKRSH₂</td>
<td>64</td>
</tr>
<tr>
<td>AAG-WMR</td>
<td>AAAGwglrrllkygkrs-NH₂</td>
<td>32</td>
</tr>
<tr>
<td>Pro-WMR</td>
<td>Ac-EEEEAAAGwglrrllkygkrs-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>L-Pro-WMR</td>
<td>Ac-EEEEAAAGWGLRRLLKYGKRSH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>AAG-WR12</td>
<td>AAAGrwrrwrrwrrwrr-NH₂</td>
<td>32</td>
</tr>
<tr>
<td>Pro-WR12</td>
<td>Ac-EEEEAAAGrwrrwrrwrrwrr-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>PEG-P18</td>
<td>PEG-AAAGkwlkflkklpflhhlak九龙-NH₂</td>
<td>&gt;64</td>
</tr>
<tr>
<td>5E-P18</td>
<td>Ac-EEEEAAAGkwlkflkklpflhhlak九龙-NH₂</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

* Upper case = L-amino acids, lower case = D-amino acids.
Table 4.3: IC₅₀ values for parent HDPs, cleaved HDPs, and pro-HDPs versus CF bronchial (CFBE) and tracheal (CFTE) epithelial cells lines, and healthy neutrophils

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CFBE</td>
</tr>
<tr>
<td>AAG-WMR</td>
<td>AAGwglrllkygkrs-NH₂</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Pro-WMR</td>
<td>Ac-EEEEAAAGwglrllkygkrs-NH₂</td>
<td>&gt;300</td>
</tr>
<tr>
<td>L-Pro-WMR</td>
<td>Ac-EEEEAAAGWGLRRLLKYGKRS-NH₂</td>
<td>&gt;300</td>
</tr>
<tr>
<td>AAG-P18</td>
<td>AAGkwklfklpkfhlhlakkf-NH₂</td>
<td>35.5†</td>
</tr>
<tr>
<td>Pro-P18</td>
<td>Ac-EEEEAAAGkwklfklpkfhlhlakkf-NH₂</td>
<td>77.3†</td>
</tr>
<tr>
<td>5E-P18</td>
<td>Ac-EEEEAAAGkwklfklpkfhlhlakkf-NH₂</td>
<td>79.4</td>
</tr>
<tr>
<td>PEG-P18</td>
<td>PEG-AAAGkwklfklpkfhlhlakkf-NH₂</td>
<td>38.7</td>
</tr>
<tr>
<td>Pro-moiety</td>
<td>Ac-EEEEAA-OH</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

* Upper case = L-amino acids, lower case = D-amino acids. †=From Results Chapter One. ND=Not determined.

Figure 4.4: Effect of 20µg/ml NE on the bactericidal activity of 5E-P18 and PEG-P18 against *P. aeruginosa* PAO1. Killing activities shown are the mean ± SEM from three independent assays carried out in duplicate. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, ** p<0.01, and *** p<0.001 compared to the pro-HDP with 0µg/ml NE.
4.2.3 PEGylation produced an inferior pro-moiety to oligoglutamic acid

A 2000 Da (polydisperse) PEG was successfully coupled to P18 with an AAAG linker using the same conditions as amino acid coupling, except for the use of 2 molar equivalents of MeO-PEG-OH and PEGA resin instead of Rink Amide MBHA resin. The PEGA resin, a poly(ethylene glycol-co-acrylamide) support unlike the polystyrene-based Rink Amide MBHA resin, allows the diffusion of macromolecules of molecular masses up to 35kDa into the polymer matrix, affording PEGylation of peptides on their solid support. The final purified product (separated on HPLC using a C5 column as opposed to a C18 column for the other peptides) showed some polydispersity, as would be expected with the PEG reagent used (see PEG-AAAG-P18 in appendix Figure A19). As with the original pro-P18, the MIC against \textit{P. aeruginosa} was increased compared to the cleaved P18 (Table 4.2). However, PEG-P18 did not show any cleavage after incubation with NE at 37°C (data not shown). This was also observed with PAO1, where the addition of purified NE did not increase the activity of PEG-P18 (Figure 4.4). Furthermore, PEG-P18 was more cytotoxic than pro-P18, with an IC\textsubscript{50} against CFBE cells of 38.7µM (Table 4.3).

4.2.4 Replacing TFA as a counter-ion yielded no improvements in \textit{in vitro} specificity

To investigate whether replacing TFA as a counter-ion would improve the host toxicity profile of HB43, an ion exchange to Cl\textsuperscript{−} was carried out. \textsuperscript{19}F NMR analysis was used to confirm the successful complete removal of TFA as a counter-ion while leaving the active peptide, HB43, unchanged (see appendix Figures A16 and A17). Comparison between the MIC values against PAO1 and the \textit{P. aeruginosa} clinical isolates found no difference in activity. In addition, there was little change in cytotoxicity against CFBE cells with IC\textsubscript{50} values of 2.8µM and 3.7µM for trifluoroacetate and chloride counter-ions respectively (Table 4.4).
Table 4.4: MIC values for HB43 chloride and TFA salts vs. *P. aeruginosa* PAO1 and clinical isolates PABH01-04. IC$_{50}$ values against CFBE41o- cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC vs. <em>P. aeruginosa</em> strains (µg/ml)</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAO1</td>
<td>PABH01</td>
</tr>
<tr>
<td>HB43 TFA</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>HB43 Cl</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

### 4.2.5 New HDP sequences yielded improved specificity

WMR and WR12 were selected for pro-HDP modification with the tetreglutamic acid pro-moiety and both were successfully synthesised. The synthesis of pro-WR12 was difficult and the yield was low due to a large number of deletion peptides formed *i.e.* peptide impurities where one or more amino acid has not been incorporated into the sequence. NE cleaved the peptides, leaving AAG-WMR and AAG-WR12 as cleavage products. As with the first generation of pro-HDPs, MIC values increased against *P. aeruginosa* from 8-32µg/ml for the fragment peptides to >64µg/ml for the pro-HDPs. The activity of AAG-WMR compared to L- and D-WMR was not decreased except in the case of the isolate PABH04, indicating that the additional AAG residues and change to D-stereochemistry were generally not detrimental to activity (Table 4.2).

The activity of both fragment peptides against PAO1 was maintained in NaCl concentrations ranging from 50 - 250µM (Figure 4.5) and the activity of the pro-HDPs was increased with the application of 20µg/ml NE, increasing from 12.1 ± 3.8% to 93.8 ± 2.8% for 3.125µg/ml pro-WMR (p<0.0001) (Figure 4.6).
Figure 4.5: The effect of NaCl on the bactericidal activity of WMR, AAG-WMR, and AAG-WR12 (12.5µg/ml) against *P. aeruginosa* PAO1. Values shown are the means ± SEM for three independent assays carried out in duplicate.
**Figure 4.6**: Effect of 20µg/ml NE on the bactericidal activity of pro-WMR and pro-WR12 against *P. aeruginosa* PAO1. NE was added to assays containing 3.125µg/ml pro-HDPs. Killing activities shown are the mean ± SEM from three independent assays carried out in duplicate. NE alone had no killing activity (data not shown). Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, ** p<0.01, and *** p<0.001 compared to the pro-HDP with 0µg/ml NE. ns = not significant.

Because of the difficulties in synthesis and the poor synthetic yield, pro-WR12 was not investigated further as a candidate. Studies with pro-WMR showed that activity in 300µM NaCl was greatly increased when 25% v/v CF BAL fluid was included in assays, e.g. from 8.4 ± 6.8% to 86.4 ± 0.9% with BAL fluid CF004 (p = 0.0004) (Figure 4.7). HPLC and MALDI-TOF MS analysis of pro-WMR incubated with 50% v/v CF004 (without NaCl) indicated that the cleavage product was the same as with purified NE, *i.e.* AAG-WMR. Cleavage was complete after 3h (see appendix Figure A27). Conversely the incubation of pro-WMR with two sarcoidosis non-CF BAL fluids (with no NE activity detected) resulted in no cleavage of the peptide after 3h incubation, similar to incubation with PBS (appendix Figures A34-36). After incubation all samples were filtered using a Centrisart centrifugal filter with a 10kDa cut-off (Sartorius, Ireland) to remove large proteins before analysis. Pro-WMR was
incubated with 5µg/ml purified CG, and was found to be unchanged after 1h incubation (appendix Figure A45).

![Graph showing P. aeruginosa killing activity (%) for different BAL fluids](attachment:figure4.7.png)

**Figure 4.7**: Effect of 25% (v/v) CF BAL fluids on the bactericidal activity of pro-WMR (25µg/ml) against *P. aeruginosa* PA01 in the presence of 300mM NaCl. Values shown are the means ± SEM for three independent assays where activity due to CF BAL fluid alone was subtracted. Statistical analyses were carried out using an unpaired two-tailed t-test, *** denotes p<0.001 compared to the pro-HDP in 300mM NaCl without BAL fluid.

As expensive D-amino acids represent a potential economic barrier to the large-scale production of peptide therapeutics, an all-L-amino acid version of pro-WMR was synthesised. Although both D- and L- forms had comparable MICs (Table 4.1), when incubated with purified NE or CF BAL fluid, the active L-sequence as well as the pro-moiety, was cleaved. In addition, the bactericidal activity of L-pro-WMR against PA01 was not increased with the addition of NE (Figures 4.6 and appendix Figures A30 and A31). To evaluate whether CF BAL fluid could cleave the pro-moiety after the linker, the peptide pro-GG-WMR (Ac-EEEEGwglrrlkygkrs-NH₂) was synthesised and also incubated with CF BAL fluid. After 3h the peptide remained intact (see appendix Figure A33).
4.2.6 Pro-HDPs do not stimulate IL-6/IL-8 release

At sub-IC$_{50}$ concentrations of pro-WMR and AAG-WMR (up to 100µM), negligible levels of the pro-inflammatory cytokines IL-6 and IL-8 were released from CFBE cells compared to the positive control (50µg/ml LPS). Similarly, no release was observed with the pro- and cleaved peptides of HB43 and P18 (Figure 4.8).

**Figure 4.8:** Cytokine release of IL-8 (A) and IL-6 (B) from CFBE cells in response to incubation with pro- and active peptides at sub-IC$_{50}$ concentration for 24h. Cytokines were measured using a Human Pro-inflammatory Panel V-PLEX Plus Kit. LPS concentration is 50µg/ml, control represents cells alone. Values shown are the means ± SEM for three independent experiments. TNF-α and IL-1β were also measured but the cells did not produce significant amounts in response to any stimulus.
4.2.7 Pro-WMR demonstrates low human cell cytotoxicity

Both pro-WMR and the cleavage product, AAG-WMR, displayed low cytotoxicity against CFBE cells, with an IC$_{50}$ over 300µM, but solubility issues precluded the extension of the concentration range beyond 300µM for pro-WMR (Table 4.3). The released pro-moiety (synthesised from an Ala-Wang resin to maintain the C-terminus carboxyl) Ac-EEEEAA-OH also demonstrated no cytotoxicity over the concentration range tested. Similarly the IC$_{50}$ against CFTE cells was >600µM for AAG-WMR and >300µM for pro-WMR. This was higher than that for the previous lead pro-HDP, pro-P18, with IC$_{50}$ values against CFTE cells of 55.9µM and 4.7µM for pro- and AAG-P18, respectively.

The haemolytic activity of pro-WMR and its cleavage products was compared to pro-HB43, pro-P18, and their cleavage products. No haemolysis was seen up to 500µM (the maximum concentration investigated) for pro-WMR and AAG-WMR (Figure 4.9A), compared to significant concentration-dependent haemolysis with the other pro-HDPs (Figure 4.9B/C). Since the pro-HDPs are designed for cleavage by a neutrophil-derived enzyme, the toxic effects of the cleaved active peptides against purified neutrophils was also investigated. The 3h IC$_{50}$ for AAG-P18 was found to be 9.2µM and >300µM for AAG-WMR (Table 4.3).
Figure 4.9: Haemolytic activity in response to HDPs. Haemolysis is expressed as % Haemolysis relative to the 0.1% Triton-X control after 24h for pro-WMR and AAG-WMR (A), pro-HB43 and AG-HB43 (B), and pro-P18 and AAG-P18 (C). Values shown are the means ± SEM for three independent experiments.
4.2.8 AAG-WMR kills pre-formed biofilms

Exposure of PAO1 biofilms, formed under flow for 48h, to concentrations of AAG-WMR above its MIC for 6h resulted in bacterial cell death which was concentration-dependent over the concentrations range investigated. Using confocal microscopy, the relative fluorescence of live cells to dead cells decreased with increasing peptide concentration. Percentage biofilm viability decreased from 87.6 ± 4.4% for media alone to 28 ± 1.6% for 256µg/ml AAG-WMR (p = 0.0002). Representative confocal images are provided of each biofilm chamber including the positive control of 50% ethanol in media (Figure 4.10).
Figure 4.10: Viability of a 48h PAO1 flow biofilm exposed statically to AAG-WMR in PBS for 6h. % viability represents the fluorescence of SYTO® 9 green (live) relative to propidium iodide (dead) viewed under a 40x oil immersion lens. Biofilm images taken of each assay condition are representative (A). Values shown are the means ± SEM for three independent experiments (B). Statistical analyses were carried out using an unpaired two-tailed t-test, ** denotes p<0.01, and *** p<0.001 compared to the negative control (PBS alone). The positive control is 50% ethanol in PBS. ns = not significant.

4.3 Discussion

We previously demonstrated in Results Chapter One, using the pro-HDPs pro-HB43 and pro-P18, how an oligoglutamic acid modification could be used to target the activity of HDPs while limiting the cytotoxicity. These
results have been published (211). However there were cytotoxicity concerns with these pro-HDPs and this required consideration. The focus of this chapter was on exploring additional methods of improving selectivity and evaluating the potential pro-HDP therapeutics more fully in terms of cytotoxicity, haemolytic potential, and immunogenicity.

We have investigated here whether elongating the oligoglutamic acid sequence beyond four residues offers further advantages. Pro-P18 was chosen as the HDP for this purpose as it showed significant antimicrobial activity at 6.25 µg/ml (Figure 3.7) and had a net charge of +3 which could be further reduced; for comparison purposes the net charge of active Bac8c is +4. The synthesis of these elongated peptides proved difficult with five glutamic acids requiring multiple couplings and the aid of microwave activation. Glutamic acid modification beyond five residues was not attained. While the five glutamic acid pro-P18 showed reduction of antimicrobial activity (restored by NE), and reduced cytotoxicity compared to AAG-P18, there was no discernible advantage over the tetraglutamic acid pro-P18.

When these results are taken in conjunction with the difficulty in synthesis and the increased resource demands, the addition of further glutamic acids does not seem advantageous. The other rationale for additional reductions in net charge, that the slightly acidic conditions of the CF lung might protonate glutamic acid and compromise the net charge-reducing prodrug effects, was not supported by the results, with three pro-HDPs maintaining their high MICs in pH 5 and 6. This is likely to originate from the difference (greater than 1 pH unit) between the pKa of the side-chain carboxyl of glutamic acid and the lowest pH tested. In the CF lung the protective effects of the pro-moiety would not be expected to be compromised by pH. For these effects to become significant the pH may need to decrease to pH 4 (the pKa of glutamic acid carboxyl being 4.1), which is not biologically relevant for CF (197, 198).

PEGylation is a well-established method of prodrug delivery, particularly in the delivery of cytotoxic agents such as paclitaxel and larger biological agents such as erythropoietin (Mircera®); allowing a reduction in activity, reduction in cytotoxicity and controlled delivery (147). Unfortunately,
PEGylated P18 only demonstrated the first characteristic. While the 2000 Da PEG reduced the antibacterial activity of P18, this was not reversed by the addition of NE, with the enzyme unable to cleave the linker group. In addition, no benefit was demonstrated in terms of reduced cytotoxicity, with an IC$_{50}$ against CFBF cells of 38.7µM compared to 35.5µM for AAG-P18. It may be that the PEG is not large enough to provide adequate reductions in cytotoxicity but still so large that it sterically hinders the cleavage of the linker by NE. Improvements in both cytotoxicity and enzymatic release of the active peptide might be achieved with the combination of a larger PEG and a longer linker; however, this would mean a much larger final product and therefore lower drug loading, which may be a disadvantage, especially considering these improvements can be made with the much simpler oligoglutamic acid pro-moiety. The already large size of the molecule before adding a long PEG is illustrated by Figure 4.2. Previously, the effect of PEG size on HDP release has been noted. Nollman et al produced a series of HDP-PEG conjugates with various linker groups. The release of the active HDP by the target enzyme (Trypsin) was far more efficient with the 750 Da PEG than the 5000 Da PEG (for one peptide, 70% after 1h and 20% after 4h respectively). They also noted a reduction in antimicrobial activity, albeit one more modest than found here e.g. MIC values of 0.7µM vs. 2.8µM with and without Trypsin respectively for a 750 Da prodrug (158). Another study, comparing a 600 and 2200 Da PEG found a correlation between increasing PEG length and a reduction in HDP antimicrobial activity and haemolysis. PEGylation was also found to potentially reduce cytotoxicity (155). Similarly, PEGylation of the HDP CaLL produced a modest reduction in MIC and cytotoxicity (156). Importantly, none of these studies fully quantified the IC$_{50}$ against human cells, making the actual reduction in cytotoxicity hard to evaluate. The full determination of cytotoxicity used here may give a more accurate picture of the effect of 2000 Da PEG on a HDP such as P18.

In a manner that has been noted previously by Pini et al (199), replacement of the TFA salt in HB43 had no effect on in vitro antimicrobial activity or cytotoxicity, with MIC and IC$_{50}$ values near-identical for both the TFA and Cl$^-$ salts. This would indicate that for the purposes of in vitro evaluation of HDPs
there is no benefit to ion exchange and that the salt has negligible effect on the activity, even for a peptide like HB43 with high cytotoxicity and moderate net charge. The toxic effects of this peptide against host cells at low concentrations (Table 4.4) are a major barrier to its use in vivo and peptides with similar or better antimicrobial activity and lower cytotoxicity are available. Counter-ion replacement is still worthwhile for in vivo studies, as the removal of TFA does not seem to reduce antimicrobial activity and may reduce variability in response to the HDPs (199).

The most success in improving the selectivity of the pro-HDPs was seen in the replacement of the active HDP itself. A search for new sequences from the large HDP library, based on desirable characteristics identified in Results Chapter One, produced two new candidates, WMR and WR12. As expected, they both demonstrated excellent salt-resistance. The two candidates were synthesised as pro-HDPs, and produced similar cleavage patterns in response to NE as seen with the first generation. The cleaved, active peptides had lower MICs against *P. aeruginosa* than the pro-HDPs but activity for both was less than previously reported in the literature, i.e. MIC values of 64µg/ml vs. 3.3µg/ml for L-WMR (205), and 32µg/ml for AAG-WR12 vs. 11 ± 5µg/ml for WR12 against PAO1 (209). It is possible that the modifications to WR12 may adversely affect the MIC, although for WMR the additional alanine and glycine residues did not affect activity. The differences in MIC may be due to the use of different strains and assay conditions. Greater salt tolerance was observed for AAG-WR12 and AAG-WMR compared to the previous generation of cleaved HDPs. This is a favourable characteristic for a HDP as CFTR dysfunction has been linked to increasing salt concentrations in the ASL (121), although this remains the subject of debate (5, 122-124). In addition, high salt tolerance would facilitate the delivery of these HDPs with hypertonic saline, the inhalation of which improves lung function in CF (73, 119).

The synthesis of pro-WR12 was challenging, requiring multiple coupling cycles for each amino acid after the active sequence i.e. Ac-EEEEEAAAG and resulting in low yields. A number of contaminating deletion peptides, i.e. peptides where a coupling step has been missed and in which an amino acid
is absent, reduced the final yield of pure pro-WR12 to near 1mg (the theoretical yield being 145mg). This precluded cytotoxicity studies and allowed only limited antibacterial assays (Figure 4.11).

**Figure 4.11:** 214nm RP-HPLC of crude pro-WR12. The number of peptide peaks from RT 21-23min indicates a series of deletion peptides arising from inefficient couplings. A crude product like this is exceedingly difficult to purify by HPLC and the majority of the crude product are peptides with an undesired sequence (A). Absorption spectrum of crude pro-WR12, high absorbance at 214nm and 280nm indicates the impurities are also peptides containing tryptophan (B).

This was in contrast to the synthesis of pro-WMR, which was less complex and had a much higher yield. In 25% v/v CF BAL fluid pro-WMR performed better than both pro-HB43 and pro-P18, with near full bactericidal activity in BAL fluid but little activity in its absence, characteristics not seen with the others (Figure 3.9). This is significant because components of BAL such as proteases, mucins, and extracellular DNA may inactivate other HDPs (77,
Complete cleavage of pro-WMR to the active HDP was observed using HPLC after 3h incubation with 50% v/v CF BAL fluid, but no conversion was observed in non-CF BAL fluid, devoid of NE activity. This demonstrates the enzyme-targeting afforded by the pro-moiety and linker. A prodrug of WMR with an un-cleavable GG linker was unchanged after 3h incubation with CF BAL fluid, indicating that AAAG is the only point cleavable by the BAL fluid enzymes. The lack of cleavage observed with purified CG, purified PE in Results Chapter One, and the observed inability of PR-3 to cleave AAA in the literature strengthens the case for the specificity of the linker for NE (50).

The cost of production of peptide drugs is greatly increased by the use of non-natural amino acids such as the D-amino acids; as a result their necessity in the design was investigated. The use of an all-L-pro-WMR, while providing low antimicrobial activity and cytotoxicity (Tables 4.1 and 4.2), resulted in cleavage of the active sequence by NE, both purified and in CF BAL fluid and therefore precluded its use in this disease model. The HPLC and MALDI-TOF MS analyses indicated multiple cleavage sites, ruling out the simple substitution of one or two amino acids with D-isomers, which would be an alternative approach to reducing costs and the issue of increased side effects with a prolonged peptide half-life (212). This degradation in CF samples has been seen previously with the HDP P-113, where 10% v/v CF sputum completely cleaved the peptide after 10min, removing all activity against P. aeruginosa. The addition of SLPI or A1AT had only a modest protective effect. The D-peptide, on the other hand, remained stable and retained activity in CF sputum even after one week’s incubation (76). For CF at least, it appears that an all-D-active sequence is essential to survive the challenging proteolytic conditions.

Cytotoxicity is one of the major issues that have limited the progress of HDPs as therapeutics. Despite the improvements previously seen with HB43 and P18 after pro-HDP modification, some cytotoxic effects were evident based on their low IC\(_{50}\) values against CFBE cells (50.8\(\mu\)M and 77.3\(\mu\)M respectively). Their toxic effect on neutrophils, from which large amounts of the target enzyme are derived, was also unknown and to our knowledge, has not been investigated with HDPs before. Pro-WMR shows superiority to the
previous generation of pro-HDPs, exhibiting lower cytotoxicity against CFBE cells, CFTE cells and neutrophils, and lower haemolysis against erythrocytes. This is in agreement with the low cytotoxicity against Vero cells and haemolysis originally seen with L-WMR by others (205, 206).

The cleaved peptide, AAG-WMR was not as active against *P. aeruginosa* as AG-HB43 or AAG-P18 (Table 3.1) but the improvements in toxicity compensate for this. For example, it was observed in this study that AAG-P18 demonstrated high toxicity against neutrophils with an IC\(_{50}\) of 9.2µM. It is therefore likely that, upon delivery to the CF lung, neutrophils would be subjected to a high concentration of the active peptide and a large proportion would be killed. While CF is a neutrophil-dominated disease and the resultant high levels of NE contribute to morbidity (39, 40), it may be unfavourable to kill immune cells when a patient is suffering from a potentially severe infection. The necrosis of neutrophils in CF also leads to the release of extracellular DNA which increases mucous viscosity and facilitates bacterial attachment (19). In comparison, pro-WMR and AAG-WMR had no measurable cytotoxicity in any cells tested. The lack of haemolysis is important as microbleeds in CF lead to the release of erythrocytes, the lysis of which can release haemoglobin, which is converted to haem by NE and acts as an inducer of IL-8 release and as an iron source for *P. aeruginosa* (32).

The immunomodulatory properties of many endogenous HDPs such as LL-37 are well-documented (110), however, the effects of exogenous peptides on immune function are less clear. The rationally-designed IDR HDPs, although devoid of antimicrobial activity, demonstrate anti-inflammatory effects and are protective against infection. The mechanism may involve interaction with intracellular targets or via direct receptor interaction (213). One might expect the d-HDPs, such as those used here, would be unable to interact with receptors and have immunomodulatory effects, but nonetheless it has been demonstrated that d-LL-37 can stimulate far higher IL-8 release from keratinocytes than L-LL-37, arguing against the necessity of structure-specific binding to receptors for cytokine release (115). At concentrations below their IC\(_{50}\) values (up to 100µM for the WMR peptides) both pro- and
cleaved HDPs did not induce significant IL-6 or IL-8 release from CF bronchial epithelial cells. This is a desirable characteristic as IL-8 is a potent chemoattractant for neutrophils (13). The lack of pro-inflammatory cytokine response to the pro-HDPs is likely due to the lack of an immune response to the active HDP sequence. However, if an immunomodulatory HDP was modified to a prodrug, there is the possibility that the addition of the pro-moiety could reduce the effects and this should be considered when investigating the consequences of pro-HDP modification. For example, it has been observed with one HDP that PEGylation can reduce its ability to inhibit LPS-induced NF-κB activation of macrophages (155).

As discussed in Results Chapter One, when acute P. aeruginosa infections progress, adaptation can occur and the bacteria may form a biofilm as the infection becomes chronic. The biofilm matrix consists of 50-90% extracellular polymeric substances such as polysaccharides and nucleic acids that can resist mechanical forces and decrease the penetration of antibiotics (14). The reduced susceptibility of P. aeruginosa in a biofilm is well documented (17, 19, 20, 182) and the activity of HDPs is often greatly reduced against them. Despite this, a number of HDPs have been shown to have antibiofilm activity including LL-37 against 24h biofilms pre-formed in flow cells (182) and a shortened variant of chicken cathelicidin, F2,5,12, against Staphylococcus epidermidis biofilms (189). The short peptide 1037, despite having a very high MIC against P. aeruginosa, was shown to reduce biofilm formation at low concentrations (187). Short D-amino acid peptides have been developed that are able to disperse flow PAO1 biofilms at low concentrations (2.5µg/ml) and can protect both Caenorhabditis elegans and Galleria mellonella from biofilm infection from a variety of pathogens. These peptides’ antibiofilm activity is based on translocating into cells and disrupting biofilm signalling molecules, an effect which unlike membrane disruption, may be stereo-specific i.e. depending on amino acid conformation (108). AAG-WMR was able to greatly reduce the viability of a 48h pre-formed biofilm to near the level of the positive control after 6h of treatment, albeit at the high concentration of 256µg/ml, eight times its MIC against PAO1. While comparisons of the results between the flow biofilm used here
and the static biofilms of Results Chapter One are not easily made, AAG-WMR would seem to be more effective than the previous generation of active HDPs. Despite the relatively high concentration required, the concentration-dependent biofilm killing in conjunction with its low cytotoxicity would make pro-WMR a promising candidate for treating *P. aeruginosa* infection in an *in vivo* model. Further investigations with this pro-HDP will be required to fully evaluate its utility in CF and the advantage of the prodrug modification. This study demonstrates that simple modifications to HDPs can produce a potential therapeutic agent that may be suitable for use in difficult-to-treat conditions and that many of the shortcomings of HDPs such as protease-lability and cytotoxicity are not insurmountable.
5. Results Chapter Three: Evaluating the nebulisation of pro-WMR

5.1 Introduction

5.1.1 Rationale
Several antibiotics used to treat infection in CF are now delivered by the inhaled route, which has many advantages over systemic delivery. The combination of HDPs and nebulisation has been investigated in several studies as a means of delivery in in vivo models. However, the characterisation of the peptide spray has not been extensively studied and the compatibility of HDPs with this system has not been investigated. This chapter describes the evaluation of the delivery of pro-WMR using existing nebulisation technology to determine whether the characteristics of the aerosol spray are compatible with inhaled delivery of the peptide to the bronchioles and whether this delivery platform is feasible in CF.

5.1.2 Inhaled delivery
The delivery of peptides to their targeted site of action presents a series of challenges. Most peptide therapeutics are delivered by injection as issues with stability, half-life, and epithelial absorbance mean that they often display poor bioavailability by other routes. When the requirement is only for local action, however, the number of available routes of administration increases; the majority of non-injected peptides operate locally (214). Should a pro-HDP progress to more detailed clinical studies, it is envisaged that it would be delivered locally to the lung via inhalation, allowing high drug concentrations and minimal systemic exposure (134). Direct pulmonary delivery is extremely attractive for HDPs; for example sputum levels of tobramycin are over 1000 times higher when delivered by inhalation versus the intravenous route (215). Inhalation also bypasses first pass metabolism and the requirement for
gastrointestinal absorption (which is poor with many peptide drugs) (216). If one considers insulin to be a peptide (with 51 residues, it is referred to as both a peptide and a protein depending on the definition) then the repeated attempts to deliver it systemically via the lung underlines the potential for inhaled delivery. While Pfizer’s insulin (Exubera®) was a commercial failure, other companies have continued research and Mannkind’s Afrezza® has demonstrated superiority in efficacy over subcutaneous injection in phase III clinical trials. Its particle diameter of 2µm allows systemic delivery with inhalation of a dry powder (214). In CF, the delivery of medicines such as antibiotics, corticosteroids, and bronchodilators is commonly via nebulisation. Dornase alfa, colistin, and tobramycin are all delivered this way (217).

However, the large number of drug treatments required and extensive inhalation times mean that patients have a high treatment burden. The low adherence to treatment regimens that may follow can often contribute to the failure of antimicrobial therapies (15). Ideally, any new treatment should limit the increase in patient burden and therefore pro-WMR would ideally be combined with the fastest delivery method possible. The current standard of care for inhaled anti-pseudomonal antibiotics is tobramycin (300mg in 5ml), delivering high concentrations of drug to the endobronchial space and minimising systemic exposure. The typical delivery time is 12-15min by jet nebuliser, and this relatively lengthy time period can negatively affect compliance (218).

The physicochemical properties of the inhaled drug, as well as the formulation, can affect its deposition in the lung. Particle size is crucial, a mass median aerodynamic diameter (MMAD) (the diameter at which 50% of the particles by mass are larger and 50% are smaller) of between 1 and 5µm is required for lower airway deposition. At size ranges lower than this, the particle is likely to be exhaled, and at higher size ranges it will physically impact the throat and only lower amounts will reach the lung (219). More specifically, particle diameter below 3µm is generally used to target the alveoli, the thinness of the epithelium there making it an attractive target for systemically-active peptides (220). However, this is not the target for pro-HDPs. Newman et al investigated the delivery of carbenicillin with two
different nebulisers to treat *P. aeruginosa* and the results illustrate the important of particle size. One nebuliser produced droplets with a mean MMAD of 3.2µm and lower lung deposition of 0.81mg, compared to 7.3µm and 0.44mg with the other nebuliser. As would be expected, the oropharyngeal deposition of the latter aerosol was increased (on average four times higher) (221).

Small peptides delivered to the lung are usually degraded by proteases. Those resistant to such proteolysis, such as D-amino acid peptides, could potentially be absorbed para-cellularly to the bloodstream. However, drugs such as tobramycin that are lipophilic with a net positive charge have been shown to preferentially bind to lung tissue. This effect might also be expected from the cleaved active peptide (222).

There are several different means of delivering a medicine by inhalation:

### 5.1.3 Metered dose inhalers

The first Metred Dose Inhaler (MDI) was developed in 1956. Since then MDIs have become the most widely used treatment in several respiratory diseases, including asthma and COPD. The drug is formulated with a chemically-inert hydrofluoroalkane propellant and is released as a pressurised spray upon actuation (223). MDI inhalers were originally designed for the delivery of small, highly potent drugs such as salbutamol, the dose of which is 100µg per spray. The delivery efficiency of these inhalers is low at ~12%. This is not a major limitation with potent drugs, but renders MDIs unsuitable for delivery of antibiotics where the desired dose is greater than 1mg (216). With careful adjustment of the formulation it is possible to deliver peptide drugs with MDIs. One study reported bioavailability of 18.3% with MDI delivery of the nonapeptide hormone leuprolide. However, as with salbutamol, the dose was much lower than required for HDPs, at 500µg (224).
5.1.4 Nebulisation

Conventional nebulisers consist of a compressor and a nebuliser chamber and include jet nebulisers which are commonly used with CF medication. Negative pressure draws the drug from a reservoir to the chamber where it is atomised by compressed air into a range of particle sizes. Only the smaller particles can exit the chamber, as large droplets are trapped by baffles in the chamber and recycled. The constant repetition of droplet formation and impaction can degrade large molecules and liposomes (causing their contents to leak) (225, 226). The high shear stress involved also leads to the denaturation of peptides and proteins (220). Conventional nebulisers are cheap to maintain and reliable, but are also noisy, slow, bulky, and wasteful of medication. As nebulisation continues, evaporation causes the drug solution to become more concentrated (219). Ultrasonic nebulisers use a vibrating piezoelectric crystal to cause droplets to form. They are faster and quieter than conventional systems but are unsuitable for many medications, including many of those commonly used in CF (217). The vibration of the crystal causes significant heat generation, which can be problematic for heat-labile molecules such as peptides (225).

Newer nebulisation technologies such as vibrating mesh nebulisers (VMN) have many advantages over conventional jet nebulisers, such as reduced treatment time and smaller particle size (217). As their name suggests, they generate an aerosol by the action of a vibrating mesh covered in microscopic holes, which control the droplet size of the produced spray (227). The droplets produced are optimally sized and the aerosol is low velocity, so this results in minimal throat impaction and increases delivery efficiency (228). Unlike bulky jet and ultrasonic nebulisers, VMNs have been miniaturised and can be carried with a portable power source (216). One example is the Aeroneb® Solo produced by Aerogen in Galway, Ireland (Figure 5.1), which is very compact and operates from a battery pack. VMNs represent a major improvement in convenience for patients. For example, comparing tobramycin solution delivered by jet nebuliser and VMN, a significant reduction in treatment burden is observed (218). Dornase alfa treatments times have also been reduced with a VMN. In addition, the lower residual
volume allows a larger proportion of the drug to be delivered (229). This can increase patient compliance and improve clinical outcomes. VMNs have demonstrated increased lung deposition in many cases compared to other nebulisation methods but this can be variable and depends on the system used. Based on the results of a number of studies, a Cochrane review concluded that VMNs held promise in optimising nebulisation therapy (217).

![Aerogen Aeroneb® Solo vibrating mesh nebuliser.](image)

**Figure 5.1**: Aerogen Aeroneb® Solo vibrating mesh nebuliser.

### 5.1.5 Inhaled powder

Dry powder inhalers (DPI) involve the inhalation of the drug in powder form, as opposed to an aqueous solution. They are generally well-liked by patients because they are easy-to-use, fast, portable, and do not require any reconstitution. They have not typically been used for inhaled antibiotics as the amount deliverable is small and not suitable for high-dose treatments. DPIs have also traditionally been limited in terms of delivery efficiency and drug-loading. The particle size requirement of 1-5µm for inhalation meant in the past that drug powders had poor flow characteristics and aggregated readily. The high ratios of carrier particles, e.g. lactose, required to overcome this, coupled with poor delivery efficiency, meant that higher doses of drugs were not possible (216). However, the recent development of new formulation technologies has allowed the development of antibiotic DPIs with
high drug-loading and improved lung delivery efficiencies, using technologies such as spray-drying to produce favourable particle characteristics (230). While tobramycin is typically delivered as an inhaled solution, it has been formulated for dry powder inhalation. There are several advantages to this approach over solution including reduced delivery time (4-6min), portability and convenience of an inhaler, and no requirement for an external power source. The drug is manufactured as porous spheres with an MMAD below 4µm, ensuring efficient lung deposition. Its use has been approved in the EU and US, demonstrating no inferiority to nebulised tobramycin and lower costs (215). Other antibiotics have been formulated as DPIs; including a DPI of colistimethate sodium approved for use in the EU. A ciprofloxacin DPI is also currently undergoing clinical trials (216). Mannitol, used as an osmotic agent to draw water into the airway and improve mucociliary clearance in CF, is delivered as a DPI (227). As mentioned previously, with smaller particle sizes of ~2µm it is possible to deliver peptides systemically via the inhaled route as a dry powder (214). An additional benefit of powder over solution is that the stability of the drug product is often greatly improved, for example it has been demonstrated that liposomal SLPI for aerosolisation is more stable under storage as a dry powder than as an aqueous solution (231) and parathyroid hormone has also been formulated in this manner for the same reason (232).

5.1.6 Inhaled delivery of HDPs - options and challenges

While delivering HDPs as a DPI may represent an attractive future proposition, their compatibility with the processes required to achieve high enough drug-loading would first need to be established and is beyond the scope of this project. Many chemically-labile compounds such as proteins and peptides are not amenable to the spray-drying process (223). The immediate availability of nebuliser technology makes it a more realistic route of inhaled administration; the relatively small size and fast delivery times of VMNs making them particularly attractive. The low residual volume compared to other delivery methods is an important advantage for peptides, considering their potential high costs of production (228). Establishing compatibility of pro-WMR with delivery by VMN would advance the case for
its use in CF as an anti-pseudomonal therapeutic agent. The requirements are that the peptide survives nebulisation, retains activity, and that the spray is of correct droplet size to ensure lower lung delivery (1-5\,\mu m). Particle size distribution can be quantified by cascade impaction using a next generation impactor (Figure 5.2). This entraps aerosol droplets of different sizes in each stage of the impactor. The stages have a progressively smaller pore diameter, entrapping larger droplets and, with quantification of the proportion of the dose in each stage, allowing estimation of the spray’s MMAD. Droplet evaporation can sometimes lead to an underestimation of particle size with an impactor. To combat this, one can also measure droplet diameter using laser diffraction (225). Combining the two sizing methods allows broad determination of the particle size distribution of the spray (224).
Figure 5.2: Next generation impactor (in open configuration), consisting of eight different stages that entrap particles of different sizes. Each subsequent stage has a smaller pore diameter and will entrap particles of progressively smaller sizes e.g. stages 4 and 5 have cut-off diameters of 3.3µm and 2.08µm respectively. The trapped droplets then settle on a removable cup. The final stage, the micro-orifice collector (MOC) collects particles of extremely small diameter (below 1µm). After nebulisation, the cups can be removed, washed, and analysed by HPLC. This allows accurate quantification of the droplet size distribution of the nebulised aerosol.

5.2 Results

5.2.1 Pro- and AAG-WMR are unchanged after nebulisation

A 1mg/ml aqueous solution of pro-WMR and AAG-WMR were both nebulised using an Aeroneb® Solo. This was characterised before and after
nebulisation using electrospray mass spectrometry (ESI-MS) and HPLC. Neither peptide was changed by the nebulisation process as evidenced by the comparable retention times (20.89 min vs. 20.857 min for AAG-WMR) and mass spectra (Figures 5.3 and 5.4). A comparison of the MICs of both peptides before and after nebulisation was also made. Against *P. aeruginosa* PAO1 and three CF clinical isolates, no difference in MIC was observed (Table 5.1).

**Table 5.1: MIC values for pre- and post-nebulisation HDPs vs. *P. aeruginosa* PAO1 and clinical isolates**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC vs. <em>P. aeruginosa</em> strains (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAO1</td>
</tr>
<tr>
<td>AAG-WMR</td>
<td>32</td>
</tr>
<tr>
<td>AAG-WMR (nebulised)</td>
<td>32</td>
</tr>
<tr>
<td>Pro-WMR</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Pro-WMR (nebulised)</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>
Figure 5.3: HPLC and ESI-MS of AAG-WMR before (A) and after (B) nebulisation. Retention times 20.890min and 20.857min. The signals at $m/z = 611.1, 458.6,$ and 367.1 are the $(M+1)/3,$ $(M+1)/4,$ and $(M+1)/5$ respectively for AAG-WMR.
Figure 5.4: HPLC and ESI-MS of pro-WMR before (A) and after (B) nebulisation. Retention times 22.527min and 22.450min. The signals at \( m/z = 820.8, 616, \) and 493 are the \( (M+1)/3, (M+1)/4, \) and \( (M+1)/5 \) respectively for pro-WMR.
5.2.2 The particle size distribution of the peptides is favourable for lung delivery

Analysis of the particle size of a 1mg/ml aqueous solution by laser diffraction gave the volume mean diameter (VMD) of both peptides: 3.79 ± 0.1µm and 3.8 ± 0.1µm for pro- and AAG-WMR respectively, with accompanying geometric standard deviations (GSD) of 1.76 and 1.74. The % of particles with a diameter below 5µm, i.e. the fine-particle fraction (FPF) was above 65% for both peptides (Table 5.2).

Table 5.2: VMD, MMAD, GSD and %FPF values for pro- and AAG-WMR, determined using both laser diffraction and cascade impaction

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Laser Diffraction</th>
<th>Cascade Impaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VMD (µm)</td>
<td>GSD</td>
</tr>
<tr>
<td>Pro-WMR</td>
<td>3.79 ± 0.1</td>
<td>1.76</td>
</tr>
<tr>
<td>AAG-WMR</td>
<td>3.8 ± 0.07</td>
<td>1.74</td>
</tr>
</tbody>
</table>

The results of the cascade impaction were in general agreement with the laser diffraction. The MMADs were 3.59 ± 0.23µm and 3.14 ± 0.25µm for pro- and AAG-WMR respectively (Table 5.2). The difference was not statistically significant (p = 0.0835). The mass balance of the different stages of the impactor indicated that the majority of both peptides settled in stages 4 and 5, which had a cut-off diameter of 3.3µm and 2.08µm respectively. Negligible amounts of peptide remained in the nebuliser after the run was finished (Figures 5.5 and 5.6).
Figure 5.5: Mass balance of each stage of the next generation impactor for pro-WMR. Included are stages 1 to 7, the MOC, the connector, throat, and filter stages, as well as the remaining peptide in the nebuliser. Each stage of the impactor has a smaller cut-off diameter, with a large proportion of the peptide in stages 4 and 5 indicating a particle size below 3.3 $\mu$m and 2.08 $\mu$m respectively. After nebulisation of 1ml, each stage was washed with dH$_2$O and the washings analysed by HPLC to determine drug deposition.
Figure 5.6: Mass balance of each stage of the next generation impactor for AAG-WMR. Included are stages 1 to 7, the MOC, the connector, throat, and filter stages, as well as the remaining peptide in the nebuliser. Each stage of the impactor has a smaller cut-off diameter, with a large proportion of the peptide in stages 4 and 5 indicating a particle size below 3.3\(\mu\)m and 2.08\(\mu\)m respectively. After nebulisation of 1ml, each stage was washed with dH\(_2\)O and the washings analysed by HPLC to determine drug deposition.

5.2.3 The peptides are deliverable to a model lung

Using the parameters for a healthy adult lung, both peptides were nebulised into a breathing apparatus. The quantity of peptide collected in the terminal filter (representing the beginning of the lung) was compared to the initial dose. This allowed the percentage delivery to be calculated. For pro-WMR % delivery was 42 ± 3.1%, and for AAG-WMR 47.6 ± 7.8%. The difference between both peptides was not statistically significant (p>0.05). An image of the breathing apparatus is provided in Figure 5.7. The nebulisation time for the 1ml solution was less than 3min for both peptides (170 ± 3s for AAG-WMR and 171 ± 2.3s for pro-WMR).
Figure 5.7: Image of the breathing apparatus used to determine the deliverable dose of both peptides to the lung. The parameters chosen were those of a healthy adult. An aqueous solution of peptide was nebulised by the VMN and inhaled by the apparatus through the mask. After passing through, it was collected in a gas permeable filter, representing the lung. The proportion of peptide collected in the filter was determined by HPLC.
5.3 Discussion

Both pro- and active peptide were intact and unchanged after nebulisation, maintaining their antimicrobial activity and demonstrating no degradation. This has previously been an issue when biological agents have been delivered with nebuliser systems, e.g. with rhDNAse and insulin in an ultrasonic nebuliser, where activity has been shown to be altered (233). The protein lactate dehydrogenase is labile to both heat and shear stress, which are generated in the nebulisation process. Its degradation has been demonstrated with a series of jet and ultrasonic nebulisers, with an accompanying loss of activity (to around 20% in some cases) (234). Nebulisers have been shown to degrade other biopharmaceuticals, e.g. naked siRNA has been shown to be degraded by jet nebulisation (an effect avoided by formulating it as a nanoparticle) (235). On the other hand, other therapeutics can be unscathed by nebulisation and retain activity, e.g. colistin is not degraded and has been shown to maintain its MIC after both jet and ultrasonic nebulisation (233) and using a similar VMN to that used here (Aeroneb® Pro) siRNA nanocomplexes were found to maintain their ability to knockdown luciferase (236).

The use of VMNs should reduce the degradation of biological agents such as peptides and proteins, with most of the instability of nebulisation observed with jet and ultrasonic nebulisers. However, this is not guaranteed and must be evaluated with every peptide-nebuliser combination. VMNs have a stability advantage because, unlike jet and ultrasonic nebulisers, they do not recirculate droplets. Furthermore, the generation of shear, heat, and air-liquid interface (where shear effects are most damaging) are reduced in VMNs. However, these effects are not completely absent or insignificant in VMNs. Reservoir temperatures of 40°C have been demonstrated with the Aeroneb® Go and VMNs generate (less obvious) air-liquid interfaces where new droplets are created, creating the potential for destabilising stress (237).

Identical mass spectrums and chromatograms of the peptides pre- and post-nebulisation are demonstrated here with pro- and AAG-WMR. However, HPLC alone cannot determine if a peptide remains active after nebulisation, and analysis must also include biological testing. It has been shown with the
PARI® eFlow VMN that a post-nebulisation protein may appear intact under HPLC but still be inactivated, the protein SM101 losing 50% of its potency (237). The thermal stress generated in VMNs can be reduced by pre-cooling solutions or actively-cooling the reservoir. This has been shown to reduce the aggregation and loss of activity of SM101 and lactate dehydrogenase. Cooling the reservoir solution also has the added benefit of increasing the fraction of droplets between 1 and 5µm. However, even with cooling, interfacial stress from VMNs can still degrade proteins including the previous two and even heat-stable molecules such as IgG1 (238).

The facile nebulisation of both simple aqueous peptide solutions is advantageous when one considers that other peptides, for reasons of host toxicity and instability, often require reformulation to deliver them to the lung. A common approach in nebulisation, and one that deserves special consideration, is to deliver drugs as liposomes. While formulating inhaled peptides and proteins in this way has many advantages, especially in terms of improving drug stability (226), activity (239) and limiting host toxicity (240), there are several complications associated with it. Potential difficulties one can encounter included the issue of degradation and leakage of contents which can be induced by nebulisation or after a period of storage. The high HDP concentration required for effective treatment requires a parallel high lipid content which can increase solution viscosity and hinder nebulisation. Conversely, liposomes that produce less viscous suspensions can be less stable and leak their HDP contents (240). Optimisation of the lipid content can overcome many of the leakage issues, with the behaviour of different liposomes varying considerably. With the HDP CM3, one study observed encapsulation after nebulisation which varied from 20% to 90% depending on the formulation as peptide-leakage often occurred after processing. However, the stability under nebulisation does not necessarily correlate with the nebulisation efficiency of the drug, i.e. the amount of peptide the nebuliser will emit. A liposome that is resistant to leakage under nebulisation can still be inefficiently delivered, even though all formulations might have MMAD values between 1 and 5µm. With nebulisation both leakage and delivery must be considered with each liposome formulation (241).
While liposomes can protect from protease degradation, if peptide release is too slow it can exacerbate total proteolysis, the slow released of low concentrations of active peptide may facilitate enzymatic degradation whereas this is less likely with a high bolus dose. This was observed with the 32 residue peptide calcitonin (242). Liposome leakage has been observed with SLPI delivered by jet nebulisation. The protein was encapsulated in liposomes to reduce its proteolytic inactivation, an issue which limits its clinical application. It was observed after nebulisation that its resistance to cathepsin L degradation was reduced by over 50%, a result of drug leakage from the liposomes induced by the nebuliser. However, this issue may be limited to jet nebulisers and the high encapsulation efficiency observed with SLPI, a result of the ionic interaction between the cationic protein and an anionic lipid, illustrates the potential for this technique to be used with active cationic HDPs (but perhaps not the low net charge pro-HDPs) (226). The low cytotoxicity of pro-WMR and the need for enzymatic cleavage makes the use of liposomes redundant, but while this approach is not suitable here, with the correct formulation it may serve as an alternative to the pro-HDP model. It has been applied to polymyxin B, aimed at improving the toxicity and residency time of the peptide in the lung. The importance of the liposome content is underlined by the fact that against the same \textit{P. aeruginosa} strain (ATCC 27853), one study demonstrated improved activity compared to free drug (239) while another formulation reduced its activity (antimicrobial activity being further reduced by nebulisation) (243). Interestingly, reformulating SLPI as a dry powder for DPI delivery resulted in liposomes that were better at protecting the protein from enzyme inactivation after delivery, had better stability under storage, and had an MMAD of 2.44 ± 0.12\textmu m (231).

Another consideration is the effect of liposome size on the drug distribution in the lungs. Smaller liposomes might be expected to have a negligible effect on the droplet size of the aerosol but as liposome size approaches that of the droplets, \textit{e.g.} 1-5\textmu m, it is possible that their distribution among the various droplet sizes will be affected. In other words, would the liposome distribution among the different droplet sizes of a normal aerosol be homogenous or would larger liposomes be more likely to reside in larger droplets, and as a
result would liposomes deposited in the peripheral lung differ from those in central airways? This question was investigated with liposomal amikacin, again aerosolised to treat pulmonary *P. aeruginosa*. However, the liposome size in each stage of the impactor was below 0.4µm and mostly homogenous (244). Despite the demonstration of homogeneity with amikacin, this effect should be considered with every new liposomal formulation and remains a complication of their use. An alternative to liposomal or prodrug delivery is to formulate HDPs as nanoparticles, an approach that has been used in a study with tobramycin (which is also cationic) to provide a degree of controlled release and increase the residence time of the antibiotic. This also allows the coadministration of drugs such as dornase alfa, which as stated previous, has been functionalised onto these nanoparticles. The particle size achieved (~0.5µm) would ideally not interfere with homogenous nebulisation (78).

The MMAD values for the combination of the Aeroneb® Solo and pro- and AAG-WMR (3.59 ± 0.23µm and 3.14 ± 0.25µm respectively) are within the desired range of 1-5µm for local lung delivery and compare favourably to the droplet diameter of other inhaled medicines for CF. Dornase alfa has a particle diameter of 4.2µm when delivered by jet nebuliser and 4.3µm with Omron’s MicroAir VMN (229). The particle size of the peptides is also above 3µm and so would not be in the range for reaching the alveoli (which is not the target). Peptides that are delivered beyond the endobronchial space to the alveoli would be expected to be absorbed into the blood stream (depending on their characteristics), be degraded by proteases, or be removed by alveolar macrophages (220).

MMAD is not just a function of the characteristics of the drug solution, as the nebuliser and its settings can also have a profound effect on particle size. In one study with the same tobramycin solution, for example, a range of MMADs were produced with different nebulisers, many of which were well outside the ideal range and not respirable (245). Achieving a suitable particle size with a simple aqueous solution is advantageous when one considers that additives are often required to render a drug respirable. Using a similar VMN to our own, the aerosol of a mannitol solution had a MMAD of 11.27µm. This droplet size is too large for lung delivery and co-formulation with 1% w/v
NaCl was required to lower it to a more feasible range for treatment (5.75µm). NaCl also greatly decreased the nebulisation time for 2.5ml from 477s to 298s (228). For comparison, the treatment time of pro-WMR was 171s/ml compared to 190.8s/ml for mannitol alone. While the combination of hypertonic saline and pro-WMR may be of potential benefit to CF patients (73), it is advantageous that the inclusion of any other excipients to the peptide solution would be for reasons of therapy and not formulation. The treatment time also compares well to tobramycin delivery, where delivery with a Pari® eFlow VMN was on average 156s/ml (678s/ml with a jet nebuliser) (246).

The breathing apparatus results demonstrate that a large proportion of the peptide solution can be expected to reach the lung and compares well to established therapeutics. While a larger percentage of the active peptide reached the model lung, the difference between pro- and AAG-WMR was not statistically significant. In any case it is demonstrated that the additional residues and the modification of net charge are not detrimental to the pulmonary delivery. The settings for our apparatus were those of a healthy patient, with a tidal volume of 500ml, inhalation:exhalation ratio of 1:1, 15 breaths/min, and 2l/min of supplementary gas flow. Under similar conditions (but with 12 breaths/min and no supplementary gas) the delivered dose of dornase alfa was 52% with a VMN (229). The parameters for a CF patient are different and may produce a different % delivery. Lange et al investigated the delivery of a liposomal HDP to a model adult CF lung, using a tidal volume of 620ml, inhalation:exhalation ratio of 1:1.3, and 18 breaths/min. Final delivery of the peptide dose with a jet nebuliser was 28%, while its MMAD was 2.84 ± 0.1µm (240). These parameters may form the basis of a future delivery analysis. For comparison with current in vivo efficiencies, in a group of male CF patients receiving 150mg of tobramycin from a Pari® eFlow VMN, 28.3% was deposited in the lung (246).

The successful production of a respirable aerosol with pro-WMR represents another step towards the clinical use of pro-HDPs. We demonstrate here that the prodrug modification is not detrimental to delivery and that both peptides may be given therapeutically in a way which should not significantly increase
the treatment burden on CF patients. There are scarce reports of nebulised antimicrobial peptides in the literature, and while much work has been carried out on proteins and peptide hormones such as insulin, this study represents one of the few studies into the aerosol delivery of a HDP. It is also the first, to the author’s knowledge, to investigate a HDP with a prodrug modification. There are several reports of liposomes being used to delivery cytotoxic and unstable therapeutics to the lung. For HDPs, at least, the oligoglutamic acid prodrug modification may represent a more facile delivery method, d-amino acids negating the concerns over stability in vivo and the net charge reduction tackling potential toxicity. As an alternative approach it would bypass issues of leakage and formulation, requiring only a simple aqueous solution and being feasible with existing technology to treat CF infections.
6. Results Chapter Four: An *in vivo* study into the anti-infective and toxic effects of pro-WMR

6.1 Introduction

6.1.1 Rationale

The combination of favourable *in vitro* antimicrobial activity, low host toxicity, and its compatibility with nebulisation makes pro-WMR an attractive candidate to bring forward to *in vivo* studies. While the activation of the peptide in the presence of purified NE and CF BAL fluid has been demonstrated *in vitro*, in order for it and other pro-HDPs to progress in development it will need to be confirmed *in vivo*. This chapter deals with the design and implementation of suitable animal experiments to evaluate both the antimicrobial activity and cytotoxicity of pro-WMR in a model that closely resembles CF in a human context.

6.1.2 *In vivo* studies with HDPs

Many HDPs have been evaluated using *in vivo* lung infection models, with significant bacterial reductions being demonstrated. For example, intranasally-delivered LL-37 has been shown to induce an early neutrophil response to PAO1 infection in the lungs of mice, accelerating clearance of the bacterium (247). It has also been shown to ameliorate the pneumonia induced by MRSA when delivered in advance intratracheally (248). Beyond LL-37, *in vivo* activity has been demonstrated with exogenous HDPs. A series of peptides related to HB43 delivered in high doses were shown to greatly reduce the bacterial burden of a *P. aeruginosa* lung infection in rats after nebulisation over three days (121). Similarly, intraperitoneal administration of the peptide HPA3P2 was able to greatly improve mouse survival in a sepsis model of multidrug-resistant *P. aeruginosa* infection (249). The expectation for the reduction in bacterial load may be compared to a clinical setting. During chronic *P. aeruginosa* infection, bacterial numbers...
can reach $10^7$ to $10^8$ CFU/g BAL fluid and a two log reduction after antibiotic therapy is considered a major effect (15). For this reason, and because it is a common measurement of treatment effect in animal experiments, CFU/ml BAL is used to quantify the effectiveness of the HDPs in the present study.

6.1.3 CF mouse model

The cause of cystic fibrosis may be simply explained by a number of mutations in the gene for CFTR leading to ion channel dysfunction. As detailed previously, this leads to depletion of ASL volume, impaired mucus clearance, and bacterial infection. Our understanding of the disease would predict that the simple deletion of CFTR would produce similar effects in mice, but this is not the case. Unlike in humans, the removal of CFTR activity does not lead to spontaneous lung disease, even in older mice raised outside the sterile environments commonly used in animal facilities. This is found consistently in CFTR$^{-/-}$ models and may be due to the fact that CFTR is not expressed in the lower airways of mice. Consequently, its removal will not have major pathological effects on the peripheral lung. In contrast, CFTR is common in the murine gastrointestinal tract and the CFTR$^{-/-}$ model has been found to closely mimic the intestinal pathophysiology of human patients (7).

Defective Cl$^{-}$ secretion is not the only ionic characteristic of CF. The lung epithelium (and indeed other organ systems) is characterised by increased Na$^{+}$ absorption which, in combination with chloride dysregulation, leads to depletion of water on the airway surface (250). To this end, an alternative mouse model has been developed that is characterised by Na$^{+}$ hyperabsorption as opposed to reduced Cl$^{-}$ secretion. It is based on the “low-volume” hypothesis i.e. that CFTR also regulates the epithelial sodium channel (ENaC), with dysfunction of the former leading to reduced NaCl concentrations in the ASL and a resulting reduction in osmotic force for water transport to the lumen. This is in contrast to the alternative “high-salt” hypothesis where the absence of CFTR is theorised to lead to increased
NaCl concentrations in the ASL and the inhibition of HDP activity, exacerbating bacterial infection. While evidence exists to support both hypotheses, the fact that ASL volume has been demonstrated to be lower in CF supports the “low-volume” hypothesis. Figure 6.1 demonstrates how defective Cl⁻ and Na⁺ transport reduce the height of the ASL and impair mucociliary clearance. If the “high-salt” hypothesis was correct, the increased NaCl concentrations would induce an accompanying movement of water across the lumen and increase ASL depth. This increase in ASL depth, as seen in the disease pseudohypoaldosteronism, would increase mucociliary clearance, in contrast to the situation in CF, and one would expect it would act as an increased barrier to bacterial adherence rather than facilitate it (251). The “low-volume” hypothesis also predicts that increasing the salt concentration of the ASL would induce water entry into the lumen, increase the ASL depth, and improve clearance. Supporting this is the fact that hypertonic saline has been repeatedly shown to improve mucociliary clearance and lung function in CF (73).
Figure 6.1: A demonstration of the effects of CFTR and ENaC on mucociliary clearance (MCC) based on the “low-volume” hypothesis. Under normal conditions the balance of secretory and absorptive processes ensures that the ASL is an appropriate height (~7 µm), compatible with effective MCC. In CF, defective Cl− clearance and inappropriate Na+ absorption leads to a dehydrated, compressed ASL (~3-4 µm) that collapses the cilia, prevents MCC, and promotes bacterial adherence. Taken from (251).

Mice have been generated that overexpress the β-ENaC subunit (encoded by the Scnn1b gene) which results in increased Na+ absorption, reduced airway surface volume, mucus obstruction of the lungs, neutrophilic inflammation, and, crucially, reduced clearance of a bacterial challenge compared to wild-type mice. The pulmonary disease phenotype of these mice is similar to the human disease (252). These have also been crossed with a murine NE (mNE)−/− mouse line (48) to examine the effects of mNE in a CF phenotype. The genetic deletion of mNE has been demonstrated in one study to reduce neutrophil recruitment. In addition, large quantities of mNE were found to be bound to the membranes of β-ENaC neutrophils, although free mNE was not detected in BAL fluid, potentially due to inhibition by a robust anti-protease defence. The results suggest that membrane-bound NE plays a large role in tissue degeneration in the mouse model (253). A similar observation has been made with MMP-12 levels in these mice (70). The lack of free enzyme has the potential to pose an issue for testing NE-activated...
pro-HDPs, but in mice from the same background it has been shown in some
studies active NE is expressed in BAL fluid after nasal instillation of *P.
aeruginosa* or LPS (254). Overall, there are mixed reports on whether *P.
aeruginosa* infection in these mice can induce substantial free NE activity
(255, 256).

Other CF *in vivo* models have been developed, using different species
whose lung pathology more closely resembles human CF in many respects.
CF is the first human disease to have two non-roden t knock-out models, pigs
and ferrets. Unlike in CFTR<sup>−/−</sup> mice, the lungs of both CFTR<sup>−/−</sup>
and CFTR<sup>∆F508/∆F508</sup> pigs are characterised by lung infection, inflammation, and
remodelling in the first months of life (257). The similar lung pathology allows
important observations to be made that are precluded in CF patients, e.g.
whether inflammation precedes infection, with the pig model demonstrating
no significant inflammation a few hours after birth. A barrier to using this
model however, beyond the obvious increased costs with raising enough
pigs for statistically significant data, is that 100% of CFTR<sup>−/−</sup> pigs require early
intestinal surgery for meconium ileus, *i.e.* intestinal blockage, comparable to
the surgery to clear intestinal blockages that is sometimes required in CF
patients (258).

Ferret CF models have also been developed. They are an attractive species
for modelling lung disease because they reproduce relatively quickly and
share many features of lung biology with humans, such as a similar
distribution of submucosal glands. It has been shown that CFTR<sup>−/−</sup> ferrets are
extremely susceptible to lung infection early in life. This model, however, is
also complicated by the fact that ferrets are obligate carnivores (unlike mice
and pigs) which requires special considerations for their nutritional needs.
Intestinal pathology is again an issue, with 75% having meconium ileus. This
pathology of the ferret model is severe, resembling human CF in many
respects (259). But while their susceptibility to infection and gastrointestinal
pathology makes them attractive for establishing the link between CFTR and
disease, it also means many CF ferrets die before reaching maturity (260). In
addition, the necessity of antibiotic treatment from birth complicates
experiments that investigate new therapeutic agents.
Considering all the options, the β-ENaC mouse model was the most attractive in terms of breeding speed, precedents set with other HDP treatments, parallels with human pathology, and economic considerations for the study.

As the activity of pro-WMR is enzyme-dependent, an important consideration for the study was the potential differences between human NE and mNE, which could preclude the use of a mouse CF model. A study examining some of the differences in substrate affinity demonstrated that mNE had a higher $K_{cat}/K_m$, i.e. catalytic efficiency, for the substrate Suc-AAA-pNa, nearly twice that of the human enzyme. From this, one would expect the AAAG linker of pro-WMR to be at least as labile to the mouse enzyme as to the human (168). Human NE and mNE share 69% sequence homology, in comparison to the 50% similarity between human NE and PR-3. However, there are subtle differences between the binding sites of both NE enzymes, such as the ability of mNE to bind acidic residues at P2' (261). To ensure that any antibacterial activity was due to the active peptide and not the intact pro-HDP, the lability to purified mNE would need to be quantified.

While generation of a respirable aerosol of pro-WMR was demonstrated in Results Chapter Three, the differences in breathing parameters between mice and humans means that delivery to the mouse lung via nebuliser is not used here. Discounting the differences in lung morphology which are a caveat when using in vivo models (262), the difference in breathing frequency is an order of magnitude greater. Previously, 18 breaths/min has been used as a parameter of human CF in model lungs (240) while the measured frequency of β-ENaC mice, though reduced from wildtype, is 172 breaths/min (263). Most rodents are primarily nose-breathers and, in comparison to humans, lung deposition is lower (dogs and primates have closer deposition) (264). These differences could potentially compromise the delivery of HDPs via nebulisation and, as a result, intratracheal instillation was used as an alternative delivery method. This method is commonly used for delivering HDPs to rodent lungs (248, 265).
6.2 Results

6.2.1 Murine NE cleaves pro-WMR but is less active than the human enzyme

Using HPLC it was determined that pro-WMR was cleaved by mNE. Figure 6.2 details how the peak for pro-WMR at 11.12min is not present on the chromatogram after overnight incubation with 5µg/ml of mNE, instead being replaced by a peak at 10.55min, close to the retention time for AAG-WMR of 10.46min and within the variability for the HPLC machine. This indicated that the product of pro-WMR cleavage was the same as with human NE. Using this method, the velocity of the cleavage of the pro-peptide by both enzymes was compared (see Figure 6.3) by monitoring the change in the relative area of the peaks of pro-WMR and AAG-WMR. It was seen that 5µg/ml of human NE more efficiently converted the peptide, with 50% of pro-WMR cleaved after 31.51min, compared to 98.17min for 5µg/ml of mNE.
Figure 6.2: A comparison of the retention times of pro-WMR (A), AAG-WMR (B), and pro-WMR after overnight incubation with 5µg/ml of mNE (C). The retention time of 10.55min of pro-WMR after incubation with mNE indicates cleavage to AAG-WMR. The pink line represents the absorbance at 214nm, the blue 280nm.
Figure 6.3: A comparison of the % cleavage of 500µg/ml pro-WMR by 5µg/ml of human and murine NE. The proportion of pro-WMR that was cleaved at each time-point was determined using RP-HPLC.

Using the fluorescent substrate MeOSuc-AAPV-AMC, the ability of both enzymes to cleave the NE-specific linker AAPV was compared. The cleavage of the substrate at different enzyme concentrations was analysed. The slope of each cleavage over 10min was then plotted against enzyme concentration, giving a linear relationship. The slopes of the resulting lines for human and murine NE were then compared, demonstrating that human NE was the more potent enzyme for the with a slope of 11345 compared to 4213 for mNE (arbitrary units) (Figure 6.4).
Figure 6.4: A comparison of the cleavage of the substrate MeOSuc-AAPV-AMC by different concentrations of mouse and human NE. The change in fluorescence of the substrate over 10min incubation with enzyme was analysed and plotted. The slope of each cleavage was then plotted against enzyme concentration, demonstrating a linear relationship. The resulting slopes of the lines for mouse and human NE were then compared, a larger slope indicating a more active enzyme.

6.2.2 Murine NE is produced in response to *P. aeruginosa* infection but it is insufficient for cleavage

In most of the BAL fluids collected from mice infected with *P. aeruginosa*, low levels of mNE activity were detected (maximum 0.393µg/ml). The comparison between BAL mNE levels and CFU levels is shown in Figure 6.5A. A linear relationship was not evident between both but with increasing levels of *P. aeruginosa* the levels of mNE tended to increase. When the BAL fluids were split into two groups, those with CFU/ml levels below and above 1 x 10^5, the increase in mNE levels was statistically significant, from 0.08 ± 0.02 µg/ml to 0.18 ± 0.03 µg/ml (p = 0.0341) (Figure 6.5B). The levels found were still relatively low compared to NE levels of human BAL as described in Results Chapter One.
Figure 6.5: The relationship between CFU/ml and mNE levels in mouse BAL fluid. The presence of mNE in all characterised BAL fluids versus the log(CFU/ml) of *P. aeruginosa* (n = 35) (A) and the comparison of the mNE levels of BAL fluids containing 0 to 1 x 10^5 CFU/ml (n = 14) and 1 x 10^5 to 1 x 10^7 (n = 21) CFU/ml *P. aeruginosa* (B). * denotes P<0.05.

Consistent with the low levels of mNE measured in BAL, when the pro-HDPs were incubated with β-ENaC BAL after *P. aeruginosa* infection, no cleavage was observed by HPLC after 48h incubation (Appendix figures A38 for pro-WMR and A39 for pro-P18). This was in contrast with the incubation of both pro-HDPs with sputum from a CF patient undergoing an exacerbation, where cleavage was observed after 3h. When incubated with healthy human sputum, used as a control, neither pro-HDP was cleaved (Appendix figure A40). When incubated with four fresh β-ENaC BAL fluids after *P. aeruginosa* infection, as opposed to the BAL supernatant used above, pro-WMR was not cleaved after 3h (Appendix figure A41). These four BAL fluids contained *P. aeruginosa* ranging from 5 x 10^3 to 1 x 10^7 CFU/ml and 0.07 - 0.17 µg/ml mNE.

6.2.3 Pro-WMR does not reduce the bacterial load of acute *P. aeruginosa* lung infection

To evaluate the antimicrobial activity of the pro-HDPs against *P. aeruginosa*, an acute model of lung infection was employed. Mice were intratracheally instilled with an inoculum of 2.5 x 10^7 CFU/ml of PAO1, then treated with peptide after 6h intratracheally, sacrificed 24h after infection, and their BAL
fluids analysed for mNE activity and CFU/ml PAO1. It was noted that neutrophils made up 80-90% of the cells recovered from BAL after infection and that weight loss for all groups was 5-10% (data not shown). There was a trend for β-ENaC mice to have higher CFU counts than wildtype littermates, but the differences were not statistically significant (4 x 10⁵ vs. 3 x 10⁶, p=0.087). Consistent with the lack of cleavage seen in the BAL fluids, in wildtype and β-ENaC mice, pro-WMR did not reduce the bacterial load in the lungs compared to the PBS control. AAG-P18, which had the lowest MIC against PAO1 of all the active peptides described in Results Chapter One, also did not reduce the bacterial load, but instead increased it compared to PBS, from 4 x 10⁵ to 6.6 x 10⁶ CFU/ml (p = 0.0009) for wildtype mice (Figure 6.6).

![Figure 6.6](image-url)

**Figure 6.6:** The CFU/ml of *P. aeruginosa* PAO1 recovered from BAL fluids after treatment with pro- and active HDPs. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes p<0.05, and *** p<0.001 compared to PBS alone. Lines show where comparisons have been made between treatment groups. The n number of each treatment is displayed beneath each bar. Wt denotes wildtype mice and Tg denotes β-ENaC mice.
In contrast to pro-WMR, AAG-WMR-treated mice demonstrated a reduced bacterial load, from $3 \times 10^6$ to $2.8 \times 10^4$ for β-ENaC mice. However, this reduction was not statistically significant ($p = 0.1135$). Despite the higher MIC against PAO1 for AAG-WMR (Table 4.2), the reduction in CFU/ml compared to AAG-P18 treatment for both mouse groups was statistically significant: $2.8 \times 10^4$ compared to $6.6 \times 10^6$ CFU/ml in β-ENaC ($p = 0.0187$) and $1.1 \times 10^4$ compared to $6.6 \times 10^6$ CFU/ml in wildtype ($p = 0.0329$). It must be noted however that the treatment groups were smaller for AAG-P18: $n = 3$ for β-ENaC and $n = 2$ for wildtype.

### 6.2.4 Prodrug modification reduces the toxicity of active peptides

To compare the toxicity of pro- and active peptides, wildtype mice were treated twice intratracheally with 50µg of peptide and sacrificed the next morning. Of the four mice treated with AAG-P18, three died (Table 6.1). All mice treated with pro-P18 survived but displayed significant weight loss compared to the PBS control (5.0% compared to 0.4%, $p = 0.018$) and raised BAL cell numbers ($7.8 \times 10^5$ compared to $4.8 \times 10^4$, $p = 0.008$). Both groups of mice treated with AAG-WMR and pro-WMR survived, with BAL cell counts comparable to the PBS control. However, mice treated with AAG-WMR displayed significant weight loss compared to pro-WMR (8.0% compared to 0.4%, $p = 0.0037$) (Figure 6.7). Against NE-knockout mice, two out of three AAG-P18 mice died, while all pro-P18 mice survived (Table 6.1).

**Table 6.1:** Mouse survival after two intratracheal treatments with 50µg of peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Wildtype</th>
<th>Neutrophil elastase knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>AAG-WMR</td>
<td>4 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Pro-WMR</td>
<td>4 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>AAG-P18</td>
<td>1 (3)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Pro-P18</td>
<td>4 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>ND = not determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 6.7**: The % weight loss of wildtype mice treated twice intratracheally with 50µg/ml of peptide (A) and the total BAL human cell counts after treatment (B). n = 4. Statistical analyses were carried out using an unpaired two-tailed t-test * denotes p<0.05, and ** p<0.01 compared to the PBS control. Lines show where comparisons have been made between treatment groups. AAG-P18 was not included as 3 of 4 mice died before lung lavage.

**6.2.5 Prodrug modification reduces the immunogenicity of AAG-WMR**

Cytokine analysis was carried out on the BAL fluids of the mice receiving a morning and evening dose of peptides with no infection. The release profile was variable, but statistically significant increases in cytokines were observed that correlate with the weight loss results. IL-5 and TNF-α were both increased with AAG-WMR compared to control (respectively to 12 ± 3.4pg/ml p = 0.04 and 23.2 ± 5.4pg/ml p = 0.01). Additionally, the trend was for IL-6 and KC to be elevated but this was not statistically significant (respectively to 85 ± 32.2pg/ml p = 0.055 and 28.2 ± 6.5pg/ml p = 0.1). In response to pro-P18, the trend was also for increased cytokine release, particularly for IL-6, but this was also not statistically significant (to 3350 ± 1752 pg/ml, p = 0.11).

No increase in cytokine release compared to control was apparent with pro-WMR. In comparison with AAG-WMR, the release of both KC and TNF-α was reduced with pro-WMR (3.1 ± 1.8pg/ml vs. 23.2 ± 5.4pg/ml, p = 0.01 for the latter). Additionally, the trend was for lower levels of IL-5 and IL-6 but
again these were not statistically significant ($p = 0.0765$ and $p = 0.0782$ respectively) (Figure 6.8).

![Graphs of IL-5, IL-6, KC/GRO, and TNF-α concentrations](image)

**Figure 6.8**: Wildtype mouse BAL fluid levels of IL-5 (A), IL-6 (B), KC/GRO (C), and TNF-α (D) measured after 24 h in response to 2 doses (morning and evening) of 50µl PBS control or 50µg HDPs. $n = 4$. Statistical analyses were carried out using an unpaired two-tailed t-test, * denotes $p < 0.05$ compared to the PBS control. Lines show where comparisons have been made between treatment groups. ns = not significant (with p values given above in some cases). AAG-P18 is not included as 3 of 4 mice died before lung lavage.

The levels of IFN-γ, IL-1β, IL-2, IL-4, IL-10, and IL-12p70 were all also analysed but were not found to be elevated significantly in any of the treatment groups (Appendix figure A42).
6.2.6 Cytokine release is induced by PAO1 but not aggravated further by active peptides

Cytokine analysis was also carried out on β-ENaC mice in response to PAO1 infection and subsequent treatment with peptides. It was observed in both treatment groups (AAG-WMR and AAG-P18) and the PBS control, that high levels of KC (2800-6700pg/ml) and moderate levels of IL-5 were produced. No significant difference was observed between each group (Figure 6.9). This was also seen for the cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-10, and IL-12p70 (Appendix figure A43).

**Figure 6.9:** β-ENaC mouse BAL fluid levels of KC/GRO (A) and IL-5 (B) measured after 24 h in response to 2.5 x 10⁷ CFU/ml PAO1 and 50µl PBS control or 50µg HDP 6h later. n = 4 (n = 3 for AAG-P18). Statistical analyses were carried out using an unpaired two-tailed t-test, ns = not statistically significant compared to the PBS control.

6.3 Discussion

As revealed by the experiments described in this chapter, in vivo evaluation of a pro-HDP targeted at a human enzyme requires special considerations. The activity differences between human and murine NE pose a potential roadblock in the testing of the pro-peptide activation. This issue is not unprecedented in cystic fibrosis therapeutics: in the development of the potentiator ivacaftor, the difference between mouse and human CFTR meant
that G551D and ΔF508 mice were unsuitable for testing (266). As a result, drug evaluation went directly from in vitro efficacy assays to testing in human subjects (89, 267). In the present study, the different substrate affinities of human and murine NE were taken into account in the design of the animal study, with the literature reporting a similar ability of both enzymes to cleave the AAA linker (168). We observed that 5µg/ml of both enzyme cleaved 500µg/ml pro-WMR but the rate of cleavage was slower with the murine enzyme, taking 6h to entirely convert the pro-HDP to its active form and taking over three times as long to convert 50% of the prodrug as compared to human NE.

Compounding the lower cleavage rate with the murine enzyme, the levels of active NE found in mouse BAL fluid were far lower than those previously observed in humans. While it was observed that sputum from a CF exacerbation could cleave both pro-WMR and pro-P18 after 3h, incubating these peptides with various mouse BAL fluids, sometimes for up to 48h, produced no cleavage. There was no observable activity in uninfected BAL fluids, which is consistent with previous reports of mouse NE activity. For example, β-ENaC mice have been found to have no soluble NE activity in BAL fluid due to a high endogenous anti-protease load (253). Free NE was detected in the BAL fluid after infection with PAO1 and when all treatment groups were compared there appeared to be some correlation between BAL CFU counts and NE levels. However, the levels were still much lower than that of human CF BAL fluids, the highest being 0.393µg/ml, which is very low when one considers the length of time required for 5µg/ml to cleave pro-WMR. For comparison, the human NE levels observed in Results Chapter One neared 200µg/ml. The expression of free murine NE after stimulus is again consistent with the literature. Elastase activity has been induced in the same mouse background by stimulation with LPS (268), LPS and the neutrophil chemoattractant N-Formyl methionyl-leucyl-phenylalanine (254), and agarose-encased P. aeruginosa (256). The latter appeared to induce a significant (but variable) increase in mNE after 5 days. This was a chronic model of infection in contrast to our acute model. Unfortunately the authors did not quantify the mNE level in terms of µg/ml for comparison. Similar
studies to our own, using a PAO1 suspension in PBS failed to produce active mNE in BAL fluid, a fact that the authors again suggested was due to increasing antiprotease activity in response to lung infection (255). Future studies into pro-HDP design may benefit from adopting a more chronic model of *P. aeruginosa* infection which may have the potential to produce the high levels of NE required to cleave the pro-peptide. C57BL/6 mice have previously shown to have a more muted neutrophil response to stimuli such as LPS compared to BALB/c mice (254); another option may be to investigate an alternative genetic background for the β-ENaC model.

The lack of CFU reduction with the application of pro-WMR is consistent with the low levels of mNE found in mouse lungs. The peptide would be expected to be inactive when uncleaved. The trend in wildtype mice was for the CFU count to increase with pro-WMR, which is unexpected but perhaps due to an inflammatory response to the treatment. What is more surprising is the 240 times lower CFU count in response to AAG-WMR than with AAG-P18, considering the latter has a lower MIC against PAO1 (2µg/ml vs. 32µg/ml). While an expanded AAG-P18 treatment group is required to fully elucidate the difference, the superiority of AAG-WMR was profound. However, there was significant toxicity with two doses of AAG-P18 (killing over 70% of the mice tested) which may contribute to the large difference. The peptide perhaps exacerbates lung infection by causing lung damage in its own right. Another possible explanation is the high neutrophil toxicity observed with AAG-P18 in Results Chapter Two, which could affect the host’s immune response.

The 100% survival of pro-P18-treated mice (both wildtype and NE-knockout) is evidence of the benefits of the pro-HDP model and is consistent with the reduced cytotoxicity previously noted *in vitro* (211). Consistent again with the *in vitro* cytotoxicity outlined in Table 4.3, there was less lung disease in both pro- and AAG-WMR compared to pro-P18, with lower BAL fluid host cell counts. The absence of weight loss with pro-WMR also illustrates the benefits of the prodrug model, even though the outward toxicity of AAG-WMR is low. CF patients undergoing an acute pulmonary exacerbation frequently experience acute weight loss. In mice infected with *P. aeruginosa*
this phenomenon has been demonstrated to be the result of increased energy expenditure as opposed to reduced food consumption. This is potentially related to increased pulmonary inflammation and BAL levels of the cytokines KC, TNF-α, and mip-2 have all been shown to correlate to weight loss in mice (269). The observed increase in cytokines in response to the peptides supports this, especially when one considers there was no observed significant cytokine increase with pro-WMR (which also induced no weight loss). An increase in cytokine release was not observed with AAG-WMR compared to its prodrug in CFBE cells in Results Chapter Two. The increase in KC and TNF-α may not necessarily be the result of direct immuno-stimulation but could alternatively be the indirect result of epithelial damage that the pro-HDP modification protects the lungs from. These cytokines have the potential to exacerbate inflammation in CF. TNF-α increases neutrophil chemotaxis, adhesion, and production (270), while KC is also a neutrophil chemoattractant (252). There is no structural analogue of IL-8, a potent human neutrophil chemoattractant, in mice; therefore it could not be analysed here (271). Analysis of β-ENaC BAL fluid after PAO1 infection indicated that high concentrations of cytokines were present but that no increase in inflammation was observed after treatment with AAG-WMR or AAG-P18. The pro-inflammatory effects of the peptide treatment may have not been apparent when combined with the large pseudomonas-induced inflammation. The high levels of cytokines found are consistent with those seen elsewhere. For example, 2800-6700pg/ml KC here compared to ~9000pg/ml KC in mice 24h after infection with agarose-embedded P. aeruginosa (269). The infection model produced comparable weight loss and neutrophil recruitment to that observed by others; another study with P. aeruginosa in agarose beads noted % neutrophils of 81 ± 2% and weight loss of ~11% 48h after inoculation (269). The trend for β-ENaC mice to have higher CFU counts compared to wildtype is also consistent with results seen elsewhere with PAO1 (252).

While not statistically significant compared to control, the trend was for AAG-WMR to reduce the mean bacterial burden, by 99% in β-ENaC mice and by 97% in wildtype mice. The expansion of the treatment groups may bring the
CFU reduction with AAG-WMR to statistical significance and if so it would compare favourably to the treatment of *P. aeruginosa* with other peptides. Zhang *et al* described a series of antimicrobial peptides for treating the bacterium in CF, achieving a CFU reduction of 74% and 91% after three day’s treatment with 100µg of two peptides in their chronic rat lung infection model (121). In a similar rat infection model, treatment with 500µg of the polycationic antibiotic peptide polymyxin B was able to reduce the bacterial burden by 87% and this was even more profound when encapsulated in liposomes, where the reduction was 99.5% (239). In an acute *P. aeruginosa* mouse infection model similar to ours, 10µg of LL-37 was administered immediately after infection and was able to enhance the clearance of infection, despite the low direct antimicrobial activity of the peptide. 24h after infection, the enhanced inflammatory response reduced the CFU count of the mouse lung homogenates to ~1/100th of that of the PBS control (247). In a similar manner, LL-37 and IDR-1 delivered at the same time as MRSA were able to ameliorate the lung disease induced by the bacteria. At higher doses (50-66µg/mouse) the protective effects of both peptides were lost. Survival time of the mice was also reduced compared to the MRSA-infected control, indicating a degree of host toxicity (248). Another peptide A3-APO was able to prevent *K. pneumoniae* lung infection from progressing to the bloodstream when 125µg was delivered intranasally, with some of its activity potentially due to the inhibition of inflammation. Dose-limiting toxicity was observed at 625µg/mouse (272).

The *in vivo* studies, although limited in evaluating the activity of pro-WMR due to the relatively low-NE levels of the specific infection model, demonstrate the superiority of AAG-WMR over AAG-P18 as an active peptide in CF, consistent with the results seen previously. The toxicity study also illustrates the benefit of delivering HDPs as a prodrug with the prevention of mortality with the P18 series, and the prevention of weight loss and cytokine release with the less toxic WMR series. Expansion of treatment groups and further detailed analysis will grant new insights into the pulmonary effects of the peptides; awaiting completion are the lung histology and differential cell counts. However, the work carried out so far makes a
strong case for the further development of the pro-HDP model and for pro-WMR as an anti-pseudomonal therapeutic.
7. Conclusion and Future Plans

7.1 Conclusion

The aim of this project was to develop pro-HDPs for CF with two main goals: the first was to demonstrate the utility of the NE-targeted oligoglutamic acid model for HDPs in CF, the second was to bring one of these pro-HDP therapeutics closer to the clinic by evaluating its use in vitro and in vivo. The sum of the four results chapter has achieved these goals.

Modification of five HDPs: Bac8c, HB43, P18, WMR, and WR12 has produced pro-HDPs with NE-dependent activity. In addition, reduction in cytotoxicity has been shown in four of these. The application of CF BAL fluid underlines the potential of the pro-HDPs to work in the CF lung and reinforces the potential for targeting prodrugs to NE. Some of the other enzymes relevant to CF such as PE and CG have been tested against the AAAG linker but the lack of proteolysis coupled with the concentrations several times lower than NE (which represents approximately 90% of the protease load) would render them inferior targets, should an alternative linker be used. The fact that the low levels of mNE found in the mouse BAL fluids were insufficient for cleavage emphasises this; the highest concentration found (0.393μg/ml) might be considered a high for another enzyme. While the pro-HDP model is versatile, and could be applied to other disease models such as cancer with a suitable linker, the requirement for an extracellular protease of sufficiently high concentration must be carefully considered. An appropriate combination of infectious disease/cellular dysfunction and co-localised high levels of protease would be an attractive target for HDPs. With this combination also comes the consideration of what the fate of the active sequence in the same conditions will be: it is shown that for CF, d-amino acids can provide stability in hostile proteolytic conditions where L-amino acid peptides would be quickly degraded and that this is more than likely an absolute requirement.
The development of pro-WMR not only represents a proof-of-concept for pro-HDPs, it has the potential to be used as an antibacterial therapeutic in its own right. CF provides the required high levels of protease but also an uncomplicated means of delivering the peptide. It is demonstrated here that the nebulised delivery of pro-WMR is facile and that the additional glutamic acid residues are not antagonist to the generation of a respirable aerosol. The % deliverable to a model human lung compares well to other CF therapeutics. In other disease models this delivery may not be as straightforward. For example, injection of pro-HDPs for cancer introduces the complications of circulatory proteases and the exposure of off-target sites to the HDPs. In addition, the local drug concentrations with intravenous delivery are not as high as with inhalation.

The results of the *in vivo* tests complement the *in vitro* data and demonstrate the reduction of host toxicity afforded by the pro-HDP modification. This was evident in the significant differential in outcome between AAG-P18 and the pro-HDP and also in the reduction in weight loss and cytokine release afforded by pro-WMR compared to AAG-WMR treatment. The trend is also for the less-cytotoxic AAG-WMR to reduce infection with *P. aeruginosa* in contrast to AAG-P18, which may facilitate bacterial infection by inducing lung damage, despite the fact that it has a lower MIC against the bacterium. This illustrates how the evaluation of *in vitro* antimicrobial activity and cytotoxicity may be used to predict which HDPs will perform better *in vivo*.

In many respects, while pro-HDPs show great promise in treating CF infections, CF itself is ideally suited for pro-HDPs. The predominance of one particular pathogen, *P. aeruginosa*, a high concentration of co-localised enzyme, NE, and availability for local delivery simplifies the design and delivery of the peptides. We demonstrate with pro-WMR the potential for therapy of CF, killing the pathogen and targeting the enzyme. In other biological systems the difficulties in delivery and enzyme-targeting may be compounded, but the progress made with these peptides in CF should advise design. More-labile linkers can be developed for enzymes that appear in lower concentrations and modern drug-delivery systems such as liposomes or nanoparticles may be used to further localise delivery. For CF,
these considerations are thankfully not necessary and the present work validates that pro-HDPs should form part a potential strategy for developing new therapeutics for this disease.

### 7.2 Future Plans

The most important set of experiments that must be carried out to advance the results of this project is the expansion of the animal study. While many interesting conclusions could be drawn, more work beyond analysis of the current data should be ideally undertaken. Some of the treatment groups (especially AAG-P18) were too small for complete observations to be made. For example, the trend was for AAG-WMR to produce a 2 log reduction in CFU/ml, a figure which is consistent with some of the more active in vivo HDPs, but more subjects are required to bring the result to statistical significance. In addition, while it seems that the mouse model may not be suitable for testing a NE-dependent prodrug, they must first be tested in a chronic infection model before this can be fully ruled out. The literature is conflicting on whether enough free mNE can be induced, but the most optimistic model we could adopt is to infect mice with bacteria embedded in agarose beads and treat with pro-HDP several days later (256).

There are several other interesting directions that may be taken to evaluate pro-HDPs for CF:

#### 7.2.1 Expanded clinical isolate groups

The study has focused on reference strains and four clinical isolates of *P. aeruginosa*. The bacterium is usually initially acquired from the environment but may sometimes be transferred from patient-to-patient, leading to genotypically-indistinguishable and potentially-resistant strains passing between individuals. Infection with one strain for example, Liverpool Epidemic Strain, can lead to rapid loss of lung function and increased mortality while another strain resistant to colistin has been reported in a paediatric CF clinic (273). Classical phenotypic methods usually applied to
other bacteria such as serotyping are less discriminatory for \( P. \ aeruginosa \), with the correlation with genotype being less clear (274). While clonal and epidemic strains are typically more resistant, they often do not show distinctive antibiograms which, along with other phenotypes, such as being mucoid, have been seen to vary over time (275). Despite the availability of fast and cost-effective PCR-based genotyping methods, the more laborious pulsed-field gel electrophoresis (PFGE) remains the gold standard for typing \( P. \ aeruginosa \). This is useful for examining local populations but less so for cross-sectional studies (276). The demonstration of pro-HDP efficacy against well-characterised and representative \( P. \ aeruginosa \) clones that are circulating in the Irish CF population would be important. Nearly 30 CF isolates have so far been collected and characterised from patients in Beaumont Hospital. After collection and genotyping of the CF \( P. \ aeruginosa \) isolates a comprehensive review of the susceptibility to the HDPs could be carried out using MIC assays. Recently, an analysis of the activity of WR12 against 100 CF \( P. \ aeruginosa \) strains was carried out, demonstrating the lack of intrinsic bacterial resistance to the peptide in the wider population (210).

7.2.2 Quorum sensing effects

Another interesting direction would be the effects of these HDP prodrugs on \( P. \ aeruginosa \) virulence factors at sub-inhibitory concentrations, specifically factors associated with Quorum Sensing (QS). QS describes the ability of populations of bacteria to coordinate gene expression in response to their surroundings, relying on the transmission of small diffusible signal molecules (277). Virulence factors such as rhamnolipids, elastases, and pyocyanin have been used before as indicators of QS activity and could be the subject of investigation in this study (277-282). QS is associated with much of the pathogenicity of \( P. \ aeruginosa \) infections and so any reduction would be beneficial (282). It has previously been shown that structurally-unrelated compounds such as azithromycin, ceftazidime, ciprofloxacin, and certain sesquiterpene lactones have the ability to reduce QS activity, and it has been suggested that the diversity of the compounds may indicate that membrane disruption is the source of the anti-QS activity. On the other hand, other
antibiotics do not have anti-QS activity, including tobramycin (277, 280). The effect of HDPs, which are known to disrupt membranes, on these virulence factors has not been extensively studied. The factors for investigation, elastase (LasB protease), rhamnolipid, and pyocyanin production, are associated with increased QS activity. As many reference strains can lose QS characteristics over time, clinical isolates would be used for comparison (281). The measurement of many of these factors is relatively straightforward. Elastase can be measured by incubating filtered culture supernatants in elastin congo red and measuring absorbance at 495nm, correcting for pigments already produced by *P. aeruginosa* (278, 281). Rhamnolipids can be measured by culture on M8 salt-based agar and the measurement of the blue rhamnolipid halo produced by the cultures (282). Pyocyanin can be measured at 520nm after extracting it from broths with chloroform and 0.2N HCl (279). The expression of specific QS genes after exposure to the HDPs may also be investigated using reverse transcriptase PCR (277).
8. References


86. FAQs about Combined Ivacaftor (Kalydeco™) & Lumacaftor (VX-809) Phase 3 Clinical Trials. Cystic Fibrosis Foundation Website 2014 [accessed on 20/03/15]; Available from: http://www.cff.org/research/ClinicalResearch/FAQs/CombinedKalydeco-VX-809/.
90. National Centre for Pharmacoeconomics. Cost-effectiveness of Ivacaftor (Kalydeco™) for the treatment of cystic fibrosis in patients age 6


111. Zuyderduyn S, Ninaber DK, Hiemstra PS, Rabe KF. The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. The Journal of Allergy and Clinical Immunology. 2006;117(6):1328-35. Epub 2006/06/06.


138. Bergen PJ, Li J, Rayner CR, Nation RL. Colistin methanesulfonate is an inactive prodrug of colistin against Pseudomonas aeruginosa.


146. Jallouk AP, Palekar RU, Marsh JN, Pan H, Pham CT, Schlesinger PH, et al. Delivery of a Protease-Activated Cytolytic Peptide Prodrug by


156. Morris CJ, Beck K, Fox MA, Ulaeto D, Clark GC, Gumbleton M. Pegylation of antimicrobial peptides maintains the active peptide


163. CLSI. Performance standards for antimicrobial susceptibility testing. 20th informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.


165. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm


175. Shin SY, Kang JH, Hahm KS. Structure-antibacterial, antitumor and hemolytic activity relationships of cecropin A-magainin 2 and cecropin A-


Figure A1: HPLC chromatogram and MALDI-TOF mass spectrum of Bac8c$_{2.5}$Leu. Retention time 19.09 min. The signal at $m/z = 1183.7135$ corresponds to the molecular ion.
Figure A2: HPLC chromatogram and MALDI-TOF mass spectrum of AAG-Bac8c<sub>2.5 Leu</sub>. Retention time 19.37 min. The signal at \( m/z = 1382.8251 \) corresponds to the molecular ion.
Figure A3: HPLC chromatogram and MALDI-TOF mass spectrum of pro-Bac8c$^{2,5}$Leu. Retention time 20.45min. The signal at $m/z = 2011.7845$ corresponds to the molecular ion.
Figure A4: HPLC chromatogram and MALDI-TOF mass spectrum of HB43. Retention time 21.6min. The signal at $m/z = 1456.0015$ corresponds to the molecular ion and the signal at $m/z = 1477.9773$ corresponds to $[\text{M+Na}]^+$. 
Figure A5: HPLC chromatogram and MALDI-TOF mass spectrum of AG-HB43. Retention time 24.28 min. The signal at $m/z = 1584.0674$ corresponds to the molecular ion and the signal at $m/z = 1606.0494$ corresponds to $[\text{M+Na}]^+$. 
Figure A6: HPLC chromatogram and MALDI-TOF mass spectrum of pro-HB43. Retention time 25.29min. The signal at m/z = 2285.3325 corresponds to the molecular ion; the signal at m/z = 2307.3230 corresponds to [M+Na]⁺, and the signal at m/z = 1570.6774 corresponds to the calibration standard.
Figure A7: HPLC chromatogram and MALDI-TOF mass spectrum of P18$^8\text{Leu}$. Retention time 19.33 min. The signal at $m/z = 2300.4690$ corresponds to the molecular ion; the signal at $m/z = 1571.6694$ corresponds to the calibration standard.
Figure A8: HPLC chromatogram and MALDI-TOF mass spectrum of AAG-P18$^8$Leu. Retention time 19.48 min. The signal at $m/z = 2499.7$ corresponds to the molecular ion; the signal at $m/z = 2520.6653$ corresponds to [M+Na]$^+$, and the signal at $m/z = 2537$ corresponds to [M+K]$^+$. 
Figure A9: HPLC chromatogram and MALDI-TOF mass spectrum of pro-P18 \textsuperscript{8}Leu. Retention time 19.73min. The signal at $m/z = 3128.8047$ corresponds to the molecular ion; the signal at $m/z = 3151.8130$ corresponds to $[\text{M+Na}]^+$, and the signal at $m/z = 1564.8899$ corresponds to the calibration standard.
pro-Bac8c\textsubscript{2,5 Leu} and 5µg/ml neutrophil elastase after 1h incubation. Retention times 19.07, 19.24 (AAG-Bac8c\textsuperscript{2,5 Leu}), and 20.39 min (Bac8c\textsuperscript{2,5 Leu}). The signal at \textit{m/z} = 1382.8209 corresponds to the molecular ion of AAG-Bac8c\textsuperscript{2,5 Leu}; \textit{m/z} = 1311.7871 of AG-Bac8c\textsuperscript{2,5 Leu}, and \textit{m/z} = 2013.0475 of Bac8c\textsuperscript{2,5 Leu}. The signal at \textit{m/z} = 1570.6674 corresponds to the calibration standard, and \textit{m/z} = 1404.8041 to [M+Na]\textsuperscript{+} of AAG-Bac8c\textsuperscript{2,5 Leu}. 
Figure A11: HPLC chromatogram and MALDI-TOF mass spectrum of pro-Bac8c$_{2,5}$Leu$_{2}$ and 10µg/ml pseudolysin after 1h incubation. Retention time 20.16 min (pro-Bac8c$_{2,5}$Leu$_{2}$). The signal at $m/z = 2013.0105$ corresponds to the molecular ion of pro-Bac8c$_{2,5}$Leu$_{2}$. The signal at $m/z = 2033.9878$ corresponds to [M+Na]$^+$ of pro-Bac8c$_{2,5}$. 
Figure A12: HPLC chromatogram and MALDI-TOF mass spectrum of pro-HB43 and 5µg/ml neutrophil elastase after 1h incubation. Retention times 24.04 (AG-HB43) and 25.16 min (pro-HB43). The signal at m/z = 1584.066 corresponds to the molecular ion of AG-HB43; m/z = 1657.1227 of AAG-HB43, and m/z = 2285.3325 of pro-HB43. The signal at m/z = 1606.052 corresponds to [M+Na]⁺ for AG-HB43.
Figure A13: HPLC chromatogram and MALDI-TOF mass spectrum of pro-HB43 and 10µg/ml pseudolysin after 1h incubation. Retention time 25.08min (pro-HB43). The signal at $m/z = 2285.3264$ corresponds to the molecular ion of pro-HB43. The signal at $m/z = 2307.3152$ corresponds to $[\text{M+Na}]^+$ and $m/z = 1570.6774$ corresponds to the
Figure A14: HPLC chromatogram and MALDI-TOF mass spectrum of pro-P18⁸ Leu and 5µg/ml neutrophil elastase after 1h incubation.

Retention times 19.04 (AAG-P18⁸ Leu), 19.45, and 19.91min (pro-P18⁸ Leu). The signal at $m/z = 2499.5676$ corresponds to the molecular ion of AAG-P18⁸ Leu; $m/z = 2428.5322$ of AG-P18⁸ Leu, and $m/z = 3128.8054$ of pro-P18⁸ Leu. The signal at $m/z = 1564.899$ corresponds to the calibration standard, and $m/z = 2521.5566$ corresponds to [M+Na]$^+$ for AAG-P18⁸ Leu.
Figure A15: HPLC chromatogram and MALDI-TOF mass spectrum of pro-P18 and 10µg/ml pseudolysin after 1h incubation. Retention time 19.72 min (pro-P18 $^{8}$Leu). The signal at $m/z = 3128.755$ corresponds to the molecular ion of pro-P18 $^{8}$Leu. The signal at $m/z = 1564.8798$ corresponds to the calibration standard.
Figure A16: HPLC chromatogram and $^{19}$F NMR of HB43 before TFA removal. Retention time 21.6 min. The chemical shift at -75.52 represents TFA.
Figure A17: HPLC chromatogram and $^{19}$F NMR of HB43 after TFA removal. Retention time 21.68 min.
Figure A18: HPLC chromatogram and MALDI-TOF mass spectrum of 5E-P18\textsuperscript{8 Leu}. Retention time 20.08 min. The signal at \( m/z = 3256.8176 \) corresponds to the molecular ion.
Figure A19: HPLC chromatogram and MALDI-TOF mass spectrum of PEG-P18<sup>8</sup>Leu. Retention time 21.23min. The signals between m/z = 4006.2717 and m/z = 4974.8047 corresponds to the molecular ions of the polydisperse PEG conjugates.
Figure A20: HPLC chromatogram and MALDI-TOF mass spectrum of WMR\textsuperscript{3, 6 Leu}. Retention time 19.05 min. The signal at $m/z = 1631.8450$ corresponds to the molecular ion.
Figure A21: HPLC chromatogram and MALDI-TOF mass spectrum of AAG-WMR$_{3,6}$. Retention time 20.89 min. The signal at $m/z = 1831.0861$ corresponds to the molecular ion.
Figure A22: HPLC chromatogram and MALDI-TOF mass spectrum of pro-WMR$^{3,6}\text{Leu}$. Retention time 20.19min. The signal at $m/z = 2460.2066$ corresponds to the molecular ion.
Figure A23: HPLC chromatogram and MALDI-TOF mass spectrum of AAG-WR12. Retention time 21.85min. The signal at $m/z = 2270.1965$ corresponds to the molecular ion.
Figure A24: HPLC chromatogram and MALDI-TOF mass spectrum of pro-WR12. Retention time 21.16min. The signal at $m/z = 2899.3940$ corresponds to the molecular ion.
Figure A25: HPLC chromatogram and MALDI-TOF mass spectrum of pro-WMR and 20µg/ml neutrophil elastase after 3h incubation. Retention times 20.77min (AAG-WMR), and 21.53min (pro-WMR). The signal at $m/z = 1759.9899$ corresponds to the molecular ion of AG-WMR; $m/z = 1831.0222$ of AAG-WMR, and $m/z = 2460.2058$ of pro-WMR.
Figure A26: HPLC chromatogram and MALDI-TOF mass spectrum of pro-WR12 and 5µg/ml neutrophil elastase after 1h incubation. Retention times 19.39min (AAG-WR12) and 20.33min (pro-WR12). The signal at $m/z = 2270.0989$ corresponds to the molecular ion of AAG-WR12 and $m/z = 2899.2732$ of pro-WR12.
Figure A27: HPLC chromatogram and MALDI-TOF mass spectrum of pro-WMR and 50% CF BAL CF004 after 3h incubation. Retention times 13.357min, 13.523min, 14.223min, 16.063min (BAL components, see figure A27), and 20.570min (AAG-WMR). The signal at \( m/z = 1761.1 \) corresponds to the molecular ion of AG-WMR and \( m/z = 1832.2 \) of AAG-WMR.

Figure A28: HPLC chromatogram of 50% CF BAL CF004 alone after 3h incubation. Retention times 14.097min, 15.917min (BAL components).
Figure A29: HPLC chromatogram and MALDI-TOF mass spectrum of L-pro-WMR. Retention time 24.3 min (L-pro-WMR). The signal at \( m/z = 2460.4211 \) corresponds to the molecular ion of L-pro-WMR.
Figure A30: HPLC chromatogram and MALDI-TOF mass spectrum of L-pro-WMR and 20µg/ml neutrophil elastase after 1h incubation. Retention times 21.050min (AAG-WMR), 23.690min and 26.283 (degradation fragments). The signal at \( m/z = 1760.1161 \) corresponds to the molecular ion of AG-WMR; \( m/z = 1831.1890 \) of AAG-WMR, and \( m/z \) between 970 and 1700 to different degradation fragments of L-pro-WMR.
Figure A31: HPLC chromatogram and MALDI-TOF mass spectrum of L-pro-WMR and CF BAL CF004 after 3h incubation. Retention times 13.883min, 16.007min (BAL components), 18.600min, 20.527min, 23.380min, and 26.273min (degradation fragments). The signals at $m/z$ between 900 and 1900 correspond to the molecular ion of L-pro-WMR degradation products.
Figure A32: HPLC chromatogram and MALDI-TOF mass spectrum of pro-GG-WMR. Retention time 21.765min (pro-GG-WMR). The signal at $m/z = 2305.222$ corresponds to the molecular ion of pro-GG-WMR.
Figure A33: HPLC chromatogram and MALDI-TOF mass spectrum of pro-GG-WMR and 50% CF BAL CF004 after 3h incubation. Retention times 14.250min, 16.073min (BAL components), and 21.740min (pro-GG-WMR). The signal at $m/z = 2305.3$ correspond to the molecular ion of pro-GG-WMR.
Figure A34: HPLC chromatogram of pro-WMR and 50% non-CF BAL 50 after 3h incubation. Retention time 22.987min (pro-WMR).

Figure A35: HPLC chromatogram of pro-WMR and 50% non-CF BAL 53 after 3h incubation. Retention time 22.737min (pro-WMR).

Figure A36: HPLC chromatogram of pro-WMR and PBS after 3h incubation. Retention time 22.340min (pro-WMR).
Figure A37: HPLC chromatogram and negative ESI mass spectrum of Ac-EEEEEA-OH. Retention time 16.987 min. The signal at 645.9 corresponds to the negative molecular ion of Ac-EEEEEA-OH. Please note that the molecule was too small for accurate MALDI-TOF MS analysis and a different HPLC method was used, using an elution gradient from 2% acetonitrile to 65% in 50 minutes.
Figure A38: HPLC chromatogram of pro-WMR after 48h incubation with CF BAL fluid from a β-ENaC mouse infected with $2.5 \times 10^7$ CFU of *P. aeruginosa*. Retention time 11.10min (pro-WMR), 13-20min (BAL components). The pink line represents the absorbance at 214nm, the blue 280nm.
Figure A39: HPLC chromatogram of pro-P18 after 48h incubation with CF BAL fluid from a β-ENaC mouse infected with 2.5 x 10^7 CFU of *P. aeruginosa*. Retention time 11.74min (pro-P18), 13-20min (BAL components). The pink line represents the absorbance at 214nm, the blue 280nm.
Figure A40: HPLC chromatograms of pro-WMR (A, B) and pro-P18 (C, D) after 3h incubation with human CF (A, C) and healthy sputum (B, D).
Retention times 10.38min (AAG-WMR), 11min (pro-WMR), with other sputum peaks (A). Retention time 11.05min (pro-WMR), with other sputum peaks (B). Retention times 11.43min (AAG-P18), 11.70min (pro-P18), with other sputum peaks (C). Retention times 11.73min (pro-P18), with other sputum peaks (D).
The pink line represents the absorbance at 214nm, the blue 280nm.
Figure A41: HPLC chromatogram of pro-WMR after 3h incubation with fresh CF BAL fluids from β-ENaC mice infected with $2.5 \times 10^7$ CFU of \textit{P. aeruginosa} (A-D). Retention times 10.86-10.97min (pro-WMR), with other BAL fluid peaks. The chromatograms are edited, to remove regions after 15 minutes, due to the occurrence of interfering air bubbles. The pink line represents the absorbance at 214nm, the blue 280nm.
Figure A42: 24h wildtype mouse BAL fluid levels of cytokines in response to a morning and evening dose of 50µl PBS control or 50µg HDPs. AAG-P18 is not included as 3 of 4 mice died before lung lavage. n = 4.
Figure A43: 24h β-ENaC mouse BAL fluid levels of cytokines in response to 2.5 x 10^7 CFU/ml PAO1 and 50μl PBS control or 50μg HDP 6h later. n = 4 (n = 3 for AAG-P18).
Figure A44: HPLC chromatogram of pro-WMR and 5µg/ml cathepsin G after 1h incubation. Retention time 21.683min (pro-WMR).