INVESTIGATING COPPER CHELATION WITH TOBRAMYCIN AS AN ANTI-INFLAMMATORY THERAPY IN CYSTIC FIBROSIS

Marta Gziut

The thesis is submitted in partial fulfilment of the requirements for the award of the Doctor of Philosophy of the University of Portsmouth

May 2012
UNIVERSITY OF PORTSMOUTH
SCHOOL OF PHARMACY AND BIOMEDICAL SCIENCES
Doctor of Philosophy
INVESTIGATING COPPER CHELATION WITH TOBRAMYCIN AS AN ANTI-INFLAMMATORY THERAPY IN CYSTIC FIBROSIS
Marta Gziut

ABSTRACT

Excessive neutrophilic inflammation of the airways in response to infection is characteristic for patients with CF. There is also an important but not fully understood role for platelets. Previous studies established increased copper levels in the circulation and in the sputum in CF. Inhaled tobramycin was suggested to have an anti-inflammatory effect beyond eradicating Pseudomonas aeruginosa. This study tested the hypothesis that tobramycin has anti-inflammatory and anti-oxidant efficacy due to its ability to bind copper into a copper-tobramycin complex.

A copper-tobramycin complex was synthesised and the UV-VIS spectrum analysed. Neutrophil migration through a TNF-α-stimulated human lung microvascular endothelial cell layer towards thrombin-activated platelets was measured. The role of CFTRinh-172 on neutrophil transendothelial migration was assessed. Endothelial tobramycin uptake and CFTR expression were assessed using immunocytochemistry. Endothelial oxidative stress was measured using a fluorescent indicator. Neutrophils were stimulated to measure reactive oxygen species (ROS) production and neutrophil elastase (NE) activity, spectrophotometrically.

Platelet and endothelium-derived NAP-2 and IL-8, respectively, contributed to neutrophil transendothelial migration. Copper-tobramycin was shown to be more effective than tobramycin in limiting migration of neutrophils. Both, tobramycin and copper-tobramycin accumulated in endothelial cells via a heparan sulphate-dependent mechanism, decreased intracellular ROS and increased endothelial surface CFTR expression. CFTRinh-172 failed to create an inflammatory profile in endothelium. Copper-tobramycin decreased extracellular superoxide released by activated neutrophils, and displaced NE from sites of encryption, making it more susceptible to inhibition by α1-antitrypsin.

The antibiotic tobramycin was demonstrated to be a multi-potent drug with additional anti-inflammatory and anti-oxidant properties. These effects, desirable in CF treatment, are due to copper binding.
DECLARATION

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Marta Gziut
May 2012
ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my supervisor, Dr Janis Shute for valuable guidance and ideas, which benefited me greatly throughout my PhD; for her insights and knowledge within the field of immunopharmacology; and the dedication and enthusiasm to work she had every day.

Secondly, I would like thank my supervisor, Dr David Laight for helpful advice, constructive suggestions and the time and passion he devoted to this project.

I am grateful to Dr Tom Nevell, who shared his experience and knowledge and provided his assistance to an important aspect of this study.

A special thank you to all my blood donors, without whom, this project would not have been possible; and to all the kind people who I met along the way.

I also wish to thank my loved ones for their understanding, support and unending encouragement.
1. GENERAL INTRODUCTION

1.1. Pathogenic mechanisms of cystic fibrosis
1.1.1. Epidemiology of cystic fibrosis
1.1.2. Genetic effect
1.1.3. Pulmonary infection
1.1.4. Pulmonary inflammation
1.1.5. Resolution of inflammation
1.2. Cellular contribution to airway inflammation in CF
1.2.1. Neutrophils
1.2.2. Endothelial cells
1.2.3. Platelets
1.3. Leukocyte recruitment
1.4. Leukocyte recruitment to the lung
1.5. Chemokines involved in the inflammatory process
1.5.1. Interleukin-8 (IL-8)
1.5.2. Neutrophil-activating peptide-2 (NAP-2)
1.6. Plasminogen activator inhibitor-1 (PAI-1)
1.7. Copper and its dual role in the human body
1.7.1. Copper as an essential element
1.7.2. Copper homeostasis
1.7.3. Copper and oxidative stress
1.8. Anti-copper therapy
1.9. Current treatment of cystic fibrosis
1.9.1. Reducing airway obstruction ................................................................. 36
1.9.2. Treatment of airway infection with antibiotics ................................. 37
1.9.3. Therapies directed at inflammation  .................................................. 42
1.10. Hypothesis, aims and objectives ....................................................... 43

2. CELLULAR COPPER CONTENT AND FORMATION

OF THE COPPER-TOBRAMYCIN COMPLEX ................................................. 44

2.1. Introduction ............................................................................................. 44
2.1.1. Superoxide dismutase ........................................................................ 44
2.1.2. Catalase ............................................................................................. 47
2.1.3. SOD mimetics .................................................................................. 48
2.1.4. Copper(II)-aminoglycosides .............................................................. 50
2.2. Hypothesis, aims and objectives ......................................................... 53
2.3. Materials ............................................................................................... 53
2.4. Methods ............................................................................................... 54
2.4.1. Neutrophil isolation ......................................................................... 54
2.4.2. Platelet isolation ............................................................................... 55
2.4.3. Plasma and serum sample preparation ............................................ 55
2.4.4. Endothelial cell culture ..................................................................... 55
2.4.4.1. Cell feeding .................................................................................. 56
2.4.4.2. Subculturing ................................................................................ 57
2.4.4.3. Subculturing for new flasks ......................................................... 57
2.4.4.4. Subculturing for cryopreservation .............................................. 58
2.4.5. Copper measurement by Graphite Furnace Atomic Absorption

Spectrometry (GF-AAS) ............................................................................. 58
2.4.5.1. Sample preparation for GF-AAS .................................................. 58
2.4.5.2. Sample digestion ........................................................................ 59
2.4.5.3. Instrument settings ...................................................................... 59
2.4.6. Synthesis of copper-tobramycin ...................................................... 60
2.4.7. UV-VIS Spectrophotometry ............................................................... 61
2.4.8. Microplate superoxide dismutase assay ......................................... 62
2.4.9. Hydrogen peroxide assay ................................................................. 62
2.4.10. A rapid kinetic assay for catalase activity ...................................... 63
2.4.11. Statistical analysis .......................................................................................................................... 63
2.5. Results .................................................................................................................................................. 64
  2.5.1. Development of copper analysis using the standard addition method (GF-AAS) ....................... 64
  2.5.2. Cellular copper levels ...................................................................................................................... 65
  2.5.3. UV-VIS spectrum of tobramycin, copper sulphate and copper-Tobramycin .............................. 66
  2.5.4. SOD-like and catalase activities associated with copper-tobramycin ........................................ 74
2.6. Summary of results ............................................................................................................................. 78
2.7. Discussion ........................................................................................................................................... 78

3. THE ROLE OF HEPARAN SULPHATE IN THE UPTAKE OF TOBRAMYCIN AND COPPER-TOBRAMYCIN BY HLMVEC ......................................................................................................................... 86
  3.1. Introduction ........................................................................................................................................ 86
    3.1.1. Mimicking CF endothelium ........................................................................................................... 86
    3.1.2. Glycosaminoglycans and proteoglycans in normal and CF lung .................................................. 86
    3.1.3. Heparanases ............................................................................................................................... 90
    3.1.4. GAG-Cu(II) interactions ............................................................................................................. 92
    3.1.5. The uptake of aminoglycosides .................................................................................................. 92
    3.1.6. The role of proteoglycans in aminoglycoside uptake .................................................................... 94
  3.2. Hypothesis, aims and objectives ......................................................................................................... 95
  3.3. Materials .......................................................................................................................................... 95
  3.4. Methods .......................................................................................................................................... 97
    3.4.1. Collagen IV coating of cell culture dishes ..................................................................................... 97
    3.4.2. Subculturing of HLMVEC into 8-well chamber slides .............................................................. 97
    3.4.3. Treatment of HLMVEC in 8-well chamber slides ....................................................................... 97
    3.4.4. Immunocytochemical staining of HLMVEC for GAGs ................................................................ 98
    3.4.5. Immunocytochemical staining of HLMVEC for tobramycin .................................................... 99
    3.4.6. Immunocytochemical staining of HLMVEC for CFTR ............................................................. 99
    3.4.7. Tobramycin uptake by neutrophils .............................................................................................. 100
    3.4.8. Immunocytochemical staining of neutrophils for tobramycin ................................................... 100
    3.4.9. Subculturing of HLMVEC into collagen IV-coated 96-well plates for a viability assay .................. 101
3.4.10. Treatment of HLMVEC into collagen IV-coated 96-well plates for a
viability assay............................................................................................................101
3.4.11. Endothelial cell viability assessment...............................................................102
3.4.12. Statistical analysis............................................................................................102
3.5. Results.........................................................................................................................103
3.5.1. The presence of HS on endothelial cell surface and the effect of
TNF-α, CFTRinh-172 and hepatitinase II on GAGs expression.................................103
3.5.2. Tobramycin uptake by endothelium.................................................................105
3.5.3. The role of HS in tobramycin and copper-tobramycin uptake...........................111
3.5.4. Tobramycin uptake by neutrophils.................................................................113
3.5.5. The effect of CFTRinh-172 on tobramycin uptake by endothelium...............113
3.5.6. The effect of tobramycin, copper-tobramycin, copper-sulphate and
N-acetylcysteine on endothelial CFTR.......................................................................116
3.5.7. Endothelial viability.............................................................................................121
3.6. Summary of results...............................................................................................122
3.7. Discussion...............................................................................................................122

4. THE EFFECT OF TOBRAMYCIN AND COPPER-TOBRAMYCIN ON OXIDATIVE
STRESS IN NEUTROPHILS AND ENDOTHELIUM..................................................130
4.1. Introduction..............................................................................................................130
4.1.1. Oxidative stress in the lung.............................................................................130
4.1.2. Main contributors to oxidative stress..............................................................133
  4.1.2.1. NADPH oxidase.........................................................................................133
  4.1.2.2. Myeloperoxidase......................................................................................134
  4.1.2.3. Neutrophil elastase...............................................................................135
4.1.3. Oxidative stress in cystic fibrosis......................................................................136
4.1.4. Antioxidant defence of the lung........................................................................137
4.2. Hypothesis, aims and objectives.........................................................................139
4.3. Materials................................................................................................................139
4.4. Methods..................................................................................................................139
  4.4.1. Opsonization of zymosan.............................................................................139
  4.4.2. Superoxide assay...........................................................................................140
  4.4.3. Hydrogen peroxide release of neutrophils..................................................140
4.4.4. Neutrophil elastase activity...............................................................141
4.4.5. Assessment of interference in the IL-8 ELISA with different components...141
4.4.6. IL-8 ELISA..................................................................................142
4.4.7. PMN viability assessment.................................................................142
4.4.8. Immunocytochemical staining of HLMVEC for ROS........................143
4.4.9. HLMVEC viability assessment.........................................................144
4.4.10. Statistical analysis........................................................................144
4.5. Results.............................................................................................145
4.5.1. Superoxide anion detection from neutrophils and the effect of tobramycin, copper-tobramycin, SOD and copper sulphate...............145
4.5.2. Hydrogen peroxide release by neutrophils and the effect of tobramycin, copper-tobramycin and SOD........................................148
4.5.3. Viability of neutrophils during the respiratory burst........................150
4.5.4. Neutrophil elastase activity and the effect of α1-antitrypsin..........152
4.5.5. Viability of neutrophils during neutrophil elastase activity measurement..................................................................................165
4.5.6. The effect of different compounds on IL-8 level detected by ELISA......167
4.5.7. Oxidative stress is not induced in HLMVEC by copper and/or hydrogen peroxide.................................................................168
4.5.8. TNF-α induced ROS generation in HLMVEC and the protective role of tobramycin and copper-tobramycin........................................169
4.5.9. The effect of CFTRinh-172 on ROS generation in HLMVEC and the role of tobramycin and copper-tobramycin ................................173
4.6. Summary of results........................................................................175
4.7. Discussion.......................................................................................176

5. THE EFFECT OF TOBRAMYCIN AND COPPER-TOBRAMYCIN ON NEUTROPHIL TRANSENDOTHELIAL MIGRATION...............................................185
5.1. Introduction.....................................................................................185
5.1.1. Neutrophil-platelet-endothelial cell interactions.............................185
5.1.2. In vitro model of leukocyte transendothelial migration (TEM)...........189
5.2. Hypothesis, aims and objectives......................................................191
5.3. Materials.......................................................................................192
5.4. Methods ......................................................................................................................... 192
5.4.1. Subculturing into transwells .................................................................................... 192
5.4.2. A cell culture model of neutrophil transendothelial migration (TEM) ................. 193
5.4.3. Obtaining a dose response curve to TNF-α and IL-8 PMN TEM ......................... 194
5.4.4. Setting up a CF model of MN TEM using CFTRinh-172 ..................................... 195
5.4.5. Thrombin platelet activation ................................................................................... 195
5.4.6. ELISA ....................................................................................................................... 195
5.4.7. Plasmin activity assay ............................................................................................ 195
5.4.8. PMN viability assessment ....................................................................................... 196
5.4.9. HLMVEC viability assessment ............................................................................. 196
5.4.10. Statistical analysis ............................................................................................... 197
5.5. Results ....................................................................................................................... 197
5.5.1. Neutrophil TEM towards IL-8 across unactivated endothelium ......................... 197
5.5.2. Neutrophil TEM on TNF-α-activated endothelium ............................................. 197
5.5.3. Neutrophil TEM towards thrombin activated platelets through TNF-α- stimulated endothelium compared to subentothelial IL-8 ............................................. 198
5.5.4. IL-8, NAP-2 and RANTES release by thrombin-activated platelets ............... 203
5.5.5. The effect of tobramycin and copper-tobramycin on PMN TEM ...................... 207
5.5.6. The effect of catalase and SOD on neutrophil TEM ........................................... 218
5.5.7. The effect of MnTBAP on TEM of neutrophils .................................................... 220
5.5.8. The effect of copper sulphate on TEM of neutrophils ........................................ 223
5.5.9. The effect of PAI-1 inhibitor (XRS5118) on TEM of neutrophils ...................... 224
5.5.10. The effect of CFTRinh-172 on neutrophil transendothelial migration .............. 225
5.5.11. Viability of neutrophils ......................................................................................... 236
5.5.12. Viability of HLMVEC ......................................................................................... 241
5.6. Summary of results ................................................................................................. 246
5.7. Discussion .................................................................................................................... 247

6. PRELIMINARY DATA AND FURTHER WORK .............................................................. 260
6.1. Introduction ................................................................................................................ 260
6.1.1. Normal and CF pulmonary epithelium ............................................................... 260
6.1.2. Epithelial cell lines for CF research ................................................................. 261
6.2. Aims and objectives ................................................................................................. 261
6.3. Materials .................................................................................................................. 262
6.4. Methods .................................................................................................................... 262
  6.4.1. Epithelial cell culture ......................................................................................... 262
  6.4.2. Collagen I/fibronectin coating .......................................................................... 262
  6.4.3. Other methods .................................................................................................. 262
6.5. Results .................................................................................................................... 263
  6.5.1. CFTR localization in epithelial cells ................................................................. 263
6.6. Discussion .............................................................................................................. 267

7. FROM BENCH TO BEDSIDE ...................................................................................... 272

8. APPENDIX ................................................................................................................ 282

9. LITERATURE .......................................................................................................... 286
LIST OF FIGURES

1.1. The structure of CFTR ................................................................. 2
1.2. Normal and CF airway, respectively ........................................... 3
1.3. Classification of the CFTR mutations .......................................... 4
1.4. The excessive inflammatory response in the cystic fibrosis (CF) lung 10
1.5. The action of NADPH oxidase and further dismutation of superoxide anion to hydrogen peroxide ......................................................... 12
1.6. Neutrophil effector mechanisms in the inflammatory process ....... 13
1.7. The structure of platelet .............................................................. 16
1.8. Multistep process of leukocyte recruitment from the circulation into the inflammatory sites ......................................................... 18
1.9. The structure of a complex beween t-PA and PAI-1 .................... 28
1.10. Plasminogen activation cascade ................................................ 29
1.11. Cellular copper homeostasis ..................................................... 32
1.12. Fenton and Haber-Weiss reaction ............................................. 33
1.13. Mechanisms of antibiotic action ............................................... 36
1.14. Schematic structure of aminoglycosides ..................................... 39
1.15. The decoding site in 16S ribosomal RNA .................................... 41
2.1. Human Cu/Zn-SOD structure .................................................... 45
2.2. Dismutation of superoxide by SOD ............................................ 45
2.3. Classification of SOD mimetics with regard to their catalytic activity 48
2.4. Cu^{2+}-neamine at pH 7.5 ........................................................ 50
2.5. Cu^{2+}-tobramycin complex at physiological pH ......................... 51
2.6. A representative standard addition plot ...................................... 64
2.7. UV-VIS spectrum of tobramycin at 37^0C .................................. 67
2.8. UV-VIS spectrum of a range of copper sulphate at 37^0C .............. 68
2.9. UV-VIS spectrum of copper-tobramycin at 37^0C ....................... 69
2.10. A Beer-Lambert plot for copper-tobramycin ............................. 70
2.11. Tobramycin binds copper ......................................................... 72
2.12. The effect of buffer systems on wavelength scan of copper sulphate at 37^0C ............................................................ 73
2.13. Superoxide dismutative activity of copper-tobramycin measured an
inhibition of the rate of nitro blue tetrazolium reduction


2.15. Hydrogen peroxide dismutation by tobramycin, copper-tobramycin and copper chloride in the range of 0.2 – 2 mM

2.16. Decomposition of hydrogen peroxide over time by varying quantities of catalase

2.17. Evaluation of hydrogen peroxide decomposition in the presence of tobramycin, copper-tobramycin and copper chloride (0.5 mM)

2.18. A species distribution diagram of tobramycin (L) complexes with Cu(II) for concentrations used in spectroscopic studies

3.1. Major types of glycosaminoglycans

3.2. Cell surface proteoglycans

3.3. Substrate specificities types of heparin lyases

3.4. Aminoglycosides and guanidinoglycosides evaluated for cellular uptake

3.5. HS on HLMVEC, fixed and permeabilised

3.6. HS on HLMVEC, fixed, not permeabilised

3.7. Tobramycin uptake of HLMVEC for 3.5 hours

3.8. Copper-tobramycin uptake of HLMVEC for 3.5 hours

3.9. Tobramycin uptake of HLMVEC for 24 hours

3.10. Copper-tobramycin uptake of HLMVEC for 24 hours

3.11. Tobramycin localization in HLMVEC

3.12. HLMVEC stained for tobramycin with counterstained nucleus under tobramycin and copper-tobramycin

3.13. Background correction

3.14. Immunocytochemical quantitatively analysis of tobramycin and copper-tobramycin uptake by HLMVEC for 3.5 hours, following subtracting

3.15. The effect of heparitinase II on tobramycin and copper-tobramycin uptake by HLMVEC

3.16. Tobramycin and copper-tobramycin is not taken up by resting or IL-8-activated neutrophils

3.17. The effect of CFTRinh-172 on tobramycin uptake

3.18. The effect of CFTRinh-172 on copper-tobramycin uptake

3.19. Tobramycin stain showing the effect of heparitinase II on HLMVEC
incubated with tobramycin in the presence of CFTRinh-172 and TNF-α......116

3.20. The effect of tobramycin, copper-tobramycin, NAC and copper sulphate on CFTR expression in HLMVEC..............................................................................................................117

3.21. The effect of tobramycin, copper-tobramycin, NAC and copper sulphate on CFTR expression in HLMVEC activated with TNF-α in the presence or absence of CFTRinh-172..............................................................................................................118

3.22. Z-stack of untreated HLMVEC control stained for CFTR..............................................119

3.23. Z-stack of tobramycin-treated HLMVEC in the presence of CFTRinh-172 and stained for CFTR.........................................................................................................................120

3.24. HLMVEC viability in full growth medium during 24 hours incubation..............121

4.1. Model of NADPH oxidase activation.................................................................................134

4.2. Major antioxidant pathways in the lung........................................................................138

4.3. OPZ-stimulated neutrophil respiratory burst in comparison to the spontaneous respiratory burst and reagent control.................................................................145

4.4. The effect of tobramycin and copper-tobramycin on OPZ-stimulated neutrophil respiratory burst.................................................................................................146

4.5. The effect of SOD on OPZ-stimulated neutrophil respiratory burst..................147

4.6. The effect of copper sulphate on OPZ-stimulated neutrophil respiratory burst.................................................................................................................................148

4.7. The effect of tobramycin, copper-tobramycin and extracellular SOD on hydrogen peroxide release from OPZ- and PMA-activated neutrophils at 1 x 10^7/ml.........................................................................................................................149

4.8. The effect of tobramycin on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C.................150

4.9. The effect of copper-tobramycin on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C.............151

4.10. The effect of copper sulphate on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C............151

4.11. The effect of SOD on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C.................................152

4.12. The effect of tobramycin on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min .........................................................................................................................153
4.13. The effect of copper-tobramycin on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................154

4.14. The effect of copper sulphate on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................154

4.15. The effect of potamine sulphate on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................155

4.16. The effect of tobramycin on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................156

4.17. The effect of copper-tobramycin on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................156

4.18. The effect of copper sulphate on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................157

4.19. The effect of potamine sulphate on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min..........................................................157

4.20. The effect of tobramycin and copper-tobramycin on NE activity in supernatants and cell pellets of OPZ-stimulated PMN and the effect of α1-AT.....................................................................................158

4.21. IL-8 level released from OPZ-stimulated neutrophils treated with tobramycin.................................................................................................................................159

4.22. IL-8 level released from OPZ-stimulated neutrophils incubated with copper-tobramycin..................................................................................................................160

4.23. The effect of copper sulphate on IL-8 level released from OPZ-stimulated neutrophils..................................................................................................................161

4.24. The effect of protamine sulphate on IL-8 level released from OPZ-stimulated neutrophils..................................................................................................................161

4.25. Cell pellet-associated IL-8 od OPZ-stimulated neutrophils treated with
4.26. The effect of copper-tobramycin on IL-8 level in the cell pellet of OPZ-stimulated PMNs

4.27. IL-8 level from OPZ-stimulated neutrophils incubated with copper sulphate in the cell pellet

4.28. The effect of protamine sulphate in OPZ-stimulated PMN on IL-8 level in the cell pellet

4.29. The effect of α1-AT on the level of IL-8 in supernatants and cell pellets of OPZ-stimulated neutrophils treated with tobramycin and copper-tobramycin (0.5 mM)

4.30. The effect of α1-AT (2 mg/ml) on neutrophil viability during 1 hour incubation in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4

4.31. Neutrophil viability during 1 hour incubation with protamine sulphate in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4

4.32. The effect of copper chloride and hydrogen peroxide on ROS generation in HLMVEC

4.33. TNF-α concentration curve of HLMVEC stained for ROS using DCF-DA

4.34. TNF-α time course of ROS generation in HLMVEC

4.35. Z-stack of HLMVEC stained for ROS

4.36. The effect of tobramycin and copper-tobramycin (0.01 - 0.5 mM) on TNF-α-induced ROS generation in HLMVEC

4.37. The effect of tobramycin and copper-tobramycin on reducing TNF-α-induced ROS generation in HLMVEC

4.38. The effect of CFTRinh-172 in the absence and the presence of TNF-α on ROS generation in HLMVEC

4.39. The effect of tobramycin, copper-tobramycin (0.01 – 0.5 mM), NAC (1 – 20 mM) on oxidative stress generated by CFTRinh-172 (20 µM, 30 minutes)

5.1. Platelets enhance leukocyte rolling on the endothelium

5.2. HLMVEC (400 x)

5.3. The outline of a Transwell

5.4. Dose response curve for IL-8-induced neutrophil TEM

5.5. Dose response curve for TNF-α-induced neutrophil TEM
5.6. The effect of activated platelets on neutrophil transendothelial migration on unactivated and TNF-α-activated HLMVEC .......................................................... 199
5.7. TEM of neutrophils induced by platelet ± thrombin (2 U/ml) and LPS (100 µg/ml), n≥3 .................................................................................................................... 200
5.8. IL-8 measurement in TEM of neutrophils induced by thrombin- or LPS-activated platelets with TNF-α-stimulated or unstimulated HLMVEC ................. 201
5.9. PAI-1 activity measurement in TEM of neutrophils induced by thrombin- or LPS-activated with TNF-α-stimulated or unstimulated HLMVEC ................. 202
5.10. The effect of anti-IL-8, anti-NAP-2 and PAF antagonist, WEB2086 on neutrophil TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets ........................................................................................................... 203
5.11. Platelet dose response curve to thrombin, IL-8 .............................................. 204
5.12. Platelet dose response curve to thrombin, NAP-2 ........................................ 205
5.13. Platelet dose response curve to thrombin, RANTES .................................... 206
5.14. The effect of tobramycin pre-treatment for 16 hours of HLMVEC stimulated with 10 ng/ml TNF-α on TEM of neutrophils towards thrombin-activated platelets for 3 hours ........................................................................................................ 208
5.15. IL-8 measurement in supernatants from PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets following tobramycin pre-treatment for 16 hours ........................................................................................................ 209
5.16. Measurement of PAI-1 in PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated in the presence of tobramycin treatment for 16 hours ........................................................................................................ 210
5.17. sICAM-1 measurement in PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets following tobramycin pre-treatment for 16 hours ........................................................................................................ 210
5.18. Copper-tobramycin (0.01 – 0.5 mM) effect on neutrophil TEM in the presence of 1 x 10^8 platelets/ml stimulated with 2 U/ml thrombin across EC stimulated with 10 ng/ml TNF-α ........................................................................................................ 212
5.19. IL-8 concentration in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of copper-tobramycin ........................................................................................................ 213
5.20. PAI-1 concentration in PMN TEM across TNF-α-stimulated HLMVEC
towards thrombin-activated platelets in the presence of copper-tobramycin.............................................................................................................................................................................214

5.21. sICAM-1 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of copper-tobramycin.............................................................................................................................................................................214

5.22. The effect of tobramycin and copper-tobramycin (0.01 – 0.5 mM) on PMN TEM across unstimulated HLMVEC towards IL-8................................................215

5.23. IL-8 measurement in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin.............................................................................................................................................................................216

5.24. Total PAI-1 level in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin.............................................................................................................................................................................217

5.25. Plasmin activity in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin.............................................................................................................................................................................217

5.26. sICAM-1 level of PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin.............................................................................................................................................................................218

5.27. The effect of SOD in the absence and presence of catalase on PMN TEM across HLMVEC stimulated with 10 ng/ml TNF-α towards thrombin-activated platelets.............................................................................................................................................................................219

5.28. The effect of MnTBAP (1 – 50 µM) on neutrophil TEM across TNF-α-stimulated endothelium towards thrombin-activated platelets........................................220

5.29. IL-8 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of MnTBAP...........221

5.30. PAI-1 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of MnTBAP...........222

5.31. sICAM-1 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of MnTBAP...........223

5.32. The effect of copper sulphate on neutrophil TEM towards subendothelial IL-8.............................................................................................................................................................................224

5.33. The effect of copper sulphate on neutrophil TEM on TNF-α-stimulated HLMVEC towards thrombin-activated platelets....................................................224

5.34. XR5118 effect on neutrophil TEM in response to platelets activated with thrombin across stimulated EC..................................................................................225

5.35. The effect of CFTRinh-172 on neutrophil transendothelial migration.............226
5.36. The level of IL-8 in neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in apical supernatants...

5.37. The level of IL-8 in neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in basal supernatants...

5.38. The level of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in apical supernatants...

5.39. The level of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in basal supernatants...

5.40. The level of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in endothelial cell pellets...

5.41. Plasmin activity in neutrophil TEM in the absence and presence of CFTRinh-172 measured in apical supernatants...

5.42. Plasmin activity in neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in basal supernatants...

5.43. The correlation between a percentage of migrated neutrophils and IL-8 level detected in the basal supernatant of PMN TEM...

5.44. The correlation between a percentage of migrated neutrophils and PAI-1 level detected in the basal supernatant of PMN TEM...

5.45. The correlation between IL-8 and PAI-1 level measured in the basal supernatant of PMN TEM...

5.46. Viability of neutrophils in 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS with tobramycin treatment (0.01 – 0.5 mM), n=1, in triplicate...

5.47. Viability of neutrophils in 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS in the presence of copper-tobramycin treatment (0.01 – 0.5 mM), n=3, independent experiments...

5.48. Viability of neutrophils in 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS in the presence of copper sulphate treatment (0.01 – 0.5 mM), n=3, independent experiments...

5.49. The effect of SOD (500 – 2000 U/ml) on viability of neutrophils during 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS, n=3, independent experiments...

5.50. The effect of catalase (250 U/ml) on neutrophil viability during 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in triplicate...

5.51. Viability of neutrophils during 3.5 hours incubation of PMN with...
MnTBAP (1 – 50 µM) in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in triplicate

5.52. Viability of neutrophils after 3.5 hours incubation of PMN with RPMI 1640 2.5 % (v/v) FBS, n=3, independent experiments

5.53. The effect of CFTRinh-172 on viability of neutrophils during 3.5 hours treatment in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in triplicate

5.54. Viability of HLMVEC at 3.5 hours incubation with tobramycin, copper-tobramycin, copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporine-induced apoptosis, n=1, in duplicate

5.55. Viability of HLMVEC at 3.5 hours incubation with SOD (500 – 2000 U/ml) in RPMI 1640 + 2.5 % (v/v) FBS and staurosporine-induced apoptosis, n=1, in duplicate

5.56. Viability of HLMVEC at 3.5 hours incubation with XR5118 (1 – 100 µM) in RPMI 1640 + 2.5 % (v/v) and staurosporine-induced apoptosis, n=1, in duplicate

5.57. Viability of TNF-α-activated (10 ng/ml) HLMVEC at 3.5 hours incubation with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporin-induced apoptosis, n=1, in duplicates

5.58. Viability of TNF-α-activated (10 ng/ml) HLMVEC at 3.5 hours incubation with SOD (500 – 2000 mM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporin-induced apoptosis, n=1, in duplicates

5.59. Viability of TNF-α-activated (10 ng/ml) HLMVEC at 3.5 hours incubation with XR5118 (1 – 100 µM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporin-induced apoptosis, n=1, in duplicates

5.60. Viability (%) of HLMVEC upon 24 hours with TNF-α (10 ng/ml) and CFTRinh-172 (20 µM) in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in duplicates

5.61. Viability (%) of HLMVEC treated with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS for 24 hours, n=1, in duplicates

5.62. Viability (%) of HLMVEC treated with catalase (250 U/ml) and SOD (500 – 2000 U/ml) in RPMI 1640 + 2.5 % (v/v) FBS for 24 hours, n=1, in duplicates

5.63. Viability (%) of HLMVEC for 24 hours in RPMI 1640 + 2.5 % (v/v) FBS after
treatment with MnTBAP (1 – 50 µM), n=1, in duplicates.................................246

6.1. The effect of tobramycin and copper-tobramycin on CFTR in 16HBE 14o- ....263

6.2. Z-stack of the untreated control of 16HBE 14o- with 1 µm intervals..............264

6.3. Z-stack of 16HBE 14o- treated with 0.1 mM tobramycin with 1 µm intervals
.............................................................................................................................................265

6.4. Z-stack of 16HBE 14o- treated with 0.5 mM copper-tobramycin with
1 µm intervals..........................................................................................................................265

6.5. The effect of tobramycin and copper-tobramycin on CFTR in CFBE 41o- ....266

6.6. Z-stack of CFBE 41o- treated with 0.1 mM tobramycin with 1 µm intervals...267

6.7. Intracellular locations of CFTR during maturation, plasma membrane
insertion and degradation..........................................................................................................270
LIST OF TABLES

1.1. Human chemokines
1.2. The instrumental conditions for copper measurement by GF-AAS
1.3. The summary of the copper concentration in different buffers, media and cells expressed as mean ± SEM
1.4. The summary of the pH of 12 mM tobramycin, copper sulphate and copper-tobramycin stock solutions prepared in double deionised water
1.5. The absorbance values of buffer systems at different values
1.6. IL-8 detected in different compounds measured by ELISA
1.7. The effect of different compounds on IL-8 level detected by ELISA
1.8. Adhesion molecules involved in platelet rolling along activated or inflamed endothelium and firm adhesion into inflamed endothelium
1.9. Adhesion molecules involved in leukocyte adhesion to activated platelets
1.10. The effect of tobramycin and copper-tobramycin on TEM of PMN
ABBREVIATIONS

A549 – human lung adenocarcinoma cell line
ADP – adenosine diphosphate
ASL – airway surface liquid
ATOX1 – antioxidant protein 1 homolog (yeast)
ATP – adenosine triphosphate
ATP7A/ATP7B – ATPase, Cu^{2+} transporting, alpha/beta polypeptide
β-TG – β-tromboglobulin
b558 – flavocytochrome
BALF – bronchoalveolar lavage fluid
bFGF – basic fibroblast growth factor
BM – basement membrane
BODIPY - boron dipyrromethene
BSA – bovine serum albumin
C3 – complement component 3
C5a – complement receptor 5a
Calu-3 – airway epithelial cell lines
CAP-37 – cationic antimicrobial protein-37
CAT – catalase
CCR5 – receptor C-C chemokin type 5
CCS – copper chaperone for superoxide dismutase 1
CF – cystic fibrosis
CFBE410+ – transformed human airway epithelial cells, homozygous for ΔF508
CFTR – cystic fibrosis transmembrane conductance regulator
CFTRinh-172 – 3-[(3-Trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone
CG – cathepsin G
COMMD1 – copper metabolism gene MURR1 domains
COPD – chronic obstructive pulmonary disease
CORM – carbon monoxide (CO)-releasing molecule
COX-2 – cyclooxygenase-2
COX17 – cytochrome c oxidase assembly homolog (yeast)
CPS – count per second
CR1 – complement receptor 1
CS – chondroitin sulphate
CTAP-III – connective tissue activating peptide
CTR1 – copper transporter 1
CuCl_{2} – copper chloride
CuSO_{4} – copper sulphate
CXCR1/2 – chemokine (CXC motif) receptor 1/2
ΔF508 – CFTR mutation, loss of phenylalanine at amino acid in position 508
DARC – Duffy antigen/receptor for chemokines
DCF-DA – 2′,7′-dichlorofluorescein diacetate
DMSO – dimethyl sulphoxide
DNA – deoxyribonucleic acid
DOS – deoxystreptamine
DS – dermatan sulphate
ε – molar extinction coefficient
EC – endothelial cell
ECM – extracellular matrix
EDTA – ethylenediaminetetraacetic acid
ELAM-1; CD62 – E-selectin
ELF – epithelial lining fluid
ELISA – enzyme-linked immunosorbent assay
ELR – Glu-Leu-Arg
ENA-78 – neutrophil activating peptide
ENaC – epithelial sodium channel
eNOS – endothelial nitric oxide synthase
ER – endoplasmic reticulum
ERAD – ER-associated degradation
ESAM – endothelial cell-selective adhesion molecule
FBS – fetal bovine serum
FcGR – Immunoglobulin G Fc receptor II
FEV1 – forced expiratory volume in 1 second
fMLP – N-formyl-methionyl-leucyl-phenylalanine
γ-IP-10 – gamma immune pretein-10
γ-MIG – monokine induced by interferon gamma
GA-1000 – gentamycin and amphotericin B
GAG – glycosaminoglycan
GCP-2 – human granulocyte chemotactic protein-2
G-CSF – granulocyte-colony-stimulating factor
GF-AAS – graphite furnace – atomic absorption spectrometry
GlyH-101 – N-(2-naphthalenyl)-[(3,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide
GM-CSF – granulocyte-macrophage colony-stimulating factor
GMP-140; PADGEM – P-selectin
GPCR – G protein coupled receptor
GPx/PGxe – intracellular/extracellular glutathione peroxidase
GR - glutathione reductase
Gro-α/β/γ – growth regulated oncogene
GRXs - glutaredoxins
GSH/GSSG – reduced/oxidized glutathione
5-HETE – 5-hydroxy-eicosatetraenoic acid
5-HPETE – 5-hydroperoxy-eicosatetraenoic acid
16 HBE14o¯ – human bronchial epithelial cells
H2O2 – hydrogen peroxide
HA – hyaluronic acid
HBSS – Hank’s balanced salt solution
HCAEC – human coronary artery endothelial cells
hEGF – human epidermal growth factor
HeLa – human epithelial carcinoma cells
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEPPS – 4-(2-hydroxyethyl)piperazine-1-propanesulphonic acid
HEPPSO – N-(2-hydroxyethyl)piperazine-N’-(2-hydroxy-propanesulphonic acid)
HLMVEC - human lung microvascular endothelial cells
HNO₃ – nitric acid
HOCl – hypochlorous acid
HRP – horseradish peroxidase
HS – hepan sulphate
HSP27 – heat shock protein 27
HSPG – heparin sulphate proteoglycan
HT-29 – human colon epithelial cell lines
HUVEC – human umbilical vein endothelial cells
IC₅₀ – half maximal inhibitory concentration
ICAM-1 – intracellular adhesion molecule 1
ICP-MS – inductively coupled plasma mass spectrometry
IL – interleukin
IFN – interferon
IgG – immunoglobulin G
JAM – junctional adhesion molecule
KᵩₚD, pKᵩₚD – dissociation constant
KS – keratan sulphate
LDH – lactate dehydrogenase
LERs – low expression regions
LFA-1; αLβ2; CD11a/CD18 – lymphocyte-associated function antigen-1
LPS – lipopolysaccharide
LTB₄ – leukotriene B₄
LXA₄ – lipoxin A₄
MAC-1; αMβ2; CD11b/CD18 – macrophage antigen-1
MCP-1/2/3 – monocyte chemotactic protein
MIP-1α/β – macrophage inflammatory proteins
MMP – matrix metalloproteinase
MnTBAP – manganese(III) tetrakis (4-benzoic acid) porphyrin
MnTMPyP – manganese(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride
MPO – myeloperoxidase
m/rRNA – messenger/ribosomal ribonucleic acid
MW – molecular weight
MUC5AC – mucin 5AC, oligomeric mucus/gel-forming
NAC – N-acetylcysteine
NaCl – sodium chloride
NADPH – nicotinamide adenine dinucleotide phosphate
NAP-2 – neutrophil-activating peptide-2
NBD – nucleotide binding domain
NBT – nitro blue tetrazolium
NCI-H292 – mucoepidermoid carcinoma cells from human lungs
NE – neutrophil elastase
NETs – neutrophil extracellular traps
NF-κB – nuclear factor-kappa B
NO – nitric oxide
NSAIDs – nonsteroidal anti-inflammatory drugs
O₂⁻ – superoxide anion
·OH – hydroxyl radical
OD – optical density
OPZ – opsonised zymosan
ORCC – outwardly rectifying chloride channels
PAF – platelet activating factor
PAI-1 – plasminogen activator inhibitor-1
PAR – protease-activated receptor
PBP – platelet basic protein
PBS – phosphate buffered saline
PECAM-1 – platelet endothelial cell adhesion molecule-1
PET – polyethylene terephthalate
PF-4 – platelet factor-4
PFA – paraformaldehyde
PG – proteoglycan
PGE1/2 – prostaglandin E1/2
PMA – phorbol 12-myristate 13-acetate
PMN – polymorphonuclear neutrophil
PMS – phenazine methosulphate
PRXs – peroxiredoxins
PSGL-1, CD162 – P-selectin glycoprotein ligand
R3-IGF-1 – insulin-like growth factor
Rac-1/2 – Ras-related C3 botulinum toxin substrate 1/2
RANTES – Regulated upon Activation, Normal T cell Expressed and Secreted
RBCs – red blood cells
RCL – reactive center loop
ROMK – renal outer medullary K⁺ channel
RONS – reactive nitrogen species
ROS – reactive oxygen species
RT-PCR – real time polymerase chain reaction
SA – sodium azide
SCO1/2 – cytochrome oxidase deficient homolog 1/2
SEM – standard error of the mean
SOD1, Cu/Zn-SOD – copper/zinc-superoxide dismutase
SOD2, Mn-SOD – mitochondrial manganese-superoxide dismutase
SOD3, EC-SOD – extracellular-superoxide dismutase
SLPI – secretory leukoprotease inhibitor
TEM – transendothelial migration
TGF-β1 – transforming growth factor β1
TLR4 – Toll-like receptor 4
TNF-α – tumor necrosis factor-α
TNF-R1 – tumor necrosis factor receptor 1
t-PA – tissue-type plasminogen activator
TPS-1 – thrombospondin-1
TRXs - thioredoxins
u-PA – urokinase-type plasminogen activator
UV – ultraviolet light
VCAM-1 – vascular cell adhesion protein 1
VEGF – vascular endothelial growth factor
VIS – visible light
VLA-4; α4β1 – very late activation antigen 4
vWF – von Willebrand Factor
ZO – zonula occludens
DISSEMINATION

- The 5th European CF Young Investigator Meeting, 23rd – 26th August 2011, Lille, France. The anti-inflammatory effects of tobramycin (oral presentation)
- IBBS Day 19th May 2011, University of Portsmouth. Anti-inflammatory effect of tobramycin and copper-tobramycin in the treatment of cystic fibrosis (oral presentation)
- Breakfast Club 4th March 2011, University of Portsmouth. Tobramycin and copper-tobramycin in the treatment of cystic fibrosis (oral presentation)
- The 24th Annual North American Cystic Fibrosis Conference, 21st – 23rd October 2010, Baltimore, Maryland, USA. A role for platelets in neutrophilic inflammation and the effect of a copper-tobramycin complex (poster presentation)
- IBBS Day 20th May 2010, University of Portsmouth. Investigating copper chelation as an anti-inflammatory strategy in cystic fibrosis (poster presentation)
- Faculty of Science 1st Year PhD Poster Presentation 7th July 2009, University of Portsmouth. Investigating copper chelation as an anti-inflammatory strategy in cystic fibrosis (poster presentation)
CHAPTER 1
GENERAL INTRODUCTION

1.1. PATHOGENIC MECHANISMS OF CYSTIC FIBROSIS

1.1.1. EPIDEMIOLOGY OF CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disorder in the white population, affecting approximately 1 in 2,500 live births. It is also reported in other racial groups, but less frequently. There are over 9,000 people with cystic fibrosis in the United Kingdom (UK CF Registry, 2011; Hamosh et al, 1998). The progress during the past two decades in the diagnosis and treatment of affected individuals has resulted in improved survival (FitzSimmons, 1993). The predicted mean survival for babies born in the 21st century is now over 50 years (Dodge et al, 2007).

1.1.2. THE GENETIC DEFECT

CF is caused by mutations in a single gene, located on the long arm of chromosome 7. In 1989, Kerem et al, Riordan et al and Rommens et al identified this mutation and its protein product called the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of a family of adenosine triphosphate (ATP)-binding cassette transport proteins (Hyde, 1990). It contains two nucleotide-binding domains that bind and hydrolyze ATP, two dual sets of membrane-spanning domains that form the channel, and a central regulatory (R) domain. The R domain, unique to CFTR, is highly charged with numerous phosphorylation sites for protein kinases A or C (Figure 1.1, over) (Chang et al, 1994). Studies have established that the CFTR functions not only as a chloride ion channel but is also a regulator of outwardly rectifying chloride channels (ORCC) (Schwiebert et al, 1995), sodium channels (Stutts et al, 1995), and renal outer medullary K+ channel (ROMK) or other inwardly rectifying K+ channels (McNicholas et al, 1997). Moreover, it transports ATP (Schwiebert et al, 1995), bicarbonate (Tang et al, 2009) and GSH (Linsdell & Hanrahan, 1998).
CFTR-mediated Cl⁻ secretion and epithelial sodium channel (ENaC)-mediated Na⁺ absorption are coordinated to maintain airway surface liquid (ASL) at a depth allowing cilia to beat effectively and move the mucus across the epithelial cell surface. Two defects in this process arise from the absence of CFTR protein and/or its function in CF airway epithelium. Firstly, the decreased permeability of CF epithelia results in reduced Cl⁻ secretion and therefore less salt on the apical surface. Consequently, a reduced level of water enters the lumen. Secondly, in the absence of CFTR negative regulation, ENaC activity is removed, which results in an unregulated and inappropriate absorption of salt, and therefore water from ASL. The overlying mucus consequently becomes dehydrated and it is followed by the arrest of mucociliary clearance (Figure 1.2, over) (Clunes & Boucher, 2007).

Over 1800 different mutations of the CFTR gene have been reported to the CF Genetic Analysis Consortium database (www.genet.sickkids.on.ca/cftr/).
Figure 1.2. Normal and CF airway, respectively. Defects in CFTR cause reduced chloride secretion into airways and enhanced sodium reabsorption, resulting in dehydrated airway secretions and mucostasis with accumulation of mucus plugs (Zhang et al, 2009).

The various mutations can be grouped into six distinct classes (I-VI) that include the absence of synthesis (class I), defective protein maturation and premature degradation (class II), disordered regulation, including diminished ATP binding and hydrolysis (class III), defective chloride conductance or channel gating (class IV), a reduced number of CFTR transcripts due to a promoter or splicing abnormality (class V) and accelerated turnover from the cell surface (class VI) (Figure 1.3, over) (Rowe et al, 2005). Different mutations determine varying effects on CFTR function resulting in different phenotypes of this disease (Tsui & Durie, 1992). The most common mutation is deletion of three base pairs, which determines the loss of phenylalanine at amino acid position 508 (ΔF508). It occurs in 91.5% CF patients in the UK (UK CF Registry, 2011).

The disease manifests by abnormalities in exocrine gland functions resulting in altered ion composition and increased viscosity of epithelial secretions in the sweat ducts, salivary glands, small intestine, pancreatic exocrine gland ducts, biliary tract, vas deferens and respiratory tract. Although CF is a multi-organ disease, the major cause of morbidity and mortality in CF is due to pulmonary disease (Welsh, 1995).
Figure 1.3. Classification of the CFTR mutations. Six classes of CF-related gene mutations are compared to the normal CFTR maturation pathway. A wild-type CFTR is transcribed into messenger RNA (mRNA) followed by posttranslational modifications including folding, glycosylation and trafficking via the Golgi apparatus to the cell membrane, where it functions as a chloride channel. Class I mutations, such as G542X, contain premature stop mutations that generate truncated mRNA. Class II mutations, with the most common ΔF508, are misfolded and unable to proceed to the endoplasmic reticulum, they are then ubiquitinated and degraded. Class III mutations, exemplified by G551D, reach the cell membrane but the channel is not properly activated. Class IV mutations, such as R347P, produce fully activated cell surface channels but with decreased chloride conductance. Class V mutations result in reduced abundance of CFTR, like incorrect splicing with the mutation 3849 + 10 kb C→T. Class VI mutation is characterised by lowered stability of functional but unstable CFTR in the apical membrane (Rowe et al, 2005).

1.1.3. PULMONARY INFECTION

The CF lung is normal at birth and during the first months of life (Sturgess & Imrie, 1982). However, shortly after birth, many patients become infected with bacteria and develop airway inflammation. Infections with *Staphylococcus aureus, Haemophilus*
*influenza* and *Streptococcus pneumoniae* predominate in younger patients, while the onset of chronic *Pseudomonas aeruginosa* infection is delayed (Koch & Hoiby, 1993). Chronic *P. aeruginosa* infection is closely associated with progressive pulmonary deterioration in most CF patients.

Several hypotheses have been proposed to explain CF lung susceptibility to infection with *P. aeruginosa*. Some investigators suggest that the elevated salt level in the CF ASL renders human β-defensin-1 nonfunctional, eliminating the bacterial activity of the respiratory epithelium (Goldman *et al*, 1997). A second hypothesis claims a failure of the respiratory epithelial cells in the CF lungs to ingest bacteria. Indeed, CFTR has been proposed to function as a receptor increasing clearance of *P. aeruginosa*. Lack of CFTR could directly impair host defense allowing for *P. aeruginosa* retention at the endobronchial surface (Schroeder *et al*, 2002; Pier *et al*, 1996). A third possibility is that *Pseudomonas* abundantly adheres to epithelial cells in the CF airway due to the abnormal surface properties of the cells, thus leading to infection. *P. aeruginosa* pilin, the protein responsible for *P. aeruginosa* binding, recognizes and binds to the cell surface glycolipid asialo ganglioside M1. Asialo ganglioside M1 is present at high levels in CF airway epithelial cells as a consequence of CFTR dysfunction (Saiman & Prince, 1993; Saiman *et al*, 1992).

*P. aeruginosa* isolated from the lower respiratory tract is commonly initially non-mucoid. Persistence of this organism usually coincides with increased expression of bacterial genes coding for production of alginate (uronic acid, which is a polymer of mannuronic acid and guluronic acid) under hypoxic conditions. *P. aeruginosa* grow in mucoid macrocolonies, embedded in a biofilm of alginate, which protects the organism from neutrophils and antibiotics and contributes to the establishment of chronic infection (Pedersen, 1992). Most of the recurrent episodes of worsening pulmonary symptoms termed exacerbations are due to bacterial infections (Hoiby, 1982). Beside *P. aeruginosa* identified in the sputa of the majority of CF patients, other typical CF organisms include *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia*. The late stages of lung disease may be accompanied by the presence of less common organisms such as *Stenotrophomonas maltophilia*, *Alcaligenes xiloxidants*, *Mycobacterium avium* complex or *Mycobacterium abscessus* (Coutinho *et al*, 2008). Acute infection caused by chlamydia and mycoplasma enhance progression of
bacterial infection (Petersen et al, 1981). Also, virus infection is now implicated in exacerbation and increased susceptibility to bacteria in CF due to increased production of pro-inflammatory cytokines by epithelial cells compared to control or deficiency in antiviral innate immune responses in CF cells (Singanayagam et al, 2012).

Pulmonary infection rarely spreads outside the respiratory tract in CF patients (Hoiby, 1982). However, the inflammatory response fails to eradicate it from the airways, eventually causing irreversible damage to the airways and lung parenchyma (reviewed by De Rose, 2002).

1.1.4. PULMONARY INFLAMMATION

CF is characterized by bronchial inflammation leading to bronchial wall thickening, bronchiectasis and lung tissue fibrosis. However, alveolar inflammation with remodeling has been also described in end-stage CF lungs (Ulrich et al, 2010). Pulmonary disease in patients with CF is dominated by an excessive neutrophilic inflammatory response to infection of the airways. Polymorphonuclear neutrophils (PMNs) were preferentially detected in the CF surface epithelium, where the epithelial damage is frequently described. A significantly increased T cell population was observed in the CF bronchiolar epithelium and parenchyma together with intense morphological alterations. A high number of B cells in CF lung samples were also found, suggesting interactions between T and B cells and their activation, leading to distal tissue remodelling. Plasma cells, macrophages and mast cells were located in the lamina propria and not significantly increased in CF compared to non-CF tissues (Hubeau et al, 2001). Additionally, the study of O’Sullivan et al (2006) established increased number of blood circulating platelets.

The presence of microorganisms activates epithelial and macrophage synthesis and release of several cytokines (Bonfield et al, 1995). Both LPS and N-formyl-methionyl-leucyl-phenylalanine (fMLP) synergistically induce the inflammatory response via multiple signaling pathways in vivo and in vitro. For instance, TLR4 and nuclear transcription factor-kappa B (NF-κB) signaling pathways are involved in the synergistic induction of pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) (Chen et al, 2009).
Cells migrating within tissues can navigate through complex arrays of chemoattractants in a sequential manner (Foxman et al., 1999). In a consequence, PMNs migrate sequentially through the endothelial cell (EC) layer into the interstitial space followed by a transepithelial migration into the alveolar air space (Reutershan et al., 2005).

IL-8 is the major neutrophil chemoattractant in the CF airways. Noah et al. (1997) have shown that IL-8 levels are markedly increased in children with CF compared with non-CF children with the same level of bacterial infection of the lower airways. The concentration of IL-8 is increased in the sputum, bronchoalveolar lavage fluid (BALF), and serum of CF patients, even during stable clinical conditions and the IL-8 concentration in BALF positively correlates with the percentage of neutrophils in BALF (Dean et al., 1993).

IL-8 binds with high affinity to two CXC chemokine receptors on PMNs, CXCR1 and CXCR2 (Lee et al., 1992), but neutrophils in the blood also respond to a concentration gradient of IL-8 bound to EC surfaces (Rot, 1992). In vitro studies have shown that glycosaminoglycan (GAG) side chains of proteoglycans (PGs) acts as IL-8 binding sites on human EC. In particular, the heparan sulphate (HS) side chains associated with syndecan-1, -2 and -4 bind IL-8 and are obligatory for successful migration of normal human neutrophils across the endothelium (Halden et al., 2004, Marshall et al., 2003, Kaneider et al., 2002). Binding to the GAGs may protect IL-8 from elastase-mediated degradation and prolong its half-life in the inflamed lung (Hoogewerf et al., 1997). Expression of HSPGs on the endothelium in bronchial tissue from patients with CF is increased compared to non-CF controls (Solic et al., 2005). Plasminogen activator inhibitor-1 (PAI-1), which stabilizes endothelial gradients of IL-8 and enhances cell migration was also reported to be increased in CF sputa and correlated negatively with pulmonary function (Xiao et al., 2005; Marshall et al., 2003). Previous studies demonstrated that chemokines, including IL-8, form dimers and higher order oligomers when bound to HS on EC surfaces, which increase the local concentration of chemokines for presentation to specific receptors on neutrophils in the circulation (Hoogewerf et al., 1997). It is also possible that tissue-derived IL-8 binds to the Duffy antigen on the endothelial cells of postcapillary venules and indicates a way for the chemokine transfer to the luminal surface of the endothelium. Indeed, Duffy protein
may be a necessary EC component for controlled leukocyte migration during inflammation. It was shown that in cultured human endothelial cells, Duffy protein can be induced by TNF-α and in the mouse by LPS (Chaudhuri et al, 2003).

Other neutrophil chemoattractants, such as leukotriene B4 (LTB4), fMLP, and platelet activating factor (PAF) induced neutrophil migration through not only epithelial, but also endothelial cells in a dose-dependent fashion (Casale et al, 1992). Lipoxygenase products, 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) or 5-hydroxy-eicosatetraenoic acid (5-HETE) beside LTB4, were shown to be chemotactic for neutrophils (Nagy et al, 1982). Additionally, epithelial cell neutrophil activating peptide-78 (ENA-78), previously thought to be exclusively a product of epithelium, is expressed by endothelium in inflamed human lung and other tissues as well as by cultured ECs. ENA-78 acts in concert with IL-8 to induce PMN adhesiveness to ECs (Imaizumi et al, 1997). Another chemokine, neutrophil-activating peptide-2 (NAP-2) displays 53 % identity with ENA-78. ENA-78 and NAP-2 bind to CXCR1 with low affinity, whereas CXCR2 binds all CXC chemokines with high affinity (Ahuja & Murphy, 1996).

Significantly increased concentrations of other pro-inflammatory cytokines, namely IL-1, TNF-α, IL-6, IL-17 and IL-22 have been measured in CF sputum and BALF, and are known to extend airway inflammation (Aujla et al, 2008; Bonfield et al, 1995; McAllister et al, 1995). The synthesis of these cytokines is via activation of NF-κB by cellular interaction with bacterial LPS and proinflammatory cytokines, such as IL-1 or TNF-α. CF BALF was demonstrated to be deficient in the anti-inflammatory cytokine IL-10, which presumably contributes to the excessive inflammatory response in CF compared to normal (Berger, 2002). Interestingly, it has been noted that the CFTR protein downregulates the expression of Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES) chemokine, in contrast to IL-8 and monocyte chemoattractant-1 (MCP-1). Moreover, the absence of RANTES expression in CF may determine a dysregulated chemotaxis of lymphocytes into the CF lung (Schweibert et al, 1999). In contrast, a recent study by Scapa et al (2011) presented upregulation of RANTES in nasal polyps from patients with CF.

High levels of chemoattractants and pro-inflammatory cytokines are a prerequisite for activated neutrophils to continue to accumulate in the airways. Neutrophils in sputum
release massive amounts of active proteases, mainly elastase, which overhelm local antiprotease defences including α1-antitrypsin (α1-AT), normally found in the blood, and secretory leukoprotease inhibitor (SLPI), locally made by epithelium. As CF has been characterized by defective neutrophil apoptosis (Moriceau et al, 2010), necrosing neutrophils are the major source of long-stranded deoxyribonucleic acid (DNA), which is responsible for the high viscosity of CF sputum and further impairs mucociliary clearance (Berger, 2002). Moreover, senescent and dead neutrophils were reported to release DNA, which compose neutrophil extracellular traps (NETs) binding the active serine proteases in CF sputum (Dubois et al, 2012).

Neutrophil elastase (NE) directly contributes to tissue damage by degrading structural proteins, such as elastin, collagen type I – IV, fibronectin and proteoglycans (Janoff et al, 1979). Elastase can cause prolongation of the inflammatory process by degrading complement component C3 and releasing C5a, a potent chemoattractant for neutrophils (Fick et al, 1986). Neutrophil recruitment may be further augmented by the effect of elastase on the epithelium to synthesise and secrete IL-8 (Nakamura et al, 1992). NE can cause a reduced ciliary beat frequency of the respiratory epithelium and directly damage the epithelial cells (Amitani et al, 1991). As elastase is a potent stimulator of airway gland serous cells, bacterial colonisation could be facilitated by excessive mucus production (Sommerhoff et al, 1990). Elastase may inactivate several components of the immune system (immunoglobulins, immune complexes, complement components and neutrophil cell surface receptors), thus interfering with the ability of neutrophils to opsonise and eliminate bacterial pathogens. The augmented amount of free elastase in the airways of CF patients is also related to inactivation of the two main physiological protease inhibitors, α1-AT and SLPI (Birrer et al, 1994; Berger et al, 1989).

The effect of the exaggerated neutrophil-dominated inflammation is to generate a chronic burden of reactive oxygen species (ROS) in the respiratory tract of CF patients. High sputum levels of extracellular myeloperoxidase (MPO), a PMN-derived enzyme, which converts hydrogen peroxide into HOCl, have been reported in CF patients and inversely correlated with lung function (Witko-Sarsat et al, 1994). CF patients with an acute pulmonary exacerbation were shown to have abnormally high concentrations of H₂O₂ in exhaled air, which decrease during intravenous antibiotic treatment (Jobsis et
Higher concentrations of oxidation products have also been detected in the plasma of CF patients in comparison to control subjects (Brown & Kelly, 1994). Moreover, levels of glutathione, the major local lung antioxidant, are reduced in the epithelial lining fluid as well as in the plasma of CF patients as a result of defective CFTR. Its deficiency predisposes patients with CF to oxidative tissue damage (Roum et al, 1993).

The inflammatory response to the infection in CF is excessive and persistent and sets the stage for a vicious cycle of airway obstruction, infection and inflammation that ultimately leads to lung damage and the progression of CF lung disease (Figure 1.4) (Konstan & Berger, 1997). However, the airway remodeling might be not only a consequence of repeated cycles of infection and inflammation, but also changes specific to CF, resulting from CFTR, or a protective responses to both of them. Indeed, CF airways have been characterized with the opposite structural changes in airway remodeling leading to narrowing of the small airways. Bronchiectasis, which is commonly defined as local and irreversible bronchial dilation by degradation of the bronchial tissue matrix have been observed together with widespread fibrotic changes described, especially in end-stage CF (Regamey et al, 2011).

Figure 1.4. The excessive inflammatory response in the cystic fibrosis (CF) lung. A basic genetic defect leads to airway infection and uncontrolled inflammatory response, which both contribute to a persistent polymorphonuclear neutrophil (PMN) influx. This influx is maintained by chemoattractants released by PMNs themselves and interleukin (IL)-8 production induced by high quantity of elastase in the airways. PMNs release reactive oxygen species and proteolytic enzymes, which cause structural damage. The accelerated protease burden also impairs phagocytosis, facilitating the persistence of infection. Hence, a self-perpetuating vicious circle of infection and inflammation is established, leading to irreversible lung damage. LTB₄: leukotriene B₄; DNA: deoxyribonucleic acid (De Rose, 2002).
1.1.5. RESOLUTION OF INFLAMMATION

Several cellular and molecular mechanisms terminate the acute inflammatory response. During acute inflammation, after entering the tissues, early-phase arachidonic acid-derived prostaglandin, PGE2 switches PMNs biosynthesis from leukotriene, LTB4 to lipoxin, LXA4, which promoted resolution (Levy et al., 2001). In airway epithelial cells low production of LXA4 has been reported in the airway of CF, which could explain the persistent inflammation in this severe airway disease (Karp et al., 2004). These events coincide with the biosynthesis, from omega-3 polyunsaturated fatty acids, of resolvins and protectins, which shorten the period of neutrophil infiltration by initiating apoptosis (Bannenberg et al., 2005).

Neutrophil recruitment is therefore stopped and removal of infiltrated inflammatory cells is engaged, and plays a central role in resolution of inflammation. Inflammatory cells undergo apoptosis and efferocytosis (engulfment of apoptotic cells), which is likely to represent one of the most primitive forms of phagocytosis (Vandivier et al., 2006, deCathelineau & Henson, 2003). Indeed, a distinct macrophage subpopulation, termed fibroblastic macrophages controls the dynamics of inflammation via phagocytic clearance of leukocytes (Hou et al., 2010). However, reduced apoptosis of a number of cells, including PMNs (Moriceau et al., 2010) in the airways of CF patients was reported and related with the presence of chronic infection rather than CFTR dysfunction.

However, neutrophils are eliminated even more efficiently in the transepithelial pathway, from the apical membrane of mucosal epithelium into the lumen by induction of epithelial CD55 (Louis et al., 2005). However, neutrophils in severe chronic obstructive pulmonary disease (COPD) exhibit reduced chemotaxis compared to neutrophils in mild COPD to the increased number of neutrophil chemoattractants in bronchial mucosal tissues. It results in increased attraction and retention of neutrophils in the airway wall, and therefore modulates transepithelial egression of these cells (reviewed by Persson & Uller, 2011).

Also, pro-resolution pathways may stimulate the release of anti-inflammatory mediators, such as IL-10 (Souza et al., 2007) and transforming growth factor-β1 (TGF-β1) (Bannenberg et al., 2005). IL-10 deficiency in CF is well reported (Berger, 2002). In
addition, TGF-β1-mediated signaling in CF epithelium is altered due to a CF-related reduction of protein levels of TGF-β1 signalling molecule Smad-3 (Kelly et al, 2001).

1.2. CELLULAR CONTRIBUTION TO AIRWAY INFLAMMATION IN CF

As mentioned in Section 1.1.4, the number of PMNs in the airways, T cells in the tissue (Hubeau et al, 2001) and platelets in the blood (O’Sullivan et al, 2006) is raised in CF.

1.2.1. NEUTROPHILS

Neutrophils account for 40 – 65 % of white blood cells and are found in the blood at concentrations of 3 – 5 x 10^6 cell/ml. This number can increase up to tenfold during infections. Neutrophils have a relatively short life-time in the circulation (approximately 8 – 20 hours), but this may be extended to up to several days if the cells leave the circulation and enter tissues. In the circulation, they are spherical (about 10 µm in diameter) with few cytoplasmic extrusions (Edwards, 1994).

Neutrophils play a crucial role in the immune system, acting as the first line of defense against bacterial and fungal infections. These cells use oxygen-dependent and -independent mechanisms to destroy and remove infectious agents. Oxygen-dependent mechanisms involve the intracellular production of ROS, which can be microbiocidal (Roos et al, 2003) and oxygen-independent mechanisms, which include the use of lytic enzymes and bactericidal peptides (reviewed by Witko-Sarsat et al, 2000).

The generation of microbiocidal oxidants by neutrophils results from the activation of a multiprotein enzyme complex known as the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyses the formation of superoxide anion (O_2^-). O_2^- may further dismutate spontaneously to form hydrogen peroxide (H_2O_2) (Figure 1.5). O_2^- and H_2O_2 can also undergo further reactions to form other ROS. For example, MPO in presence of Cl^- converts H_2O_2 to HOCl and further to chloramines.

\[
\begin{align*}
(1) \quad & \text{NAD(P)H} + 2O_2 \rightarrow \text{NAD(P)}^+ + H^+ + 2O_2^- \\
(2) \quad & 2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2
\end{align*}
\]

Figure 1.5. The action of NADPH oxidase (1) and further dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) (2).
Together these reduced oxygen products play an essential role in the killing of ingested microorganisms (Badwey & Karnowsky, 1980; Babior, 1978).

Preformed and stored in vesicles, granule proteins of PMNs can be released on demand during the inflammatory response. Different granule subsets with specialized functions can be distinguished (Borregaard & Cowland, 1997). Secretory vesicles have the highest tendency to release their contents, which are delivered to the endothelium during the initial interactions of the PMN with the vessel wall. Tertiary granules are released during PMN transmigration across the EC layer. Indeed, proteases such as MMP-9 allow the PMN to cross the basement membrane (BM) more effectively. Primary (azurophilic) and secondary (specific) granule contents are released in the extravascular space. Antimicrobial polypeptides in these compartments are important in bacterial clearance by direct antimicrobial activity, bacterial opsonisation and macrophage activation (Figure 1.6) (Soehnlein et al, 2008; Levy, 2004; Fleischmann et al, 1985).

Figure 1.6. Neutrophil effector mechanisms in the inflammatory process. These mechanisms are mobilized following phagocytosis of a pathogen. Complement opsonins C3b and C4b are recognized by CR1 and CR3, while IgG opsonins are recognized via the immunoglobulin receptors (FcγR). The first microbicidal pathway is oxygen-dependent and consists of the production of radical oxygen species following NADPH-oxidase complex activation, including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and, via myeloperoxidase, hypochlorous acid (HOCl) and chloramines. The second microbicidal pathway is does not depend on oxygen, but on the fusion of granules containing preformed proteins with the phagolysosome or release the granules to the extracellular medium. Serine proteases, antibiotic proteins and myeloperoxidase are stored in azurophilic granules. Specific granules contain metalloproteinases (collagenase and gelatinase) and antimicrobial proteins (lactoferrin and cathelicidin). Gelatinase is contained in tertiary granules, also called gelatinase granules (Witko-Sarsat et al, 2000).
A novel antimicrobial mechanism of neutrophils has been described by Brinkman et al (2004). Neutrophil extracellular traps (NETs) were observed as a highly decondensed chromatin structure that is formed and released by activated neutrophils. NETs are composed of nuclear DNA with histones, proteases (e.g. elastase) and AMPs. These structures bind Gram-positive and Gram-negative bacteria, as well as fungi (Urban et al, 2006). A variety of different proinflammatory stimuli have been shown to activate the formation of NETs, including H₂O₂, LPS, the mitogen PMA and IL-8. This active process depends on the generation of ROS by NADPH oxidase. During infection, ROS formation may contribute to intraphagosomal killing in live neutrophils or NET-mediated killing post mortem (Fuchs et al, 2007). However, mitochondrial, not nuclear DNA release by neutrophils and NET formation do not require neutrophil death and also do not limit the lifespan of these cells (Yousefi et al, 2009).

1.2.2. ENDOTHELIAL CELLS

An endothelium lines the entire vascular system and is composed of a monolayer of endothelial cells. In an adult, the endothelium consists of more than 1 × 10¹² cells and covers a surface area of more than 1000 m² (Jaffe, 1987). The maintenance by the endothelium of a semi-permeable barrier is particularly important in controlling the passage of macromolecules and fluid between the blood and interstitial space. The glycocalyx, a negatively charged surface coat of proteoglycans, glycosaminoglycans and adsorbed plasma proteins lining the luminal surface of the endothelium, contributes to this function (Pries et al, 2000).

Endothelial cells form a heterogenous cell population that varies not only in different organs but also in vessels of different calibres within an organ (Bicknell, 1993). Functional studies on in vitro models of lung macro- and micro-vascular ECs show that they exhibit unique characteristics. Lung microvascular endothelial cells possess a more restrictive barrier than their macrovascular counterparts and exhibit unique signalling responses to TNF-α and other agonists (Kelly et al, 1998). Well-defined site specific vascular responses are observed in the intact lung (Chetham et al, 1999). The pulmonary microcirculation is less permeable to protein and water flux as compared to large pulmonary vessels (Parker & Yoshikawa, 2002). In contrast, macrovascular
endothelial cells express more endothelial nitric oxide synthase (eNOS) and generate more nitric oxide than do microvascular ECs (Stevens et al, 2001; Geiger et al, 1997).

The vascular endothelium is versatile and multifunctional, having many synthetic and metabolic properties. Under basal conditions ECs are broadly involved in maintaining the nonthrombogenic blood–tissue barrier by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow. They produce and release many vasoactive substances, such as prostacyclin and nitric oxide, which inhibit platelet aggregation and cause vasodilation. These mediators are released in response to a range of chemical stimuli (thrombin, bradykinin, ADP), as well as changes in haemodynamic forces (changes in blood pressure or flow) (Sumpio et al, 2002).

A critical balance between endothelium-derived relaxing and contracting factors maintains vascular homeostasis. A disrupted balance predisposes the vasculature to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation, vascular inflammation, and atherosclerosis (Pober et al, 2009; Arnout et al, 2006).

The endothelium plays a key role in immune and inflammatory responses by regulating leukocyte, monocyte and lymphocyte movement into tissues. Pulmonary endothelium modulates leukocyte function by the expression of adhesion molecules, such as P-selectin (Bonfanti et al, 1989) and by release of soluble factors including prostacyclin (Weksler et al, 1977) and PAF (Bjork et al, 1983). Vascular endothelial cells possess unique and specialised storage vesicles, called Weibel-Palade bodies that contain a number of proteins important in haemostasis and inflammation. As well as P-selectin, these secretory granules contain: von Willebrand Factor (vWF) (Wagner et al, 1982), CD63 (Vischer & Wagner, 1993), endothelin (Russel et al, 1998), IL-8 (Utgaard et al, 1998; Wolff et al, 1998), t-PA (Huber et al, 2002) and PAI-1 (Xiao et al, 2005).

Vascular endothelium can be affected by a persistent inflammatory state, which can be secondary to chronic pulmonary infection, characterised in CF. Romano et al (2001) found circulating levels of vWF, t-PA, P-selectin, TNF-α and IL-6 to be significantly higher in CF patients. Those results indicate that CF patients exhibit signs of endothelial dysfunction/perturbation, which are likely to be related to a persistent inflammatory state due to chronic pulmonary infection and may play a role in the
progression of the disease. Others have suggested that changes in EC in CF are the cause of the increased inflammation (Solic et al, 2005).

1.2.3. PLATELETS

Platelets are anucleate fragments of bone marrow megakaryocytes of approximately 1 – 2 µm in diameter (Dopheide et al, 2002). Daily production of 2.5 x 10^{11} cells with a median life span of 7 – 9 days constitutes a total platelet pool of 2 x 10^{12} cells in human adults. Platelets contain at least three types of morphologically different granules: α-granules, dense granule and lysosomes. The most abundant α-granules contain platelet-derived growth factor, PF-4, coagulation factors V & XIII and fibrinogen. Dense bodies contain serotonin, nucleotides (ADP) and calcium, while lysosomes contain hydrolytic enzymes (acid hydrolases). α-granules comprise roughly 10 % of the platelet volume, which is approximately equal to the surface of the open canalicular system, an elaborate system of tunnelling invaginations of the cell membrane unique to the platelet (Frojmovic & Milton, 1982; White & Clawson, 1980) (Figure 1.7).

![Figure 1.7. The structure of a platelet (Semple, Italiano & Freedman, 2011).](image)

Platelets were shown to be a linking element between haemostasis, inflammation and tissue repair (O’Sullivan & Michelson, 2006). Their physiological role in haemostasis is determined by their ability to change shape, aggregate, and release the contents of secretory granules in response to specific agonists. Thrombin, epinephrine, adenosine
diphosphate (ADP), thromboxane A2 (TXA2), PAF, collagen and other extracellular matrix components in the subendothelium are agonists, known to activate platelets (Johnson, 1999). Platelets can be also activated by antigens, antigen–antibody complexes, microorganisms, and bacterial endotoxins, like LPS from P. aeruginosa. Release of mediators stored in platelet granules and de novo platelet synthesis of other mediators including cytokines/chemokines, enhance the inflammatory response.

Platelet-derived histamine and serotonin contribute to increased vascular permeability; ADP augments the agonist-induced oxidative burst in PMNs; platelet-derived growth factor stimulates chemotaxis for monocytes and primes eosinophils to produce superoxide anion; PF-4 predispose PMNs to adhere to unstimulated vascular endothelium, stimulates the release of histamine from basophils, and induces the adherence of eosinophils to vascular walls. Platelet basic protein (PBP) and CTAP III, the predominant β-TG variants are secreted by activated platelets. They are inactive precursors of NAP-2, which stimulates neutrophil functions, such as chemotaxis, adherence and degranulation of lysosomal enzymes. Furthermore, platelets release RANTES, which supports the recruitment and adhesion of monocytes and T cells to activated endothelium (O’Sullivan & Michelson, 2006).

Once activated, platelets mediate homeostasis and thrombosis by adhering to the vascular wall, recruiting circulating platelets and aggregating to form a thrombus, which seals the damaged blood vessel (Johnson, 1999).

Previous studies demonstrated that CF patients have an increased number of circulating platelets, increased platelet activation in response to gram-negative infection, and increased release of platelet-derived mediators, many of which can have negative pulmonary consequences (O’Sullivan & Michelson, 2006). Previous studies have also shown that CF patients have increased ex vivo platelet aggregability, increased release of TXA2, and a blunted response to PGE1-induced inhibition of platelet aggregation (O’Sullivan et al, 2005). These studies suggest a link between platelet activation and progressive impairment of CF lung function.

Given their multiple significant roles in inflammation, platelets are a potential therapeutic target in patients with CF (O’Sullivan & Michelson, 2006). Platelets also contain micromolar concentrations of copper (Lind et al, 1993).
1.3. LEUKOCYTE RECRUITMENT

Under both normal and pathologic conditions, circulating leukocytes migrate from vessels into tissues. In the systemic microcirculation, PMN influx from blood into tissue at sites of inflammation, which typically occurs in post-capillary venules, present in non-lymphoid tissues. Post capillary venules in secondary lymphoid tissue, called high endothelial venules, allow only lymphocytes to migrate into the lymph node (Cavender, 1990; Colditz, 1985).

Leukocyte recruitment across the endothelium requires a multistep cascade of adhesive and migratory events, which are mediated by three classes of adhesion receptors, selectins, integrins and adhesion receptors of the immunoglobulin superfamily. These steps are: the initial selectin-mediated rolling, chemokine-induced activation and integrin and adhesion molecule-dependent adherence and subsequent transendothelial migration (Figure 1.8) (Ley et al, 2007; Springer, 1994).

![Diagram of leukocyte recruitment](image)

Figure 1.8. Multistep process of leukocyte recruitment from the circulation into inflammatory sites. Following initial selectin-mediated capture, adhesive interactions between leukocyte and activated endothelium are required for transendothelium migration of leukocytes into inflammatory site (Ninichuk & Anders, 2008).
Rolling interactions are reversible as they are mediated by weak binding between transmembrane glycoprotein adhesive molecules called selectins with P-selectin glycoprotein ligand (PSGL-1, CD162) and other glycosylated ligands. Selectins are expressed by most leukocytes (L-selectin) and inflamed endothelium (E- and P-selectin) (McEver, 2002; Bevilacqua & Nelson, 1993). PMNs newly released from bone marrow demonstrate higher constitutive expression of L-selectin compared with older, circulating PMNs (Matsuba et al., 1997). The activity of metalloprotease is partly responsible for the loss or shedding of L-selectin from the surface of PMN. It results in active accumulation of L-selectin in the blood (Kishimoto et al., 1995). However, L-selectin autoproteolysis can also occur after exposure to inflammatory stimuli, such as LPS and TNF-α or come from physiological rolling interactions with the vessel wall (Walchek et al., 1996). In turn, P-selectin (granule membrane protein-140, CD62P) is accumulated in platelet α-granules as well as endothelial Weibel-Palade bodies (Malik & Lo, 1996). Within minutes of exposure to ECs, inflammatory mediators, including complement products, ROS or various cytokines, P-selectin is mobilized to the cell surface, where it may interact with its PMN counterpart, PSGL-1. P-selectin on activated ECs can also bind PSGL-1 on monocytes and platelets (McEver & Cummings, 1997). E-selectin (ELAM-1, CD62) is not stored but its expression and activity on ECs is induced in vitro by inflammatory cytokines (Klein et al., 1995). E-selectin mediates slow and more stable rolling (Smith et al., 2004).

After injury or infection, inflammatory cytokines activate the endothelium to express adhesion molecules and synthesize chemokines and lipid chemoattractants from both luminal and basolateral surfaces (Middleton et al., 1997). Chemoattractants are also generated in activated mast cells (IL-1β, IL-4, TNF-α) and platelets (NAP-2, RANTES, ENA-78) and delivered to ECs through exocytosis of intracellular granules or circulating microparticles. Chemokines can oligomerize and interact with specific, high affinity receptors, G protein coupled receptors (GPCRs) that modulate their functions. Many chemokines can also bind to GAGs on the EC surface, which is crucial for efficient leukocyte recruitment and protection from proteolytic cleavage (Johnson et al., 2005). During chemokine-induced activation, the low-affinity, selectin-mediated interaction are converted into the high-affinity, integrin-mediated adhesion (Ley et al., 2007).
Integrins are heterodimeric transmembrane glycoproteins present on PMNs and other blood cells and mediate cell-cell and cell-extracellular matrix adhesions. PMN binding to activated endothelium is mediated mainly by two β2-integrins: macrophage antigen-1 (MAC-1; αMβ2; CD11b/CD18) and lymphocyte-associated function antigen-1 (LFA-1; αLβ2; CD11a/CD18) and a β1-integrin, very late activation antigen 4 (VLA-4; α4β1) (Luscinskas & Lawler, 1994). LFA-1 was identified as the essential neutrophil integrin during transendothelial migration. Indeed, LFA-1 was shown to be sufficient in mediating neutrophil emigration in MAC-1-deficient mice (Lu et al, 1997). However, it also contributes to neutrophil rolling by stabilizing the transient attachment or tethering phase of rolling (Henderson et al, 2001). MAC-1 is exclusive to granulocytes and monocytes (Thylen et al, 1992). Already assembled MAC-1 is stored in PMN vesicles (Borregaard & Cowland, 1997) and can be rapidly mobilized to the PMN surface after exposure to TNF-α and bacterial LPS or fMLP (Altieri & Edgington, 1988).

Inflammatory stimuli can also promote transcription and translation of MAC-1 genes, in order to prolong integrin involvement during inflammation (Wagner & Roth, 2000). In contrast, VLA-4 is expressed on most leukocytes and has been shown to mediate attachment to ECM proteins and to function as a ligand for the endothelial VCAM-1 (Elices et al, 1990).

Although, 5 % of neutrophils were found to use the transcellular route of migration, in vitro findings pointed to the predominance of the paracellular route for neutrophils (Carman & Springer, 2004). Endothelial junctions represent the major barrier for the transmigrating neutrophil for the paracellular pathway. At least two types of junctions are involved in transmigration: adherens junctions (zonula adherens) and tight junctions (zonula occludens). Additionally, endothelial gap junctions, which are communication structures, allow the passage of small molecular weight solutes between neighbouring cells (Bazzoni & Dejana, 2004).

Adherens junctions are formed by VE-cadherins that promotes homophilic cell-cell contacts in a calcium-dependent fashion. The link between cell membrane-associated VE-cadherin and the actin filaments is mediated by intracellular catenins (Bazzoni & Dejana, 2004). VE-cadherin permits the leukocyte passage of leukocytes, as antibodies against VE-cadherin increase the permeability of endothelial cell monolayers and the rate of neutrophils extravasation in vivo (Gotsch et al, 1997). However, in vitro studies
of Shaw et al (2001) indicate that VE-catherin gaps form transiently during diapedesis. Few pathways emerged to explain these observations. One of them reports that the crucial event promoting leukocyte trafficking is the phosphorylation of tyrosine residues 658 and 731 in the cytoplasmic tail of the VE-cadherin as well as Ser 665, correlated with the barrier function of VE-cadherin (Potter et al, 2005). Also, engagement of ICAM-1 by leukocytes results in tyrosine phosphorylation of VE-cadherin (Allingham et al, 2007). Signalling events during leukocyte transendothelial migration including ROS production is particularly interesting. ROS are generated as a signal downstream of ICAM-1 engagement (Wang & Doerschuk, 2000), although the production of ROS is known to be a downstream of other EC-leukocyte receptors, such as ICAM-3 and VCAM-1 (van Buul et al, 2004; van Watering et al, 1997). HSP27 protein activates ROS production in EC and by promoting actin cytoskeleton rearrangement, facilitates TEM (Nguyen et al, 2004; Gerthoffer & Gunst, 2001). Moreover, ROS inhibits phosphatase activity by reversible oxidation of the catalytic cysteine residue and the resulting increase in tyrosine phosphorylated junctional proteins might increase TEM as an effect of junctional distruption (Sallee et al, 2006). ROS production is also increased by the activity of Rac-1, a component of vascular NADPH oxidase complex. Indeed, treatment of ECs with NADPH oxidase inhibitors and ROS scavengers was found to block lymphocyte TEM (Matheny et al, 2000).

Tight junctions form a close intercellular adhesive contact beneath the apical cell surface. They contain several transmembrane adhesive proteins of the occludin, claudins and junctional adhesion molecules (JAM) families, which are linked intracellularly to cytoskeletal signalling molecules (Bazzoni & Dejana, 2004). Members of the JAM family (JAM-A, -B, -C) have been shown to interact with leukocyte integrins and therefore are implicated in leukocyte transmigration. In particular, JAM-A as a ligand for the leukocyte β2-integrin LFA-1, under flow conditions contributed to LFA-1-dependent transendothelial migration of T cells and neutrophils as well as LFA-1-mediated arrest of T cells (Ostermann et al, 2002). Also, endothelial JAM-A mediated neutrophil extravasation in a liver model for ischemia-reperfusion injury (Khandoga et al, 2005). JAM-B was hypothesized to be involved in leukocyte rolling and firm adhesion, because it binds to VLA-4 (Ludwig et al, 2009). JAM-C has been described as a ligand for the leukocyte integrins MAC1 and αXβ2 (Zen et al, 2004; Santoso et al,
Endothelial cell-selective adhesion molecule (ESAM) is another tight junction transmembrane protein that is related to JAMs family members. ESAM knockout mice decrease neutrophil, but not lymphocyte TEM using in vivo inflammation models. Thus, both ESAM and JAM protein functions correlate positively with leukocyte TEM, reflecting their role as leukocyte counter-receptors (Wegmann et al, 2006).

In addition to these transmembrane proteins, there are also intracellular proteins, which form a complex on the cytoplasmic side of the plasma membrane underlying the tight junction. The zonula occludens components, ZO-1 and ZO-2, are the predominant cytoplasmic tight junction proteins in endothelium (Jesaitis et al, 1994; Stevenson et al, 1986). They link the cytoplasmic domain of occludin to the actin filaments and recruit signalling molecules that function during junctional regulation (Schneeberger et al, 2004; Fanning et al, 1998).

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a member of the immunoglobulin superfamily, expressed at the intracellular borders of endothelial cells, platelets, neutrophils, monocytes and T cells subsets (Newman, 1997). Muller et al (1993) show that antibodies specific for PECAM-1 inhibited emigration of monocytes or neutrophils across endothelial cell monolayer in a quantitative in vitro assay of TEM. In addition, the findings of Sachs and co-workers (2007) provide direct evidence that neutrophil-specific CD177 is a heterophilic binding partner of PECAM-1 and their mutual interaction may contribute to the neutrophil transmigration. A step in diapedesis that is distal to the step mediated by PECAM-1 is controlled by CD99. Indeed, cells blocked at the CD99-dependent step are arrested halfway through EC contacts (Schenkel et al, 2002).

However, neutrophils are also able to migrate through a transcellular pore in the cytoplasm of the EC close to, but distinct from EC junctions (Feng et al, 1998). Interestingly, TNF-α pretreatment or ICAM-1 overexpression resulted in an increase of the relative contribution of the transcellular pathway in neutrophil transmigration (Yang et al, 2005). Upon leukocyte adhesion, leukocyte attachment induced assembling of VCAM-1 and ICAM-1 on the surface of EC. This causes, in turn, local actin remodelling and formation of the membrane protrusions around the attached leukocyte in a manner dependent on cortactin (Yang et al, 2006b; Barreiro et al, 2002).
Contactin and its tyrosine phosphorylation are required for the clustering of ICAM-1 around transmigrating neutrophils (Yang et al., 2006a). Moreover, microvascular permeability of ECs is regulated by subcellular membranous structures such as caveolae, finestrae and vesiculo-vacuolar organelles, which act as a gateway for leukocytes through the body of the ECs (Dvorak & Feng, 2001). Leukocyte migration begins with the expansion of membrane protrusions into EC. Ligation of ICAM-1 triggers cytoplasmic signalling mechanisms that lead to the translocation of apical ICAM-1 to caveolae and F-actin-rich regions and to the final transport with caveolin-1 to the basal plasma membrane. It causes the formation of channels through which leukocytes can migrate (Millan et al., 2006). Molecules involved in cell migration through endothelial-cell junctions may also mediate transcellular migration (Engelhardt & Wolburg, 2004).

Leukocytes continue their migration beyond the endothelium, exhibiting motility across EC BM and pericytes. Penetration of endothelial BM takes much longer than leukocyte migration through the endothelial barrier (Ley et al., 2007). The endothelial BM is composed of protein networks of the vascular laminins, including laminin-8 and laminin-10 and collagen type IV, which interacts with nidogen-2 and HSPGs (Hallmann et al., 2005). Pericytes are cellular components of capillaries and post-capillary and collecting venules in companion to the underlying endothelium. The pericyte network in most tissues is loosely distributed around endothelial cells and embedded in the BM (Hirschi & D’Amore, 1996).

Interestingly, examination of unstimulated mouse cremasteric venules discovered regions of low expression of matrix proteins within the endothelial BM, termed low expression regions (LERs). The level of laminin-10, collagen IV, or other basement-membrane constituents is there lower than average (Wang et al., 2006; Sixt et al., 2001). Neutrophil migration through IL-1β-stimulated cremasteric venules resulted in transient enlargement of these sites. Neutrophils migrate specifically at LERs, which are co-localized with gaps between neighbouring pericytes and low protein deposition within the extracellular matrix (ECM). Besides, a variety of proteases have been related to neutrophil transmigration and LER modelling, including matrix metalloproteinase 8 (MMP8), MMP9 and neutrophil elastase (Reichel et al., 2008; Wang et al., 2006).
1.4. LEUKOCYTE RECRUITMENT TO THE LUNG

PMN trafficking in the pulmonary circulation, which takes place in end-stage CF lungs (Ulrich et al, 2010), is distinct to that in the systemic circulation. Due to the small diameter of pulmonary capillaries (2 – 15 μm), PMN transit time is increased, allowing intimate contact with the capillary endothelium (Doerschuk et al, 1993). About 50% of the capillary segments require PMNs (6 – 8 μm) to stop and change their shape to pass through. Hence, the number of PMNs within pulmonary capillary blood is 35 – 100 times higher compared to large vessels of the systemic vasculature (Doerschuk et al, 1987).

The main site of leukocyte migration in the lung is the capillary bed (Reutershan et al, 2004). During lung injury or inflammation, PMNs migrate to the following lung compartments, intravascular, interstitial and intra-alveolar area. PMNs advancing to the alveolar airspace induce lung damage, which is associated with an increased mortality (Li et al, 2002). Inflammatory challenge, such as IL-8, PAF or TNF-α, sequester neutrophils in the pulmonary microvasculature, reducing the systemic to pulmonary PMN pool (Drost & MacNee, 2002).

Selectin-mediated rolling takes place in the pulmonary venules, but most of the pulmonary capillaries are too narrow to allow rolling. Also, the involvement of selectins may be stimulus-dependent, as they have been shown to be involved in the development of lung injury induced by LPS or complement C5a deposited intratracheally (Hayashi et al, 1999; Mulligan et al, 1996). In turn, PMN adherence in the lung may occur in both CD18-dependent or CD18-independent ways in regard to the intrapulmonary stimulus. IL-1, phorbol 12-myristate 13-acetate (PMA) and LPS, elicit migration via pathways predominantly mediated by CD18, whereas complement factor, IL-8, LTB4, C5a and Gram-positive bacteria recruit PMN mostly independently of CD18 (Mackarel et al, 2000; Hellewell et al, 1994; Doerschuk et al, 1990). Moreland et al (2002) has shown the dominance of CD18 integrin MAC-1 over LFA-1 in PMN recruitment in the lung inhaled LPS model, because antibodies to MAC-1, not to LFA-1 significantly inhibited neutrophil migration. Moreover, ICAM-1, which is known to support CD18-dependent emigration in mice, was involved in neutrophil migration in the same model, suggesting that the mechanism of adhesion may be regulated by the
expression of endothelial rather than neutrophil adhesion molecules. In addition, VLA-5 may mediate CD18-independent PMN migration (Burns et al, 2003).

1.5. CHEMOKINES INVOLVED IN THE INFLAMMATORY PROCESS

Chemokines are small heparin-binding polypeptides, produced by a variety of cells, whose main role is to regulate leukocyte migration during physiological and pathological conditions (Oppenheim et al, 1991). Chemokine activity is mediated by 7-transmembrane spanning GPCRs, which trigger different signalling pathways and gene expression changes within the responding cell (Taub et al, 1995). Chemokines are classified based on a cysteine motif conserved within their structure. The subgroups include CXC, CC, C and CX3C families (Oppenheim et al, 1991). The role of most chemokines still needs to be deciphered, however the members of CXC and CC families are inflammatory agents (Castellani et al, 2007).

The CXC family of cytokines, in which the first of two N-terminal cysteines are separated by another amino acid residue, induces chemotaxis in PMNs and T-cells, whereas the CC chemokine family, in which the first two cysteines are in juxtaposition, induces chemotaxis in monocytes, eosinophils, basophils and T cell subpopulations (Baggiolini et al, 1994; Schall et al, 1994). Moreover, members of the CXC chemokine family are known to be involved in angiogenesis, which has been related to the Glu-Leu-Arg (ELR) motif at the NH2-terminal cysteine. In general, ELR-positive chemokines, bind to PMNs and acts as angiogenic factors and ELR-negative, bind to lymphocytes and are angiostatic (Table 1.1) (Strieter et al, 1995).

<table>
<thead>
<tr>
<th>CXC chemokines</th>
<th>IL-8; PBP → β-TG → CTAP-III → NAP-2; Gro-α; Gro-β; Gro-γ; PF-4; ENA-78; γ-IP-10; GCP-2; γ-MIG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC chemokines</td>
<td>MCP-1; MCP-2; MCP-3; MIP-1α; MIP-1β; RANTES; eotaxin; l-309</td>
</tr>
<tr>
<td>C chemokines</td>
<td>lymphotactin</td>
</tr>
</tbody>
</table>

Table 1.1. Human chemokines. → indicate N-terminal processing resulting in different biological activity (Rot, 1996).
1.5.1. INTERLEUKIN-8 (IL-8)

IL-8 (CXCL8) or neutrophil-activating peptide-1 (NAP-1) is secreted from activated monocytes, fibroblasts, endothelial cells and T cells (Strieter et al, 1989; Gregory et al, 1988; Strieter et al, 1988; Walz et al, 1987) and is a potent chemoattractant for neutrophils (Baggiolini et al, 1989; Larsen et al, 1989). IL-8 has the highest specific activity as a granulocyte activating protein. *In vitro* effects on neutrophils include a transient increase of intracellular Ca\(^{2+}\) concentration, the release of azurophilic, specific and gelatinase granules, respiratory burst, chemotaxis and shape change (Baggiloni et al, 1989).

IL-8 has three binding domains: a high affinity binding domain, which mediates the binding to specific receptors on PMNs, the dimer interface, where IL-8 molecules binds to each other to form high molecule weight multimers and the GAG-binding domain (Kuschert et al, 1998; Hoogewerf et al, 1997; Lee et al, 1992).

The high-affinity binding domain is located at the N-terminus of IL-8 and it is formed of residues 4, 5 and 6, which are absolutely essential for the binding of IL-8 to CXCR1 and CXCR2. The C-terminal α-helix is probably important for stabilizing the three-dimensional structure (Clark-Lewis et al, 1991). The interaction of IL-8 with the high-affinity receptors mediates neutrophil chemotaxis and activation (Lee et al, 1992).

Hammond and co-workers (1995) suggested that CXCR1 is involved in mediating IL-8-induced chemotaxis at the site of inflammation at high IL-8 concentration, while CXCR2 contributes to the initiation phase of PMN migration, distant from the site of inflammation, where IL-8 concentration is at low (pg/ml) level. Additionally, IL-8 binds to a Duffy antigen/receptor for chemokines (DARC) on red blood cells, which prevents leukocyte stimulation by IL-8 released into the blood (Darbonne et al, 1991).

A low-affinity binding domain on IL-8, the dimer interface, is a region where IL-8 molecules bind to each other to form dimers and tetramers. IL-8 exists as a non-covalent homodimer of 8 kDa (Hassfurther et al, 1994). However, cell surface GAGs were shown to induce polymerization of chemokines, increasing their local concentration and therefore enhancing IL-8 concentration on the surface of endothelial cells *in vitro* (Hoogewerf et al, 1997).
Another low-affinity binding domain on IL-8, the GAG-binding domain is composed of a group of basic amino acids located in the C-terminal α-helix and in the proximal loop (Kuschert et al, 1998). IL-8 selectively binds to GAGs with an increased affinity: CS < DS < HS < heparin (Kuschert et al, 1999). IL-8 interactions with GAGs enhance neutrophil migration and protect IL-8 from damage by lytic enzymes released from the migrating cells in vitro (Webb et al, 1993). In the lung, IL-8 binding to GAGs promotes dimerization of IL-8, increasing the amount of IL-8 binding to lung tissue in vivo (Frevert et al, 2003).

1.5.2. NEUTROPHIL-ACTIVATING PEPTIDE-2 (NAP-2)

Neutrophil-activating peptide-2 (NAP-2, CXCL7) is generated during cleavage of the connective-tissue-activating peptide III (CTAP-III) and platelet basic protein (PBP). CTAP-III constitutes one of several molecular variants of β-thromboglobulin-antigen (β-TG), a group of chemokines that differ by their degree of N-terminal truncation. Along with platelet factor 4 (PF-4, CXCL4), CTAP-III represents the major preformed chemokines stored in platelet α-granules and released by exocytosis upon stimulation. CTAP-III and PBP are considered as pro-forms of NAP-2, because they do not have the capacity to activate neutrophils (Walz & Baggiolini, 1990; Walz & Baggiolini, 1989).

CTAP-III is converted to its active derivate by limited truncation by enzymatic proteolysis and removal of 15 amino acids from the N-terminus of the precursor (Brandt et al, 1991). CTAP-III was shown to be rapidly cleaved by chymotrypsin, cathepsin G, trypsin and chymase, serine proteases found in neutrophils, monocytes and mast cells (Schiemann et al, 2006; Car et al, 1991).

NAP-2 belongs to the family of ELR-positive chemokines, similar to IL-8, that potently activate neutrophils through interaction with CXCR1 and CXCR2 (Petersen et al, 1994). NAP-2 stimulates neutrophil chemotaxis almost as potently as IL-8 over a wide range of concentrations (Ludwig et al, 1997). NAP-2 was shown to stimulate release of the lysosomal and secondary granule enzymes upon cytochalasin-B treatment. Moreover, NAP-2-induced degranulation may be considerably enhanced by TNF-α- priming (Brandt et al, 1992; Walz & Baggiolini, 1989). NAP-2 up-regulates the adhesion molecule MAC-1, but down-regulates the surface expression of L-selectin on neutrophils (Detmers et al, 1991). Additionally, NAP-2 and CTAP-III induced CXCR2-
dependent neutrophil adhesion to human ECs, while only NAP-2 was shown to induce transmigration across monolayers of cultured HUVEC (Schenk et al., 2002).

1.6. PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1)

PAI-1 is a specific serine protease inhibitor of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). The molecular weight of this glycoprotein is around 54 kDa, depending on protein glycosylation at the asparagine residues (Asp209 and Asp265) (Gils et al., 2003). The main storage site of non-active PAI-1 is platelet α-granules, where it is released to the plasma (Nordenhem & Wiman, 1997). Under physiological conditions, PAI-1 can be constitutively secreted from a number of cells, such as platelets (De Taeye et al., 2005).

PAI-1 belongs to the serpin superfamily of serine protease inhibitors, which share unique structural features, such as a five-stranded β-sheet motif and a flexible reactive center loop (RCL). PAI-1 exists in several conformations, including the active and inactive forms as well as latent stages and the conformational changes associated with the RCL have been linked to the inhibitory function of PAI-1 (Ibarra et al., 2004; Nar et al., 2000). The binding of PAI-1 to t-PA and u-PA is mediated by the exosite interactions between the surface-exposed variable region-1 or 37-loop of the t-PA and the distal RCL of PAI-1 (Figure 1.9) (Ibarra et al., 2004).

![Figure 1.9. A model structure of a complex between t-PA and PAI-1. The α-helices, β-sheets, and loops are depicted in red, yellow, and gray respectively. Components of the catalytic triad and the variable region 1 loop of t-PA, as well as the P1 residue (Arg346) and the distal P4’ (Glu350) and P5’ (Glu351) residues of PAI-1 are represented as ball-and-stick models (Ibarra et al., 2004).](image-url)
PAI-1 is stabilised by binding to vitronectin, which may prolong its half life in vivo and in vitro (Declerk et al, 1988). Also, the protease specificity of PAI-1 toward thrombin is substantially increased in the presence of heparin (Ehrlich et al, 1991). PAI-1 is involved in the plasminogen activation cascade, where it is a key inhibitor of fibrinolysis and proteolysis (Figure 1.10).

![Plasminogen activation cascade](image)

Figure 1.10. Plasminogen activation cascade (Wyrzykowska & Kasza, 2009).

Under pathological conditions, large amounts of PAI-1 are secreted by ECs and other cells in response to inflammatory cytokines, such as TNF-α. High PAI-1 plasma levels are consistently found in patients with severe sepsis and acute or chronic inflammatory diseases, such as atherosclerosis. PAI-1 is upregulated by inflammatory cytokines and may therefore be regarded as a marker for an ongoing inflammatory process (Binder et al, 2002). A correlation between an increased level of PAI-1 and TNF-α was determined in chronic inflammatory diseases, including CF, COPD and asthma (Xiao et al, 2005). Other inflammatory mediators, IL-1β and IL-6, were found to increase PAI-1 expression by 40 and 30-fold in the HepG2 cell line (Healy & Gelehrter, 1994). PAI-1 is known to be synthesized in response to numerous factors, such as TGF-β (Sato et al, 1990), thrombin (Cockell et al, 1995), insulin (Nordt et al, 1994) or oxidation products (Dichtl et al, 1999).
1.7. COPPER AND ITS DUAL ROLE IN THE HUMAN BODY

Copper has two roles in the human body. It is both an essential element required for copper-dependent enzyme activity and also a pro-oxidant, contributing to human diseases.

1.7.1. COPPER AS AN ESSENTIAL ELEMENT

Since the late 1930s, copper has been recognised as an essential trace metal required for normal metabolic processes in living organisms. As it is an element, dietary intake and absorption are crucial (Linder, 2001). The level of copper intake that has been recommended as safe and adequate is 1.5 – 3 mg/day for adults, but many Western diets contain much less than this. Foods are believed to represent the major source for higher animals and humans, although water and nutritional supplements can contribute considerable quantities of the metal (Failla, 1999).

The total copper content in the human body is approximately 100 mg and the highest concentration is found in the liver, brain, heart and kidneys. However, due to the very large masses of muscles and bones, they contain approximately 50 % of the total copper in the body. The relatively large amounts of copper in the liver contribute 10 % to the total amount of copper in the body (Osterberg, 1980).

On entering the interstitial fluid and blood plasma from interstitial cells, copper is initially bound to albumin and transcuprein in the portal blood and general circulation. Most of this bound copper is then rapidly deposited in the liver. About 65 % is bound to ceruloplasmin in the liver during its synthesis, but albumin can bind and release copper outside the liver. A small amount of plasma copper is also associated with small peptides and amino acids. Copper from the blood is distributed into the liver, kidney and other tissues (Linder & Hazegh-Azam, 1996).

The role of copper as a cofactor for important enzymes has been well established. This includes cytochrome c oxidase (the terminal enzyme in electron transport and respiration), Cu/Zn superoxidase dismutase and ceruloplasmin (which removes superoxide and other potentially harmful radicals), as well as tyrosinase (producing melanin pigment), lysyl oxidase (which cross-links elastin and collagen), dopamine-
monooxygenase (crucial for catecholamine production), and peptidyl glycine α-amidating monooxygenase (necessary for modification of neuropeptide hormones). Most of these enzymes catalyze oxidation/reduction reactions that have molecular oxygen as a co-substrate. Indeed, the chemistry of copper makes it an ideal participant in redox reactions, as it easily cycles between cuprous \( \text{Cu}^{+} \) (Cu I) and cupric \( \text{Cu}^{2+} \) (Cu II) states (Linder, 2001). In biological systems, including water, copper tends to be in a cupric state. At physiological pH, there is little free copper in solution (Linder & Hazegh-Azam, 1996). Results from Rae et al (1999) indicate that intracellular free copper is limited to less than one free copper ion per cell. Moreover, calculated amounts of ionic copper suggested to be present in plasma are \( 10^{-18} \) M. Ionic copper has a particularly high affinity for other molecules (ligands) and, in consequence, all measurable copper in biological systems exists as complexes and chelates composed of copper bonded to organic components (Sorenson, 1987). However, extracellular copper is not controlled as tightly and is elevated in some pathological conditions, including cancer and inflammation (Linder, 1991).

In the liver cells, copper is mainly found in the nuclear, granular, microsomal and soluble fraction. Under physiological conditions and normal copper intake, the microsomal and nuclear fractions contain only minor amounts of copper, about 10 % of the total copper. The main part of copper, about 60 %, is found in the soluble fraction. In the granular fraction, which includes both mitochondria and the lysosomes, there is about 20 % copper (Osterberg, 1980).

1.7.2. COPPER HOMEOSTASIS

Although adequate copper levels are essential for normal metabolism, excess copper can be toxic to cells. The cellular requirements for copper depend on tightly regulated network of proteins such as the copper transporter 1 (CTR1), supporting copper uptake across the plasma membrane and the copper chaperones, a collection of proteins that deliver copper to specific target enzymes (Figure 1.11, over) (Wang et al, 2011).

CTR1 primarily mediates copper uptake into eukaryotic cells in mammals. This high affinity copper permease is an integral membrane protein that is structurally and functionally conserved from yeast to human (Puig et al, 2002; Zhou & Gitschier, 1997). A single CTR1 polypeptide contains 3 major domains: an extra-cytoplasmic N-terminus,
3 transmembrane helices and cytosolic C-terminal tail. The N-terminal domain, rich in Met and His residues, has been implicated in copper binding (Puig et al, 2002).

Figure 1.11. Cellular copper homeostasis. The CTR1 protein plays an important role in copper uptake. The copper chaperones COX17 and CCS deliver copper to CCO and SOD1, respectively. Additional copper chaperones (SCO1 and SCO2) involved in copper incorporation into CCO is not shown. ATOX1 copper chaperone deliver copper to the ATP7A and ATP7B proteins, which then transport copper to copper dependent enzymes in the trans-Golgi network. Increased concentrations of copper stimulate the endocytosis and degradation of CTR1 and the exocytosis of ATP7A and ATP7B to post-Golgi vesicles or the plasma membrane (Wang et al, 2011).

Once copper enters the cell via CTR1 protein, it can be transported to various distinct cellular locations. In eukaryotic cells, copper is delivered and incorporated into specific protein targets via the action of copper carrier proteins known as “metallochaperones” (Pufhal et al, 1997). One of them, COX17, acts in the delivery of copper to mitochondrial cytochrome oxidase (Glerum et al, 1996); ATOX1, escorts copper strictly to transport ATPases in the secretory pathway (Lin et al, 1997; Lin & Culotta, 1995); while CCS, copper chaperone for superoxide dismutase 1 (SOD), mediates delivery and incorporation of copper into cytosolic SOD1 (Culotta et al, 1997).

The bioavailability of intracellular copper is also regulated by copper exporters, ATP7A and ATP7B, whose main role is transporting copper to newly synthesized cuproenzymes en route through the secretory pathway and copper export from cells. Both transporters are mainly located in the final compartment of the Golgi network.
polarized cells, ATP7A and ATP7B undergo copper stimulated trafficking toward opposing membranes: ATP7A to the basolateral membrane and ATP7B to the subapical vesicles. Although ATP7A and ATP7B share similar biochemical functions, their tissue and developmental expression is different. ATP7A functions in a wide range of tissues that play critical roles in copper physiology, including copper absorption from the intestine and copper transport into the cerebral spinal fluid and cuproenzymes in the Golgi membranes of various tissues. Accordingly, loss of ATP7A function causes a lethal disorder of lack of copper called Menkes disease. In contrast, ATP7B is more selectively expressed in the liver, kidney, mammary epithelial cells, brain and eyes. Therefore, ATP7B mutation results in symptoms of hepatic and neuronal copper toxicosis seen in Wilson disease due to its specialized roles in these tissues (Wang et al, 2011).

1.7.3. COPPER AND OXIDATIVE STRESS

Unbound Cu (II) in the presence of a reducing agent, such as ascorbate, is reduced to Cu (I), which catalyzes Haber-Weiss-Fenton reaction to produce ROS (Figure 1.12) (Biaglow et al, 1997). For this reason, copper has been largely involved in the onset of oxidative stress (Valko et al, 2005) and in excess of cellular needs, copper can be cytotoxic. ROS are responsible for lipid peroxidation in membranes, direct oxidation of proteins and cleavage of DNA and RNA molecules (Tapiero et al, 2003).

\[
\begin{align*}
(1) & \quad \text{Cu}^{2+} + \text{O}_2^- \rightarrow \text{Cu}^+ + \text{O}_2 \\
(2) & \quad \text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \cdot\text{OH} \\
(3) & \quad \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2
\end{align*}
\]

Figure 1.12. The Haber-Weiss-Fenton reaction generates \cdot\text{OH} (hydroxyl radicals) from \text{H}_2\text{O}_2 (hydrogen peroxide) and superoxide (O_2^-) (1 and 2). It is followed by the Fenton reaction, which involves reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^+) (2) and net reaction (3).

The generation and action of ROS are major contributors to the development of a variety of pathologies such as ischaemia-reperfusion injury, chronic inflammation, Alzheimer’s disease, cancer, depression and aging (Tapiero et al, 2003; Rael et al, 2007). Generation of ROS was also suggested as a mechanism for the increased IL-8 secretion by endothelium observed with post-ischaemic reperfusion injury (Bar-Or et al, 2003). In inflammation and cancer, plasma copper and ceruloplasmin concentrations rise and the rates of synthesis and secretion of ceruloplasmin in the
liver are enhanced. This occurs through enhanced transcription of ceruloplasmin mRNA in hepatocytes. The increased concentrations of this protein in the circulation most likely provide additional extracellular protection from oxygen radicals to cell membranes during activation of leukocytes, macrophages and other immune cells that release them. In addition, the elevated ceruloplasmin in inflammation would provide additional copper for uptake by cells in normal tissues (Linder & Hazegh-Azam, 1996).

Copper levels are elevated in the circulation and in sputum in CF. Copper levels in plasma of adult men with cystic fibrosis are 1205.9 ± 255.4 µg/l (19.14 ± 4.05 µM), compared to healthy controls, 808.8 ± 80.0 µg/l (12.84 ± 1.27 µM) (Percival et al, 1999), while copper in sputum in CF was measured to be in the range 14.5 – 30.1 µg/l versus control, 3.0 – 16.4 µg/l (Gray et al, 2010).

There is evidence implicating transition metals as mediators of inflammation and cytotoxicity via oxidative mechanisms. Ambient air particles from a variety of locales contain ionisable metals and produce ROS in aqueous solution (Frampton et al, 1999). Kennedy et al (1998), for instance, studied air pollution particles collected in Provo, Utah. They stimulated a human EC line with metals contained in particulates. Zinc, lead and ferric ions had no effect on cytokine production, only copper dramatically stimulated secretion of the proinflammatory chemokine IL-8. However, Rice et al (2001) compared the proinflammatory effects of intratracheally instilled, equimolar, soluble forms of six metal sulphates in vivo in rats: vanadium, nickel, copper, iron (II), manganese and zinc. Copper was revealed to be the most proinflammatory metal, as it consistently induced early (4 hours post-instillation) and high airway neutrophilia even at the low dose. Copper was also found to be the most cytotoxic, as indicated by LDH activity and total protein concentrations in BALF. Eventually, copper induced expression of macrophage inflammatory protein-2 (MIP-2) mRNA at earlier sampling times compared to other treatments. Copper also initiates transcription of genes coding for fundamental proinflammatory cytokines via an NF-κB-dependent pathway through production of ROS, which could explain the mechanism contributing to the induction of leukocyte influx and cytotoxicity by copper (Perschini et al, 2006).
1.8. ANTI-COPPER THERAPY

Anti-copper drugs that have been developed to treat Wilson’s disease, include tetrathiomolybdate, zinc, penicillamine, and trientine. Tetrathiomolybdate has proven effective in numerous animal models of cancer, retinopathy, fibrosis and inflammation and with non-Wilson’s patients, tetrathiomolybdate inhibits angiogenesis, fibrosis and inflammation while avoiding clinical copper deficiency (Brewer, 2005). Lowering copper levels by a modest amount leaves adequate copper for use by cells to make vital copper-containing enzymes, such as cytochrome oxidase. The decrease of the copper concentration appears to involve inhibition of several proangiogenic cytokines, as well as inhibition of NF-κB, a master switch for transcription of many cytokines.

Bleomycin is a cytostatic drug employed in treatment of cancer, however it can cause side effects, such as pulmonary fibrosis, that may progress to fibrosis. Tetrathiomolybdate therapy is associated with inhibition of TNF-α expression and reduction of TGF-β protein levels in the lungs of bleomycin treated mice as well as MCP-1 and smooth muscle function, which have been shown to be important in the pathogenesis of fibrosis in the bleomycin model (Brewer et al, 2004). Since 1950, D-penicillamine has been used as a form of immunosuppression therapy to treat rheumatoid arthritis and its effect involves copper chelation. D-penicillamine reduces the number of peripheral blood T cells, inhibits macrophage function, reduces IL-1 and rheumatoid factor level and prevents collagen from cross-linking (Brewer, 2005).

A proinflammatory role for copper has been demonstrated as copper induced IL-8 synthesis and release from EC, which suggests that copper chelators, such as penicillamine may be useful as an anti-inflammatory therapy. Such a mechanism was demonstrated using human lung microvascular endothelial cells in cell culture, which showed that penicillamine significantly inhibited T cell transendothelial migration in response to platelet-derived RANTES (MacGregor et al, 2008, abstract).

Tobramycin and other aminoglycosides are able to chelate copper (Jezowska-Bojczuk et al, 1998). Tobramycin is widely used as antibiotic in the treatment of CF with Pseudomonas infection, having an indirect effect on inflammation by reducing the bacterial burden. However, the study of Ramsey et al (1999) indicated beneficial anti-
inflammatory effects of tobramycin beyond antibacterial activity. This project now investigates the potential direct anti-inflammatory effects of tobramycin.

1.9. CURRENT TREATMENT OF CYSTIC FIBROSIS

Understanding the pathogenesis of CF lung disease may provide opportunities for treatment, which resulted in significant improvements in life expectancy and quality of life over the past 50 years (Voynow et al., 2005). The successful therapy of CF aims at reducing airway obstruction, controlling airway infection and inflammation and improving nutritional status (Davis et al., 1996). Nowadays, therapeutic strategies targeting the cause of CF disease rather than symptomatic treatment are under development. More precise therapies focused on the basic defect, including manipulation of ion transport, activation of mutant CFTR and gene therapy are of particular interest (Becq et al., 2011).

1.9.1. REDUCING AIRWAY OBSTRUCTION

A characteristic feature of CF is the plugging of airways with thick and sticky airway secretion, a combination of mucus and pus, which requires a physical clearance procedure (Davis, 2006). Daily chest physiotherapy was demonstrated to maintain pulmonary function better over 3 years compared with deep breathing and cough alone, as shown in a prospective, controlled study by Reisman et al. (1988). Bronchodilators, such as β-adrenergic agonists or cholinergic blockers are usually used prior to chest physiotherapy to dilate small airways and facilitate mucus clearance (Davis et al., 1996). Mucolytics increase the expectoration of sputum by reducing its viscosity or hypersecretion (Del Donno & Olivieri, 1998). In particular, two mucolytic agents, administered by nebulisation are able reduce sputum viscosity in CF, N-acetylcysteine (NAC) and recombinant human DNase (Pulmozyme). NAC reduces disulfide bonds in mucins and decreases sputum viscosity in vitro. Additionally, as an antioxidant, NAC may have beneficial effects in the inflamed milieu in the CF airway (Voynow et al., 2005; Moldeus et al., 1986). Also, a reduction in viscosity was found in purulent sputum incubated in vivo with bovine pancreatic DNase I (Chernick et al., 1961). It therefore led to the development of inhaled bovine and human recombinant DNase I. However, this treatment improved pulmonary function in CF patients with only mild lung disease (Geller, 1997) and did not eliminate exacerbations of respiratory
symptoms (Fuchs et al, 1994). It was subsequently proposed to add human recombinant DNase in hypertonic saline with mannitol and denufosol (Pettit & Johnson, 2011).

1.9.2. TREATMENT OF AIRWAY INFECTION WITH ANTIBIOTICS

Antibiotics are a key therapy responsible for increased survival and improved quality of life in CF. Antibiotics can be grouped by their function into five groups: inhibitors of cell wall synthesis, inhibitors of protein synthesis, inhibitors of membrane function, antimetabolites and inhibitors of nucleic acid synthesis (Figure 1.13) (Pratt & Cornely, 2004). Aminoglycosides, which belong to the group of inhibitors of protein synthesis are the most common antibiotics prescribed to CF patients.

Antibiotic therapy is used to treat an acute exacerbation of bronchitis when signs and symptoms are consistent with worsening infection (Voynow et al, 2005). However, early in CF, most bacteria are susceptible and antibiotics can successfully treat infection. Treatment with an anti-staphylococcal agent following the diagnosis of CF through newborn screening may lead to reduced colonisation with P. aeruginosa and fewer hospitalisation during the first two years of life (Weaver et al, 1994). Indeed, an aggressive approach to the early treatment of P. aeruginosa with a combination of

Figure 1.13. Mechanisms of antibiotic action. Tobramycin acts on the translation process and affects protein synthesis by inhibiting the 30S RNA subunit (Pratt & Cornely, 2004).
inhaled colistin and oral ciprofloxacin resulted in reduced colonisation rates as well as better pulmonary function and fewer days of hospitalisation (Frederiksen et al, 1997). Inhaled therapy with antibiotics such as tobramycin, colistin, and aztreonam is prescribed for long-term treatment to improve lung function by hindering the growth of colonized bacteria (McCoy et al, 2008; Westerman et al, 2004; Pai & Nahata, 2001).

Because of the altered pharmacokinetics of many antibiotics, patients with CF often require higher doses and/or more frequent dosing intervals than what is usually recommended. In order to minimise complications, the duration of antibiotic therapy depends on clinical response and monitoring of adverse effects (Davis et al, 1996). For example, inhaled tobramycin is inhibited by sputum components, such as DNA, therefore, high concentrations of antibiotics are used with avoiding systemic toxicity.

Aminoglycoside antibiotics (aminoglycosides) form a large class of clinically important antibiotics with an efficacy in particular against Gram-negative bacteria, such as: *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus* species, *Klebsiella* species and *Serratia* species and *Enterobacter* species (Spelman et al, 1989). Most of the aminoglycosides are naturally occurring substances and are readily obtained from actinomycetes of either genus *Streptomyces* (labelled “-mycin”) or *Micromonospora* (labelled “-micin”). A key structural feature of the aminoglycoside antibiotics is a 1,3-diaminocyclohexanetriol termed streptamine or, in case where the 2-hydroxy function is deoxygenated as in most aminoglycosides, 2-deoxystreptamine (DOS) (Ring B in the structure in Figure 1.14, over). This central position of 2-deoxystreptamine suggests a crucial role for the biological activity of aminoglycosides (Busscher et al, 2005).

There are two classes of aminoglycosides that contain a 2-deoxystreptamine core, the 4,5- and 4,6-disubstituted 2-deoxystreptamine antibiotics. The 4,5-disubstituted class consists of neomycin, paromycin and ribostamycin. The largest group of the 4,6-disubstituted class consists of kanamycin, gentamycin, tobramycin and amikacin (Figure 1.14, over) (Recht et al, 2001).
Aminoglycosides carry a positive charge under physiological conditions (pH 7.4). The amine groups on ring A are the most basic (pK$_a$ ~ 9.6), whereas those on rings B (pK$_a$ ~ 5.6 to ~ 8.8) and ring C (pK$_a$ ~ 7.5) are closer to physiological pH (Gokhale et al., 2007). The presence of these positively-charged amine groups promotes aminoglycoside binding to negatively charged moieties of phospholipids, lipopolysaccharides and outer membrane proteins in Gram-negative bacteria. It is thought that binding of aminoglycosides causes displacement of Mg$^{2+}$ and Ca$^{2+}$ ions linking neighbouring lipopolysaccharide molecules. This process damages the outer membrane and enhances its permeability. The initial electrostatic surface binding is followed by energy-dependent phase I of bacterial uptake. During that phase, only a small quantity of antibiotic molecules crosses the cytoplasmic membrane. The binding of antibiotic to the interface between smaller (30S) and larger (50S) ribosomal subunits results in misreading of mRNA and production of misfolded proteins. Some of these proteins are
incorporated in the cytoplasmic membrane, leading to the loss of membrane integrity. This is followed by a cascade of events known as energy-dependent phase II uptake. During this phase, additional quantities of aminoglycoside are moved across the damaged cytoplasmic membrane. Consequently, antibiotics accumulate in the cytoplasm and bind to receptors on the 30S subunit of the bacterial ribosome, which plays an essential role in providing high-fidelity translocation of genetic material, and inhibit ribosomal protein synthesis. Higher concentration of the aminoglycoside dictate more rapid onset of energy-dependent phase II uptake and subsequent bacterial death (Lode, 1998; reviewed by Vakulenko et al., 2003).

2-deoxystreptamine-containing aminoglycosides bind to the ribosomal decoding site at the internal loop of the 16S RNA that comprises three unpaired adenine residues. Two of the adenines (A1492 and A1493) are flexible and may acquire positions inside the RNA loop or project into a site at the ribosome that accommodates the hybrid between messenger RNA (mRNA) and the A-site-bound transfer RNA (tRNA). Decoding of mRNA involves direct contacts between the flexible adenine “sensors” of the decoding site and the mRNA-tRNA codon-anticodon hybrid. Aminoglycoside binding at the decoding site displaces the flexible adenines from the RNA interior and locks them in a “flipped-out” state that closely resembles the conformation during mRNA decoding. As a result, the difference between cognate and near-cognate tRNA-mRNA interactions is reduced, leading to diminished translational fidelity. Over time, accumulation of truncated or improperly folded proteins leads to bacterial cell death (Figure 1.15, over) (Hermann, 2007).

Several mechanisms have been suggested for bacterial resistance to aminoglycosides. The main ones are: decreased antibiotic uptake and accumulation, modification of the ribosomal target, efflux of antibiotic or enzymatic modification of aminoglycosides (Vakulenko et al., 2003).

Aminoglycosides could be used in combination with another drug (an antipseudomonal penicillin or cephalosporin) to obtain a synergistic effect (a positive interaction between two anti-infective drugs that results in greater efficacy than the sum of their two individual effects) and to minimise the risk of resistance (Michea-Hamzehpour et al., 1986; Lode, 1998).
Aminoglycoside antibiotics do not easily cross normal human biologic membranes in physiological conditions and, in consequence, are not absorbed from the gastrointestinal tract, because of its poor lipid solubility. Thus they are typically administered intravenously, topically, ophthalmologically or intramuscularly. Aerosolization may be used when the drug is required in the airways, such as in CF (Lode, 1998).

The killing potential of aminoglycosides is dependent on the concentration and increases with increasing levels of the antibiotic. Importantly, aminoglycosides exhibit the post-antibiotic effect, which has been classically described as a persistent suppression of bacterial growth after a limited exposure to antimicrobial agents (Novelli et al, 1995). Aminoglycosides continue to kill bacteria even after the drug has been removed (Buntenz et al, 1981). This bactericidal activity of aminoglycosides favours one-a-day dosing. High single doses per day are used to maximise peak levels, avoiding sustained high levels of the drugs (Gilbert, 1991; Rao et al, 2006).

Aminoglycosides are excreted almost entirely by glomerular filtration without prior metabolism due to their high polarity and low protein-binding. Nephrotoxicity of
aminoglycosides is associated to the accumulation of small portions of the drug dose in the renal cortex followed by mostly reversibly renal impairment (Mingeot-Leclercq, 1999; Fischel-Ghodsian, 2005). Beside nephrotoxicity, aminoglycosides can cause irreversible ototoxicity in a dose-dependent manner that damages hair cells in the inner ear. Experimental evidence in animals has indicated that ROS may be one factor responsible for the development of aminoglycoside ototoxicity (Bates, 2003). Kalinec and co-workers (2005) indicated that the production of ROS in the cochlea during aminoglycoside therapy elicits apoptosis of hair cells via a complex signalling pathway.

General bacterial resistance by compound modifying enzymes and drug toxicity are the main limitations of aminoglycoside antibiotic therapy. The poor oral absorption is a secondary concern since these antibiotics are used in clinical settings for the treatment of closely monitored patients suffering from serious infections. A great number of efforts have been made to reduce the resistance and toxicity of aminoglycosides by focusing on modifications of the natural products or synthesis of aminoglycoside mimetics (Hermann, 2007).

1.9.3. THERAPIES DIRECTED AT INFLAMMATION

Antibiotic therapy reduces the inflammatory stimulus, however inflammation significantly contributes to the lung destruction in CF. Only few therapies for CF lung disease are specifically and directly aimed at reducing inflammation.

Although studies have not shown any particular benefit of long-term inhaled steroids for people with CF, they are used to treat CF patients, who also suffer from asthma or allergic bronchopulmonary aspergillosis. They are generally not recommended because of the potential for serious side effects including growth retardation in children, diabetes, cataracts, osteoporosis and decreased ability to fight lung infections (Davis et al, 1996).

Nonsteroidal anti-inflammatory drugs (NSAIDs) do not carry the same risk of side effects, making them more appropriate for long-term use. High concentrations of ibuprofen reveal specific activity against neutrophils, such as inhibition of neutrophil migration and release of lysosomal enzymes (Davis et al, 1996). In patients with CF, high doses of ibuprofen administered orally twice daily inhibited neutrophil
recruitment to a mucosal site in vivo (Konstan et al, 2003). A four year trial of ibuprofen demonstrated the improvement in lung function in patients 5 to 12 years of age, but did not in older patients (Konstan et al, 1995). However, the Cystic Fibrosis Foundation recommends ibuprofen as a treatment option for CF patients, who are at least 6 years old and have an FEV1 (forced expiratory volume in 1 second) of 60% or greater.

Azithromycin, a macrolide antibiotic was shown to exhibit anti-inflammatory activity in animal and humans. Azithromycin has been shown to affect several pathways of the inflammatory process, including neutrophil migration, the oxidative burst in phagocytes and the production of proinflammatory cytokines (Legssyer et al, 2006; Shinkai et al, 2006; Tsai et al, 2004; Anderson et al, 1996). Two small randomised controlled trials (Southern et al, 2003) as well as three well-designed randomised controlled trials have demonstrated a small but significant improvement in respiratory function with azithromycin compared with placebo (Southern et al, 2004).

1.10. HYPOTHESIS, AIMS AND OBJECTIVES

The hypothesis of this study is that tobramycin binds copper to become a copper-tobramycin complex with anti-oxidant properties, that inhibits neutrophilic and endothelial inflammatory responses.

OBJECTIVES:

- to study platelet-neutrophil-endothelial cell interactions in the recruitment of neutrophils across the blood vessel wall and to determine the role of each cell type
- to investigate the effect of the aminoglycoside antibiotic, tobramycin, and the copper-tobramycin complex on transendothelial migration of neutrophils
- to propose the mechanism of action of the drug

The overall aim of the study is to indicate the therapeutic potential of tobramycin in respiratory disorders, such as cystic fibrosis, beyond its known antibacterial role.
CHAPTER 2
CELLULAR COPPER CONTENT AND FORMATION
OF THE COPPER-TOBRAMYCIN COMPLEX

2.1. INTRODUCTION

2.1.1. SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) is a blue copper protein, first isolated from bovine erythrocytes by Mann and Keilin in 1939. SOD catalyses the conversion of the superoxide (O$_2^-$) to hydrogen peroxide and oxygen, according to the equation:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Superoxide dismutase is widely distributed within mammalian tissues (McCord & Fridovich, 1969). There are three different mammalian SODs: intracellular copper/zinc-SOD (Cu/Zn-SOD, SOD1), predominantly located in the cell cytoplasm; mitochondrial manganese-SOD (Mn-SOD, SOD2), present in the mitochondria and cytoplasm of the cells; and extracellular-SOD (EC-SOD, SOD3), located mainly outside the cells, where it is bound to matrix proteins, such as collagen and elastin (Marklund, 1982; Weisiger & Fridovich, 1972; McCord & Fridovich, 1969).

Intracellular SOD1 has been shown to obtain catalytic copper ion through interaction with the cytosolic copper carrier CCS, a copper chaperone for SOD1 (Culotta et al, 1997). The human CCS metallochaperone harbors a central, polypeptide region, which serves in target recognition of SOD1, while smaller segments at the N- and C-terminus of CCS facilitate the binding and release of copper into SOD1 (Schmidt et al, 1999). The Cu/Zn-SOD structure with its active site is shown in Figure 2.1 (over). The SOD molecule is a dimer with a molecular weight of 31,500 Da and it is composed of two subunits, each containing a Cu$^{2+}$ and Zn$^{2+}$ ion (Keele et al, 1971). The zinc ion is bound to three histidine residues and one aspartate residue and the copper atom is bound to four histidine residues. The two metal ions are connected via a histidine bridge (imidazole ring) (Richardson et al, 1975).
Superoxide is thought not to bind with the enzyme active site, but is guided near its active site using a specific form of electrostatic attraction, referred to as a cationic funnel (Livesay et al, 2003). The first superoxide attraction near the enzyme is followed by an electron jump from the superoxide to the active site. As a result, the histidine bridge breaks, and the copper ion moves slightly away, whereas the rings on the copper structure rotate about 20 degrees towards the zinc. The attraction of the second superoxide near the active site takes place, when the reduced copper is re-oxidized. Then, the original electron jumps back to the second superoxide molecule along with two protons, and one molecule of hydrogen peroxide is formed (Figure 2.2) (Hough & Hasnain, 1999).

\[
\begin{align*}
O_2^- + Cu^{II} + N\equiv N \equiv Zn^{II} & \rightarrow O_2 + Cu^{I} + N\equiv N \equiv Zn^{II} \\
O_2^- + Cu^{I} + N\equiv N \equiv Zn^{II} + 2H^+ & \rightarrow H_2O_2 + Cu^{II} + N\equiv N \equiv Zn^{II}
\end{align*}
\]

Figure 2.2. Dismutation of superoxide by SOD1. Kinetic studies of SOD have indicated that the copper ion cycles between the +1 and +2 oxidation states in two irreversible reactions each of which is first order with respect to O$_2^-$ (Smirnov & Roth, 2006).
Previous studies demonstrated that cytokines or oxidative stress had no effect on lung Cu/Zn-SOD mRNA or activity in vivo or in vitro, in contrast to SOD2 (Chang et al, 1993; Visner et al, 1990). Mitochondrial SOD2 forms a homo-tetramer and binds one manganese ion per subunit. SOD2 is an important antioxidant enzyme, which catalyzes the conversion of superoxide radicals to H$_2$O$_2$ and molecular oxygen in the mitochondria, playing a key role in protecting cells against oxidative damage (Weisiger & Fridovich, 1973).

SOD2 is highly inducible by oxidants and cytokines. Indeed, SOD2 mRNA, protein and activity are increased in cultured pulmonary epithelial and endothelial cells, fibroblasts, granulocytes and lung adenocarcinoma cells (A549) by oxidants, reducing agents, IL-1, TNF-α and LPS (Akashi et al, 1996; Chang et al, 1995; Das et al, 1995; et al, 1990). Moreover, TNF-α was shown to significantly increase SOD2 activity and mRNA in a dose- and time-dependent manner (Warner et al, 1991). In contrast, peroxynitrite and dexamethasone appeared to inactivate SOD2 (reviewed by Kinnula & Crapo, 2003).

Extracellular SOD3 is also a cuprozin enzyme with a molecular weight of 135,000 Da, genetically related to SOD1, because it shares 40 – 60 % similarity with SOD1 (Zelko et al, 2002). A fundamental property of the secretory tetrameric form of SOD3 is its in vivo affinity for heparin and analogues, mediating attachment to HSPGs located on cell surfaces and in the connective tissue matrix (Sandstorm et al, 1992).

SOD3 activity has been reported to be remarkably higher in the lung than in other organs. Bronchial and alveolar epithelium, epithelial cells lining intrapulmonary airways, alveolar macrophages and endothelial cells lining arteries and veins synthesize SOD3. Immunohistochemical studies revealed that SOD3 is located in the ECM and expressed in airway epithelial cell junctions and around the surface of vascular and airway smooth muscle cells (reviewed by Kinnula & Crapo, 2003).

Direct oxidative stress does not affect SOD3 expression, but it is activated by cytokines, such as IFN-γ and TNF-α in alveolar type II pneumocytes and fibroblasts (reviewed by Kinnula & Crapo, 2003). Because SOD3 exerts an important protective role in the vascular wall, the vasoactive factors, such as histamine, vasopressin, serotonin and
heparin markedly increase enzyme levels in the cultured arterial smooth muscle cells. Increased concentrations of SOD3 also prevent the degradation of NO by oxygen radicals. TGF-β in fibroblasts and platelet-derived growth factor and epidermal growth factor in vascular smooth muscle cells downregulate expression and excretion of SOD3 (Zelko et al, 2002). Moreover, proteolytic removal of the heparin-binding domain of SOD3 has been suggested to be important regulatory mechanism in the distribution of SOD3 within the ECM (Enghild et al, 1999).

2.1.2. CATALASE

Catalase is present in nearly all living organisms. Catalase serves to protect the cell from the toxic effects of hydrogen peroxide by decomposing it into molecular oxygen: $2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$ (Loew, 1990). In Eucaryota, catalase is localized mainly in peroxisomes, but a small amount of catalase was also shown in mitochondria, the endoplasmic reticulum and cytoplasm (Yamamoto et al, 1988). The highest activity of catalase was detected in hepatocytes, kidney, erythrocytes, bone marrow, mucosal membranes and the smallest in connective tissue (Aebi et al, 1968). In contrast, human vascular smooth muscle cells and endothelial cells lack catalase activity and are susceptible to hydrogen peroxide (Shingu et al, 1985).

All catalases studied so far are tetrameric. Each subunit (molecular weight around 60,000 Da) is formed by a single polypeptide chain with haem as a prosthetic group at the catalytic centre (Deisseroth & Dounce, 1970; Schroeder et al, 1969). Catalase monomers from certain species also contain one tightly bound NADP per subunit. This NADP may serve to protect the enzyme from oxidation by its H$_2$O$_2$ substrate (Eaton et al, 1995).

More than 300 catalase sequences from different organisms are now available, divided among monofunctional catalases, bifunctional catalase-peroxidases and manganese-containing catalases (Chelikani et al, 2004). Catalases present specific activities ranging from 20,700 to 273,800 units per milligram of protein and maximal turnover rates ranging from 54,000 to 833,000 per second. The effective concentrations of common catalase inhibitors, cyanide, azide, hydroxylamine, aminotriazole and mercaptoethanol varied over a 100- to 1000-fold concentration range and sensitivities to heat inactivation also varied (Switala & Loewen, 2002).
2.1.3. SOD MIMETICS

Low molecular weight SOD mimetics differentiate into organic complexes that chelate metal ions and compounds that do not contain metal ions, but also neutralise superoxide radicals. It was remarked that certain transient metal complexes, especially manganese and copper, react with the superoxide anion radical in the way reminiscent of native SODs. The SOD mimetics, which are transient metal complexes distinguish three huge groups: metalloporphyrins, salens and macrocyclic metal complexes (Figure 2.3). SOD mimetics could be also divided into compounds with solely SOD activity (group I) and compounds with both SOD and catalase activity (group II) (Wozniak & Czyz, 2008).

Figure 2.3. Classification of SOD mimetics with regard to their catalytic activity. Green colour identifies mimetics with solely SOD activity (group I) and blue – compounds with both SOD and catalase activity (group II). The end product of pseudoenzymatic activity of group I SOD mimetics is, according to the disproportionation reaction: $2H^+ + O_2^- \rightarrow H_2O_2 + O_2$, hydrogen peroxide and superoxide ion. In group II SOD mimetics, the second disproportionation follows the first one: $H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$, where hydrogen peroxide is dismutated to water and oxygen. There is a debate, whether mimetics with solely SOD activity are safe and do not trigger a cytotoxic level of hydrogen peroxide (modified from Wozniak & Czyz, 2008).

Many metal-containing macrocyclics (group I) inhibit neutrophil infiltration to sites of inflammation and release of proinflammatory cytokines (Salvemini et al, 1999), which could be attributed to their marked superoxide SOD activity. Current interest in copper complexes originates from their potential use as anti-microbial, anti-viral, anti-
inflammatory and anti-tumor agents as well as enzyme inhibitors, or chemical nucleases.

Cu(II) complexes with non-steroidal anti-inflammatory drugs (NSAIDs) are more active than their parent drugs and exhibit reduced gastrointestinal toxicity compared to the uncomplexed drug. Further, many of these complexes, including that of copper ibuprofenate complex show enhanced anti-inflammatory and anti-ulcerogenic activity (Weder et al, 2002).

Additionally, it has been shown that Cu(II)-curcumin complex possesses SOD activity, free radical neutralizing ability and antioxidant potential (Barik et al, 2005), while uncomplexed curcumin, a natural polyphenol derived from the plant Curcuma longa, has been described in several systems as a potent antioxidant and anti-inflammatory agent with anticancer potential (Aggarwal et al, 2003).

The peptides, Ac-HisValHis-NH₂ and Ac-HisValGlyAsp-NH₂, related to the active site of the Cu/Zn-SOD enzyme formed stable complexes with copper even at low pH, but the interactions with zinc resulted in formation of a precipitate, indicating polypeptide complex formation (Boka et al, 2004). Both, copper(II)-Ac-HisValHis-NH₂ and copper(II)-HisValHis systems exhibited catalytic activity toward the dismutation of superoxide anion, but the saturated coordination sphere of the metal ions in both systems results in low reactivity as compared to the native enzyme.

All porphyrin SOD mimetics (group II) have low catalytic activity, which is lower than 1% of the total catalytic activity of native catalase; however it is sufficient to protect cells from toxicity of high concentration of hydrogen peroxide. Manganese(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), a metalloporphyrin, is an effective free radical scavenger (Faulkner KM et al, 1994). For instance, it inhibited LPS-induced TNF-α production by macrophages and therefore decreased inflammation (Tumurkhuu et al, 2007). Manganese(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) is characterized by an easy penetration through the cellular membranes. It appeared to be an efficient antioxidant, which decreases release of malonyl dialdehyde, a marker of oxidative stress in a global cerebral ischaemia, preventing lipid peroxidation and DNA fragmentation induced by anoxia (Sharma & Gupta, 2007).
Two commercially available salen-manganese mimetics, EUK-8 and EUK-134 are characterized by a great stability and activity of SOD and catalase, scavenging almost all known forms of ROS. However, those reactions are reversible being a potential source of ROS. Therefore, EUK-8 and especially EUK-134 could contribute to the cell damage (Fucassi et al, 2007).

2.1.4. COPPER(II)-AMINOGLYCOSIDES

Aminoglycosides form the strongest complexes with \( \text{Cu}^{2+} \), compared to iron(III), nickel(II), cobalt(II) and zinc(II) (Szczepanik et al, 2004a). Moreover, the chelate formation stabilizes \( \text{Cu}^{2+} \) coordination to the aminoglycoside ligand (Patwardhan & Cowan, 2011). Several aminoglycoside antibiotics bind \( \text{Cu}^{2+} \), including gentamicin (Jezowska-Bojczuk et al, 1998b), kanamycin A (Szczepanik et al, 2004a), lincomycin, (Jezowska-Bojczuk et al, 2001b), kasugamycin (Jezowska-Bojczuk & Lesniak, 2001), tobramycin (Jezowska-Bojczuk et al, 1998a) and the semi-synthetic, amikacin (Jezowska-Bojczuk et al, 2001a).

The ability of aminoglycosides to coordinate metal ions is primarily governed by sugar ring substitutions on the 2-deoxystreptamine ring. Vicinal amine and hydroxyl groups within the 2-deoxystreptamine ring can form a potential metal chelating motif. The simplest 4-substituted aminoglycoside is neamine, which has 2\(^{-}\)-amine and 3\(^{-}\)-hydroxyl juxtaposed to each other (ring A) and thus can effectively form a metal chelate (Figure 2.4). Water molecules that complete an octahedral geometry occupy the remaining coordination sites around copper (Gokhale et al, 2007, Sreedhara et al, 2000).

![Figure 2.4. Cu\(^{2+}\)-neamine at pH 7.5 (Sreedhara et al, 2000).](image-url)
The proposed structure of the Cu\textsuperscript{2+}-tobramycin complex at physiological pH is schematically shown on Figure 2.5, with the central Cu\textsuperscript{2+} metal ion and a N\textsubscript{2}O\textsubscript{2} coordination sphere comprising 3''-NH\textsubscript{2}, 4''-O', 4'-OH and 6'-NH\textsubscript{2} from rings A and C (Jezowska-Bojczuk et al, 1998).

![Figure 2.5. Cu\textsuperscript{2+}-tobramycin complex at physiological pH (Jezowska-Bojczuk et al, 1998).](image)

The positive charge, which the aminoglycosides carry on their amine functions, influences their chemical and therapeutic behaviour. The proton binding abilities of amino groups determine their interactions with the negatively charged residues of nucleic acids and with biological membranes. Individual aminoglycosides differ from each other with the amount of the groups undergoing ionization and not all of them are protonated at physiological pH (Kozlowski et al, 2005).

Szczepanik et al (2004a) tested the effect of the aminoglycoside antibiotic kanamycin A on Cu\textsuperscript{2+} homeostasis. In order to examine whether the drug was capable of disturbing the Cu\textsuperscript{2+} content in blood plasma, the ability of kanamycin A to remove metal ions from saturated N-terminal binding site of human serum albumin was tested. The formation of an NH\textsubscript{2}→Cu\textsuperscript{2+} charge transfer transition characteristic for the Cu\textsuperscript{2+} complex of aminosugar antibiotics was observed. This fact, simultaneously with the decrease of the d–d band intensity of Cu\textsuperscript{2+}-HSA, suggests that under favorable conditions Cu\textsuperscript{2+}-aminoglycoside complex formation might be possible \textit{in vivo}, especially in the case of high antibiotic concentrations in plasma, which may occur during

The oxidative properties of copper centres in metalloproteins are well known. Also, many cupric complexes of aminoglycosides induce the formation of ROS (Szczepanik et al, 2004b). Although cyclic volumetric studies indicated that kanamycin A and amikacin are redox-inactive, their copper(II) complexes exhibited irreversible reduction and oxidation peaks in a wide pH range (Szczepanik et al, 2004b; Jezowska-Bojczuk et al, 2001a). Among aminoglycoside antibiotic complexes with copper, Cu$^{2+}$-neomycin B, generated hydroxyl radicals most effectively. Indeed, Cu$^{2+}$-neomycinB has the highest charge, because of six amino groups within its complex (Szczepanik et al, 2003). The hydroxyl radical is the most powerful oxidizing agent in biological system. Therefore their formation constitutes potential pro-mutagenic and pro-carcinogenic properties (Szczepanik et al, 2004b).

In the presence of H$_2$O$_2$, the Cu(II)-amikacin complex oxidizes 2$\prime$-deoxyguanosine to its 8-oxo derivative. Production of 8-oxo-guanosine suggests that these complexes may oxidize nucleobases within DNA molecules and the consecutive impairment of bases may lead to deleterious results, such as mutations. This process seems to be pH dependent, showing thereby that the complex present around physiological pH is the most efficient 2$\prime$ deoxyguanosine oxidizing agent (Jezowska-Bojczuk et al, 2002).

However, some physiologically ubiquitous compounds like histidine or ATP appeared to disturb complex formation between Cu$^{2+}$ and aminoglycoside and thus prevent DNA from damage. Both amino acid and nucleotides at physiological concentrations were found to drastically decrease the amount of strand breakages. Similar observations came from the experiment including magnesium ions. The magnesium binding to the phosphate residues protects DNA against the complex activity (Sczepanik et al, 2004c).

In the circulation, Cu(II)-kanamycin A and other copper-aminoglycoside complexes may exert influence on blood cells and generate various immune reactions, such as cytokine production, which are messenger proteins that regulate a wide variety of cellular functions. Szczepanik et al (2004a) demonstrated that Cu(II)-kanamycin A complex was able to induce TNF, interferon (IFN) and IL-10 in human peripheral blood leukocytes. The effect on the cytokine release was dose and time dependent and an
inter-dependence between IL-10 and TNF stimulation was found. In addition, the Cu(II)-aminoglycoside system can act as a moderate inducer of TNF-α, IFN-α/β and IL-10 released from human leukocytes. The research of Szczepanik et al (2004a) suggests therefore that in vivo Cu^{2+} coordination by aminoglycosides may support the pro-inflammatory process.

However, so far, tobramycin or Cu(II)-tobramycin complex has not been shown to have any pro-inflammatory effect in vivo or in vitro. Conversely, tobramycin has been suggested to have an anti-inflammatory effect beyond its known anti-pseudomonal activity in the treatment of CF patients (Ramsey et al, 1999). More recently, an anti-inflammatory role of tobramycin, related to its clinical efficacy as an inhalation therapy in chronic Pseudomonas aeruginosa airway infection, was confirmed in vitro by Nakamura et al (2011). A high dose of tobramycin (500 µg/ml) was demonstrated to decrease MUC5AC gene expression and protein production in NCI-H292 cells stimulated by LPS. Moreover, tobramycin complex with copper was shown to possess SOD-like activity (Shute & MacGregor, 2009, abstract).

2.2. HYPOTHESIS, AIMS AND OBJECTIVES

It is hypothesized that, in vitro, tobramycin binds copper and becomes a copper-tobramycin complex.

The aim of this research is to investigate a potential anti-inflammatory activity of tobramycin associated with copper binding. The initial objective was to measure the copper level in endothelial cells, neutrophils and platelets. The further objective was to prove that tobramycin binds copper in vitro and readily becomes a copper-tobramycin complex. Moreover, the SOD and catalase activity of copper-tobramycin was investigated.

2.3. MATERIALS

Human lung microvascular endothelial cells (HLMVEC) were purchased from Lonza Ltd. (Slough, Berkshire, UK), which also supplied endothelial cell culture basal medium, growth supplements and trypsin/EDTA and trypsin neutralising solution.
75 cm² culture flasks were obtained from Triple Red Ltd. (Long Crendon, Buckinghamshire, UK).

Greiner-Bio-One Ltd. (Stonehouse, Gloucestershire, UK) provided the following consumables for blood collection: Vacuette EDTA K3, lithium heparin tubes, clot activator tubes, butterfly needles 23 G x 23 cm, Vacuette holdex holder.

Lymphoprep™ was purchased from Axis Shield Diagnostics Ltd. (Huntington, UK). Macrodex™ (Dextran 70, 6 % w/v) was from Baxter Healthcare, Thetford, UK. Phosphate buffered saline (PBS), Hank’s Balanced Salt Solution (HBSS), RPMI 1640 and distilled water were obtained from GIBCO, Invitrogen Ltd. (Paisley, UK). Invitrogen also provided Amplex Red Neuraminidase (Sialidase).

Trypan blue solution (0.4 %), dimethyl sulphoxide (DMSO), heat inactivated fetal bovine serum (FBS), tobramycin sulphate, copper (II) chloride, copper standard for AAS (Tracecert™), ammonium nitrate, hydrogen peroxide 30 % (v/v), bovine liver catalase, bovine erythrocytes superoxide dismutase, nitro blue tetrazolium (NBT), NADH and phenazine methosulphate (PMS) came from Sigma-Aldrich Inc. (Poole, Dorset, UK). Copper sulphate, sodium chloride and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Fisher Scientific also provided polypropylene Falcon tubes, 14 ml (17 x 100 mm) capacity, a trace metal grade nitric acid and colour-fixed pH indicator sticks.

Complete mini® protease inhibitor (double strength) was from Roche Ltd. (Wewlyn Garden City, Hertfordshire, UK).

2.4. METHODS

2.4.1. NEUTROPHIL ISOLATION

Neutrophils were isolated from EDTA-anticoagulated venous blood (18 ml) from healthy donors. Red blood cells (RBCs) were removed by sedimentation by mixing blood in a 2:1 ratio with 9 ml of 6 % (v/v) Dextran 70 (Baxter Healthcare) for 45 minutes. Leukocyte-rich supernatants were underlayered with an equal volume of Lymphoprep (Axis Shield Diagnostics) and centrifuged at 450 x g for 30 minutes at
room temperature. The upper layers were discarded and RBCs remaining in the granulocyte pellet were subjected to hypotonic lysis with 5 ml of 0.2 % NaCl for 45 seconds, followed by 5 ml 1.6 % NaCl. After centrifugation at 800 x g for 10 minutes at 4°C, hypotonic lysis for 30 seconds was carried out. Neutrophils were pelleted at 200 x g for 5 minutes at 4°C. Cells were finally re-suspended in appropriate buffer/medium, depending on the experiment, HBSS (- Ca/Mg) + 20 mM HEPES, pH 7.4 or RPMI 1640 containing 2.5 % (v/v) FBS. The cells were then counted using a haemocytometer and diluted to the required density. Cell viability was assessed by trypan blue exclusion to be above 95 % and purity above 98 %. Contaminating cells were mostly eosinophils and rarely monocytes.

2.4.2. PLATELET ISOLATION

Platelets were isolated from 18 ml EDTA-anticoagulated normal venous blood. Platelet-rich supernatant was prepared by adding 0.1 volume of 0.15 M NaCl plus 77 mM EDTA, pH 7.4 to whole blood and centrifuging at 200 x g for 15 minutes at 20°C. The platelet-rich plasma was harvested and platelets were pelleted at 2500 x g for 15 minutes at room temperature and the pellet was washed first with PBS (- Ca/Mg) containing 10 mM EDTA and secondly with PBS (- Ca/Mg) without EDTA. Cells were finally re-suspend in appropriate buffer, counted using trypan blue and haemocytometer and diluted to the required cell number.

2.4.3. PLASMA AND SERUM SAMPLE PREPARATION

Plasma samples were prepared in lithium-heparin Vacuette tubes by a centrifugation at 1300 x g of whole blood. Serum samples were prepared in serum clot activator Vacuette tubes and left for clotting for 30 minutes at room temperature. Then the samples were centrifuged at 1300 x g for 10 minutes, clear supernatants removed and stored at -20°C before copper measurement.

2.4.4. ENDOTHELIAL CELL CULTURE

Human lung microvascular endothelial cells (HLMVEC) were maintained in culture medium consisting of endothelial basal medium (EBM-2), 5 % (v/v) FBS, 0.04 % (v/v) hydrocortisone, 0.4 % (v/v) human fibroblastic growth factor (hFGF-2), 0.1 % (v/v)
vascular endothelial growth factor (VEGF), 0.1 % (v/v) insulin-like growth factor (R3-IGF-1), 0.1% (v/v) ascorbic acid, 0.1 % (v/v) human epidermal growth factor (hEGF), 1 % (v/v) gentamycin and amphoteracin B (GA-1000). The cell cultures were maintained at 37°C in 5 % CO2 and 95 % air. Experiments were performed up to passage 10 with cells at 90 to 100 % confluence.

HLMVEC were seeded in 75 cm² flasks at a density of 5000 cells/cm². The number of flasks was calculated using the following formulas:

\[
\text{Max no of cm}^2 \text{ that can be plated} = \frac{\text{No of cells available} \times \text{Percent viability}}{\text{Recommended seeding density}}
\]

\[
\text{Max no of flasks that can be set up} = \frac{\text{Max no of cm}^2 \text{ that can be plated}}{\text{Effective growth area of the flask}}
\]

For seeding, 10 ml of fresh medium was added to each flask, which was placed into the incubator for 5 minutes to warm up. The cryovial with HLMVEC was removed from the liquid nitrogen. In a sterile field, the cap was briefly twisted a quarter turn to relieve the internal pressure, then re-tightened. Holding the cryovial, the bottom ¾ of the cryovial was dipped in a 37°C water bath and swirled gently for 1 – 2 minutes until the contents were thawed. The cryovial was removed immediately and transferred to a sterile field, where the equilibrated flasks were waiting, ready to seed. The cells in the cryovial were re-suspended with a gentle, slow and steady up and down pipetting motion. An equal amount of cell suspension was dispensed into each flask. The caps were replaced and the vessels gently rocked to evenly distribute the cells. Each flask was labeled with the cell type and density, medium type, passage number and date. The cell density was checked under the microscope and the culture vessels were returned to the incubator, where cells were allowed to adhere overnight.

2.4.4.1. CELL FEEDING

One day after seeding, the old medium was removed and the same volume of warm and fresh growth medium was added per flask. Every other day, the cells were fed with a larger volume of medium as they became more confluent:
If cells are: | Then feed them:
---|---
Under 25% confluent... | 1 ml per 5 cm² (15 ml for T-75)
From 25 – 45% confluent... | 1.5 ml per 5 cm²
Exceeding 45% confluence... | 2 ml per 5 cm²

Feeding the cells was continued until 60 – 90% confluence.

2.4.4.2. SUBCULTURING

The cells were subcultured when they were 60 – 90% confluent and contained many mitotic figures throughout the flask. The growth medium was aspirated from all flasks and the cells were washed with 5 ml/flask of HBSS (- Ca/Mg). This step allowed removing the excess of medium, which contains complex proteins that neutralize trypsin. The cells were covered with 2 ml/flask warmed trypsin/EDTA (Lonza, UK) and the flasks left in the incubator for approximately 10 minutes. The flasks were rapped against the palm of the hand few times to release the cells from the culture surface. The cell layer was examined microscopically. The trypsinization was continued until approximately 90% of the cells were rounded up. Trypsin/EDTA was neutralized with an equal volume of warmed trypsin neutralising solution (Lonza, UK) and rinsed around. The detached cells were quickly transferred into a sterile 50 ml Falcon tube. The flasks were rinsed with 5 ml of HBSS (- Ca/Mg) to collect residual cells. The harvested flasks were examined under the microscope to make sure the harvest was successful. The harvested cells were pelleted at 220 x g for 5 minutes. After centrifugation, the supernatant was discarded and the cell pellet re-suspend in full growth medium. To count the cells, 10 µl of the cell suspension was mixed well with 90 µl trypan blue solution. Then, 10 µl of the mixture was added per chamber of haemacytometer. The cell number was calculated using the following formula:

\[ C_n = n \times 10 \times 10^4 / \text{ml}, \text{ where:} \]
\[ n = \text{cell number}, 10 = \text{dilution factor}, 10^4 = \text{a constant factor} \]

2.4.4.3. SUBCULTURING FOR NEW FLASKS

To subculture for new flasks, the total number of flasks to inoculate was determined by using the equation shown in section 2.4.4 as the number of flasks needed depends
upon cell yield and seeding density. 1 ml growth medium was transferred for every 5 cm² surface area of the flask (15 ml/75 cm² flask). The vessels were placed into the incubator to warm up. The calculated volume of cell suspension was dispensed into the prepared flasks. The flasks were gently rocked to promote even distribution and placed to the incubator.

2.4.4.4. SUBCULTURING FOR CRYOPRESERVATION

To subculture for cryopreservation, a cryopreservation solution was prepared. It contained: 80 % (v/v) growth medium, 10 % (v/v) FBS and 10 % (v/v) DMSO. HLMVEC were diluted to 2 x 10⁶/ml in cryopreservation solution and split into cryogenic vials (1 ml/vial). Each vial was labeled with the cell name, cell density, passage number and date of subculturing. Cryogenic vials were placed on a polystyrene base in -80°C freezer for no longer than 12 – 24 hours. The vials were finally transferred to liquid nitrogen.

2.4.5. COPPER MEASUREMENT BY GRAPHITE FURNACE – ATOMIC ABSORPTION SPECTROPHOTOMETRY (GF-AAS)

2.4.5.1. SAMPLE PREPARATION FOR GF-AAS

HLMVEC were grown in 75 cm² flasks until confluent and lifted with trypsin/EDTA as described in Section 2.4.4.2. The cells from each of three flasks were counted and lysed in 1 ml of 1 % (v/v) Triton X-100 in water, containing double strength of protease inhibitor (Roche).

Neutrophils were isolated from healthy donors as described in Section 2.4.1. They were re-suspended in HBSS (- Ca/Mg) or RPMI 1640 containing 2.5 % (v/v) FBS at 1 x 10⁷ cells/ml. 4 and 5 preparations of neutrophil suspensions (1 ml) were made in HBSS (- Ca/Mg) and RPMI 1640 with FBS, respectively.

Platelets were isolated from healthy donors as described in Section 2.4.2 and re-suspended in PBS (+ Ca/Mg) at a concentration of 1 x 10⁹ cells/ml. Platelets were then activated with thrombin in the concentration range 0 – 3 U/ml at 37°C for 30 minutes in a total volume of 1 ml. The material was centrifuged at 2500 x g for 15 minutes in 4°C. 1 ml supernatants were stored at -80°C. The cell pellets were lysed in an equal
volume of 1 ml of 1% (v/v) Triton X-100 in water, containing double strength protease inhibitor (Roche).

2.4.5.2. SAMPLE DIGESTION

The digestion system for copper analysis was adapted from Alcock (1987). 1 ml of neutrophil suspension at 1 x 10^7 cells/ml, HLMVEC and platelet lysates were transferred into trace metal free polypropylene, round bottomed tubes. HNO₃ at 0.1 M final concentration was added per 1 ml sample using 10 M stock (10 µl). As sample controls, 1 ml of HBSS (- Ca/Mg), PBS (- Ca/Mg), RPMI 1640 with 2.5% (v/v) FBS, EGM and 1% (v/v) Triton X-100 in water was transferred into trace metal free polypropylene, round bottomed tubes (Fisher), followed by addition of 10 µl trace metal grade 10 M HNO₃. Furthermore, 1 ml of 50 ppb (50 µg/l) copper standard was prepared to serve as a copper recovery control. The samples were frozen at -80°C and freeze-dried. The dried powder was dissolved in 0.5 ml of 30% (v/v) H₂O₂ and the mixture left to dry at 50°C two times. This step was repeated again with 65°C drying temperature. Then, the dry residue was dissolved in 200 µl of 10 M HNO₃ and dried at 65°C. That prepared material was reconstituted in 1 ml of 0.1 M HNO₃ before copper measurement.

2.4.5.3. INSTRUMENT SETTINGS

Thermo Scientific iCE 3300 AA Graphite Furnace - Atomic Absorption Spectrometry (GF-AAS) was used to measure the copper concentration in samples including plasma, serum, platelets and neutrophils obtained from healthy volunteers. The copper was also measured in endothelial cells and cell culture medium and buffers.

All glassware used for acid, standards and ammonium nitrate preparation and sample cups for samples measurement were washed with 0.1 M HNO₃ overnight. Working standard solutions were prepared manually in 10 ml glass volumetric flasks in the range 10 – 50 µl/ml in copper free 0.1 M HNO₃ (100 – 500 µl/10 ml). Final standard solutions were also prepared in 10 ml glass volumetric flasks in the range 10 – 50 µg/l in copper free 0.1 M HNO₃ using 10 – 50 µl/ml solutions (10 µl/10 ml, 1/1000 dilution). Plasma and serum samples were diluted 1/50 and all the other samples were analysed after re-constitution in 0.1 M HNO₃ without further dilution.
All determinations were performed under the following conditions (Table 2.1) according to the GF-AAS instruction manual. The absorbance of each sample was measured at a wavelength of 324.8 nm three times. The average number of three measurements was calculated when the % of differences did not exceeded 10. Ammonium nitrate in 5 g/l concentration was used as a matrix modifier (a substance added to enhance sensitivity or repeatability of the measurement).

<table>
<thead>
<tr>
<th>SPECTROMETER PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
</tr>
<tr>
<td>Background Correction</td>
</tr>
<tr>
<td>Lamp Current</td>
</tr>
<tr>
<td>Number Of Resamples</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FURNACE PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase</td>
</tr>
<tr>
<td>Ashing</td>
</tr>
<tr>
<td>Atomization</td>
</tr>
<tr>
<td>Cleaning</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAMPLING PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Volume</td>
</tr>
<tr>
<td>Martix Modifier</td>
</tr>
<tr>
<td>Std Additions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CALIBRATION PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration Mode</td>
</tr>
<tr>
<td>Concentration Units</td>
</tr>
<tr>
<td>Acceptable Fit</td>
</tr>
</tbody>
</table>

Table 2.1. The instrumental conditions for copper measurement by GF-AAS.

The copper level was measured using the standard addition method and the sample concentration was calculated by the instrument and expressed in µg/l. Moreover, a fit of at least 0.995 was accepted.

2.4.6. SYNTHESIS OF COPPER-TOBRAMYCIN

This method was developed from the method described in Sreedhara, Freed & Cowan (2000). Tobramycin sulphate (0.025 mmols, 0.1169 g) was dissolved in 5 ml UHQ water in a sterile 15 ml Falcon tube on a rota mixer for 5 minutes. Then copper sulphate
(0.025 mmols, 0.0624 g) was weighted and added to the solution. The lid was replaced firmly and the cap sealed with paraffilm and the tube left rotating overnight at room temperature. A clear dark blue solution was formed. Because free tobramycin interferes in the binding with copper-tobramycin to DNA it is important to remove the free, uncomplexed tobramycin from the solution. Ethanol (3.3 ml) was added to a final concentration of 40 % (v/v) in the reaction mixture. This precipitated the copper-tobramycin as a blue solid and left the uncomplexed tobramycin in solution. The blue copper-tobramycin precipitate was recovered by centrifugation at 2000 x g for 10 minutes at 10°C. The supernatant was removed and discarded. The precipitate was washed in absolute ethanol by adding 5 ml of ethanol to the tube, mixing vigorously to break up the pellet and placing on the roller for 3 hours. The precipitate was recovered by centrifugation, as before (2000 x g for 10 minutes at 10°C) and washed again in 5 ml absolute ethanol for 3 hours on the roller. Finally the precipitated copper-tobramycin was recovered by centrifugation at 2000 x g for 10 minutes, the supernatant was discarded and the pellet dried under vacuum for 1 hour at 30°C.

2.4.7. UV-VIS SPECTROPHOTOMETRY

The stock solutions of tobramycin, copper-sulphate and copper-tobramycin were prepared in double deionised and sterilized water. 12.5 mM tobramycin was prepared by dissolving 11.69 mg of tobramycin sulphate (MW 467.51) in 2 ml of water. 12.5 mM copper sulphate was prepared by dissolving 6.24 mg copper sulphate pentahydrate (MW 249.6) in 2ml water. Copper-tobramycin (MW 531) was prepared with 13.28 mg in 2 ml water to obtain 12.5 mM copper-tobramycin stock. Serial 1:2 dilution of tobramycin, copper-tobramycin and copper-sulphate were prepared in double deionised water from 12.5 mM to 0.024 mM. The pH of stock solutions was measured using colour-fixed pH indicator sticks. A wavelength scan was performed on Uvikon 923 Double Beam UV/VIS Spectrophotometer supplied by NorthStar Scientific Ltd. The measurement was carried out using 0.5 cm pathlength quartz cuvettes placed at 37°C and each reading was subtracted from the water control.

Copper-tobramycin concentrations were plotted against absorbance values. The molar extinction coefficient (ε) was calculated for the maximal absorption wavelength of
\( \lambda = 250 \text{ nm} \) using the Beer-Lambert law. Also, a dissociation constant \((K_D)\) and \(pK_D\) were calculated using appropriate formulas.

2.4.8. MICROPLATE SUPEROXIDE DISMUTASE ASSAY

The assay was modified from the procedure developed by Ewing & Janero (1995). Superoxide dismutase activity of the copper-tobramycin complex (0.001 – 500 µM) was determined by using its ability to inhibit the reduction of nitrotetrazolium blue (NBT) by superoxide ions generated by the aerobic mixtures of NADH and phenazine methosulphate (PMS). The extent of NBT reduction was followed spectrophotometrically by measuring the formation of formazan at an absorbance of 560 nm. This assay was performed in a 96-well plate with a total reaction volume of 100 µl. The complete reaction system consisted of 50 µM NBT, 78 µM NADH and 3.3 µM PMS (final concentrations) in 1 x PBS (- Ca/Mg) at pH 7.4 and 25°C. An endpoint optical density measurement at 560 nm was obtained after 5 minutes of incubation by using MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA). For comparative purposes, the activity of native superoxide dismutase from bovine erythrocytes (200 U/ml) and tobramycin (0.001 – 500 µM) was also measured under the same experimental conditions. The assay was performed in triplicate.

2.4.9. HYDROGEN PEROXIDE ASSAY

Hydrogen peroxide was detected by Amplex Red Neuraminidase (Sialidase) Assay (Molecular Probes). 10 mM stock solution of Amplex Red reagent (Component A) was prepared by dissolving the contents of the vial of Amplex Red reagent (0.26 mg) in 100 µl DMSO (Component B). 1 x working solution of Reaction Buffer was prepared by adding 4 ml of 5 x Reaction Buffer stock solution (Component E) to 16 ml of water. 100 U/ml stock solution of horseradish peroxidase (HRP) was prepared by dissolving the contents of the vial of HRP (Component C) in 200 µl of 1 x Reaction Buffer. 20 mM \( \text{H}_2\text{O}_2 \) working solution was prepared by diluting 23 µl of 3 % (v/v) \( \text{H}_2\text{O}_2 \) into 977 µl water. The sample stocks (tobramycin, copper-tobramycin and copper sulphate), were prepared 100 x concentrated in water and diluted in 1 x Reaction Buffer. 50 µl of each sample was pipetted per well of a 96-well plate. An \( \text{H}_2\text{O}_2 \) standard curve was prepared using 20 mM \( \text{H}_2\text{O}_2 \) working solution to obtain 200, 100, 50, 25, 12.5, 6.25, 3.125 µM \( \text{H}_2\text{O}_2 \) standards in water. 1 x Reaction Buffer served as blank. 50 µl of each \( \text{H}_2\text{O}_2 \)
standard was pipetted per well of a 96-well plate in duplicate. 100 µM Amplex Red reagent containing 0.2 U/ml HRP was prepared and 50 µl of this solution was added per well to begin the reaction. The plate was incubated at 37°C for 30 minutes, protected from light. The absorbance was measured at 550 nm.

2.4.10. A RAPID KINETIC ASSAY FOR CATALASE ACTIVITY

Catalase activity was measured by the method modified from Beers & Sizer (1952). Hydrogen peroxide at 1 M was prepared from 30 % (v/v) hydrogen peroxide stock (8.8 M) by diluting 0.1 ml stock with 0.78 ml in PBS (- Ca/Mg), pH 7. This was then diluted to the final concentration of 10 mM (1/100 dilution). 1000 U/ml of catalase stock was prepared in water (1 ml) and diluted to obtain 1, 5 and 10 U/ml final concentrations. 50 mM stocks of tobramycin, copper tobramycin, copper sulphate (100 µl) were also prepared in water to obtain 0.5 mM concentration of appropriate drug in 0.7 ml, the total sample volume in the cuvette (1/100 dilution). Additionally, 10 mM MnTBAP stock was prepared in TRIS buffer, pH 7.4 (100 µl) to be verified for its catalase activity. 1 and 50 µM final concentration of MnTBAP was tested.

The measurement was carried out in auto-rate mode on the Uvikon 923 Double Beam UV/VIS Spectrophotometer supplied by North Star Scientific Ltd. in 0.5 cm pathlength quartz cuvettes placed at 37°C against water as a control. A reading of 10 mM hydrogen peroxide solution was followed by a reading of each sample (0.7 ml). The samples were scanned immediately at λ=240 nm every 12 seconds within 2 minutes in duplicate.

2.4.11. STATISTICAL ANALYSIS

All statistical analyses were performed on GraphPad Prism 4 version 4. The results were expressed as average plus/minus SEM. One-way ANOVA and Dunnett’s or Tukey’s multiple comparison post hoc test were performed on the data. A paired t-test was also used for data analysis where appropriate. The p values less than 0.05 were considered as statistically significant.
2.5. RESULTS

2.5.1. DEVELOPMENT OF COPPER ANALYSIS USING THE STANDARD ADDITION METHOD (GF-AAS)

The concentration of copper in each sample was determined using a standard addition method. This method is recommended for instrumental analysis, such as GF-AAS. The standard addition method is commonly used to determine the concentration of an analyte that is in a complex matrix, like biological fluids. The matrix of blood and cell lysates may contain other components that interfere with the analyte signal causing inaccuracy in the determined concentration, therefore it is reasonable to apply the standard addition method with GF-AAS.

A separate calibration curve was performed for every sample analysed. To obtain a standard addition plot (Figure 2.6) and also determine the sample concentration, the instrument was programmed to measure the absorbance signal in three repeat measurements of the blank (0.1 M HNO₃), the measured sample and the copper standard (10 – 50 µg/l). At least three different preparations of each sample have been analysed.

![Figure 2.6. A representative standard addition plot. Blue diamonds represent copper standards (10 – 50 µg/l), red square is the blank (0.1 M HNO₃), which has OD 0.032 and green triangle is the analysed sample. The horizontal blue line shows the background subtraction. The sample concentration was calculated by using the graph formula: y = 0.0027x + 0.0686, where y = sample OD – blank OD. The sample concentration was measured here was 13.16 µg/l (arrow).](image-url)
2.5.2. CELLULAR COPPER LEVELS

Normal serum and plasma copper levels were measured using GF-AAS and found to be 1262.73 ± 211.16 µg/l and 991.08 ± 229.01 µg/l, respectively (n=7).

The copper level in endothelial cells, platelets and neutrophils was calculated after subtraction of the copper concentration measured in medium or buffer that the cells were re-suspended in before they underwent the digestion procedure. HLMVEC contained 23.37 ± 1.11 ng copper/10^6 cells (n=3), while platelets contained 14.33 ± 2.49 ng copper/10^9 cells (n=8). The copper level in supernatants of thrombin-activated platelets was below the level of detection (n=8). Also, the copper concentration in neutrophils of healthy volunteers was below the level of control medium, HBSS (- Ca/Mg) (n=4) or RPMI 1640 containing 2.5 % (v/v) of FBS (n=5).

The full growth medium, EGM (containing 5 % v/v FBS), used to grow HLMVEC had 38.48 ± 1.01 µg/l copper. RPMI 1640 supplemented with 2.5 % (v/v) FBS was found to have 23.11 ± 1.98 µg/l copper.

The buffers that were used in experimental procedures described throughout this study were HBSS (- Ca/Mg) and PBS (- Ca/Mg). The level of copper in these buffers was very similar and was determined to be: 24.85 ± 1.84 µg/l for HBSS (- Ca/Mg) (n=3) and 29.30 ± 6.00 µg/l for PBS (- Ca/Mg) (n=3). Moreover, copper concentration was also measured in 1 % (v/v) Triton X-100 lysis buffer, which had 6.02 ± 1.12 µg/l copper (n=3).

Furthermore, 50 µg/l (50 ppb) copper standard was prepared and analysed as a sample after the freeze-drying and digestion procedure. The measured copper concentration was 45.73 ± 2.30 µg/l, which corresponds to 91.45 ± 4.59 % sample recovery (n=3).
### Table 2.2. Summary of the copper concentration in different buffers, media and cells expressed as a mean ± SEM.

<table>
<thead>
<tr>
<th>Material Type</th>
<th>n</th>
<th>Copper [µg/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGM (5% FBS)</td>
<td>3</td>
<td>38.48 ± 1.01</td>
</tr>
<tr>
<td>RPMI 1640 + 2.5% FBS</td>
<td>3</td>
<td>23.11 ± 1.98</td>
</tr>
<tr>
<td>PBS (- Ca/Mg)</td>
<td>3</td>
<td>29.30 ± 6.00</td>
</tr>
<tr>
<td>HBSS (- Ca/Mg)</td>
<td>3</td>
<td>24.85 ± 1.84</td>
</tr>
<tr>
<td>Triton X-100 in water</td>
<td>3</td>
<td>6.02 ± 1.12</td>
</tr>
<tr>
<td>Serum</td>
<td>7</td>
<td>1262.73 ± 211.16</td>
</tr>
<tr>
<td>Plasma</td>
<td>7</td>
<td>991.08 ± 229.01</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>9</td>
<td>Undetectable levels above medium control</td>
</tr>
<tr>
<td>Platelets</td>
<td>7</td>
<td>14.33 ± 2.49 [ng/10^9 cells]</td>
</tr>
<tr>
<td>HLMVEC</td>
<td>3</td>
<td>23.37 ± 1.11 [ng/10^6 cells]</td>
</tr>
</tbody>
</table>

2.5.3. **UV - VIS SPECTRUM OF TOBRAMYCIN, COPPER SULPHATE AND COPPER-TOBRAMYCIN**

In order to investigate whether tobramycin forms a complex with copper sulphate in *in vitro* assay conditions, tobramycin, copper sulphate and copper-tobramycin solutions in water in a range of concentrations, were scanned using UV-VIS spectrophotometry. The pH of all solutions prepared were measured and the results are shown in the table below (Table 2.3).

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double deionised water</td>
<td>6.5</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>7.5</td>
</tr>
<tr>
<td>Copper-tobramycin</td>
<td>6.0</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 2.3. The summary of the pH of 12.5 mM tobramycin, copper sulphate and copper-tobramycin stock solutions prepared in double deionised water.

Figure 2.7 (over) demonstrates a representative UV-VIS wavelength scan of a range of tobramycin concentrations (1.5625 – 12.5 mM) measured against water blank. This scan was divided into two spectra, in the UV and VIS range (Figure 2.7A and Figure 2.7B, respectively) and the scale was adjusted for the peaks to be distinct. Tobramycin
shows very little absorbance at any wavelength. However, an interesting absorption maxima for the two highest tobramycin concentrations, 12.5 mM (absorbance 0.051) and 6.25 mM (absorbance 0.008) were observed at $\lambda=245$ nm. This is the absorbance maxima similar to the one, characteristic for copper-tobramycin (see Figure 2.19) and it could suggest trace metals contamination of tobramycin with copper. A single peak on tobramycin VIS spectrum has not been identified.

![UV-VIS spectrum of tobramycin at 37°C](image)

Figure 2.7. UV-VIS spectrum of tobramycin at 37°C. A range of tobramycin (1.5625 – 12.5 mM) was scanned across 200-900 nm using quartz cuvettes. Representative scans of: A) UV spectrum; B) VIS spectrum of tobramycin.

Copper sulphate in a range of concentrations (0.024 – 12.5 mM) was also scanned across the whole spectrum. UV and VIS spectrum of copper sulphate are presented in
Figure 2.8A and 2.8B, respectively. No obvious peak can be distinguished on the UV spectrum, whereas the VIS spectrum showed peaks with absorbance maxima at $\lambda=812$ nm for 12.5 mM (absorbance 0.068) and 6.5 mM (absorbance 0.035).

![Graph showing UV-VIS spectra of copper sulphate](image)

Figure 2.8. UV-VIS spectrum of a range of copper sulphate at 37°C. A range of copper sulphate (0.024 – 12.5 mM) was scanned across 200-900 nm using quartz cuvettes. Representative scans of: A) UV spectrum; B) VIS spectrum of copper sulphate.

Copper-tobramycin complex synthesized as described in Section 2.4.6, was dissolved in water and a wavelength scan of this complex was performed. Figure 2.9A and 2.9B (over) demonstrate characteristic peaks for copper-tobramycin in both UV and VIS range of the spectrum, respectively. Although, the UV-VIS wavelength scan of copper-tobramycin was carried out in the concentration range of 0.024 – 12.5 mM, copper-tobramycin at the concentration of 12.5 mM was excluded from the UV spectrum due
to an absorbance above 4 at the absorbance maxima at wavelength $\lambda=250$ nm. That high absorbance value for the sample could not be measured accurately by the instrument, and was outside the linear range for detection of the complex (see Figure 2.10).

![UV-VIS spectrum of copper-tobramycin at 37°C. A range of copper-tobramycin (0.024 – 12.5 mM) was scanned across 200-900 nm using quartz cuvettes. Representative scans of: A) UV spectrum; B) VIS spectrum of copper-tobramycin.](image)

Copper-tobramycin was found to have a distinct profile and a much higher absorbance value at $\lambda=250$ and $\lambda=706$, compared to the same concentration of tobramycin (Figure 2.7) and copper sulphate alone (Figure 2.8). Moreover, the copper-tobramycin complex absorbed much more strongly at the wavelength of $\lambda=250$ than at $\lambda=706$ nm.
For instance, 6.25 mM copper-tobramycin concentration elicited absorbance 3.239 at \( \lambda = 250 \text{ nm} \) and only 0.052 absorbance at \( \lambda = 706 \text{ nm} \). The dependency of copper-tobramycin absorbance values on copper-tobramycin concentration are presented in Figure 2.10, for a single experiment.

![Figure 2.10. A Beer-Lambert plot for copper-tobramycin. A) Copper-tobramycin complex at \( \lambda = 250 \text{ nm} \); B) Copper-tobramycin complexes at \( \lambda = 706 \text{ nm} \).](image)

It was of interest to find out how quickly the copper-tobramycin complex formed and how stable it was. For this reason, tobramycin sulphate and copper sulphate (12.5 mM) were mixed in 1:1 ratio directly in the quartz cuvette and the complex formation (6.25 mM final concentration) was monitored every two seconds for 1 minute. The same spectrum (Figure 2.9, red line) was obtained. An immediate change of colour
from pale to dark blue was observed. This experiment revealed that copper-tobramycin complex is formed instantly. The maximal absorption at $\lambda=250$ of copper-tobramycin (3.356 nm) appeared in 2 seconds and was constant within 1 minute of measurement. Furthermore, no change of absorbance or colour of the complex was observed within months of keeping the complex at $4^\circ$C and re-analysing.

By using the Beer-Lambert law, which is the linear relationship between absorbance and concentration of an absorbing species, the extinction coefficient for copper-tobramycin complex was calculated. The general Beer-Lambert law is usually written as: $A = \lambda bc$, where: $A$ is the measured absorbance, $\lambda$ is a wavelength-dependent absorptivity coefficient, $b$ is the path length and $c$ is the analyte concentration. When working in concentration units of molarity, the Beer-Lambert law is written as: $A = \varepsilon bc$, where: $\varepsilon$ is the wavelength-dependent molar extinction coefficient with units of M$^{-1}$ cm$^{-1}$. For the calculations a range of concentrations was used rather than a single concentration.

The extinction coefficient was calculated for the copper-tobramycin complex only at $\lambda=250$ nm to be 1062 M$^{-1}$cm$^{-1}$. However, Heyrovská (1996) shown that the non ideal thermodynamic properties of strong electrolytes in aqueous solutions at all concentrations are due to partial dissociation and hydration. The degree of dissociation of copper-tobramycin complex was calculated to be 13.1%. Therefore, the value of 1062 M$^{-1}$cm$^{-1}$ was further corrected by the following factor of 1.157 and the new extinction coefficient was calculated for the complex as 1229 M$^{-1}$cm$^{-1}$ (see Appendix).

In order to calculate the dissociation constant of the copper-tobramycin complex and therefore examine the ability of tobramycin to bind copper, a range of tobramycin concentrations was titrated to the constant concentration of copper sulphate. Figure 2.11 (over) shows a wavelength scan of this complex, made by mixing tobramycin at 0.0244 – 0.7813 mM concentration with 0.7813 mM copper sulphate.

The UV part of the copper-tobramycin spectrum (Figure 2.11A) was used for further calculations of dissociation constant. Indeed, the absorbance values in UV spectrum
are much higher and therefore more accurate compared to the same values in VIS range of the spectrum.

A dissociation constant ($K_D$) and $pK_D$ value was calculated by using the following formulas:

$$K_D = \frac{[M][L]}{[ML]}$$


$K_D$ for copper-tobramycin complex was calculated to be 0.05 mM and $pK_D$ was 4.301 (see Appendix).

![Figure 2.11. Tobramycin binds copper.](image)

Figure 2.11. Tobramycin binds copper. UV-VIS spectrum of a range of tobramycin (0.3906 – 6.25 mM) additions to a constant concentration of copper sulphate (6.25 mM) at 37°C. A) UV spectrum; B) VIS spectrum.
It was previously reported that copper binds to HEPES (Hegerschweiler & Saltman, 1986). Moreover, the research of Patwardhan et al (2011) showed different values of copper-tobramycin ε and $K_D$, what may be related to their use of HEPES buffer. Therefore, the effect of HEPES buffer systems was examined for copper sulphate (3.125 mM) as a source of copper (Figure 2.12). Because HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 was chosen in some further experiments reported here, separate components of this buffer were tested. The solvents were: water, HBSS (+ Ca/Mg), 20 mM HEPES, HBSS (+ Ca/Mg) containing 20 mM HEPES. The pH of the solutions was adjusted to 7.4.

Figure 2.12. The effect of buffer systems on wavelength scan of copper sulphate at 37°C. A) Copper sulphate at the UV range. B) Copper sulphate at the VIS range.
Copper sulphate dissolved in water gave a very little absorbance at both wavelengths, \( \lambda = 250 \) and \( \lambda = 706 \) nm, which were 0.092 and 0.009, respectively, which was comparable to the previous results presented in Figure 2.9. (0.126 and 0.002, respectively). HBSS buffer increased the copper sulphate absorption maxima to 0.512 nm at \( \lambda = 250 \) of the spectrum. HEPES buffer was observed to have a large effect on absorbance, increasing values at \( \lambda = 250 \) nm, which were: 2.187 for HEPES buffer alone and 2.514 for HEPES addition to HBSS buffer (Table 2.4). At the VIS range the absorbance signal increase gave values of 0.254 and 0.109, respectively. These results suggest a non specific interference of HBSS buffer, which was increased on addition of HEPES. These results also confirm that water was the best solvent used in the examination of UV-VIS spectrum in the conditions tested.

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>( \lambda = 250 ) nm</th>
<th>( \lambda = 706 ) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.092</td>
<td>0.009</td>
</tr>
<tr>
<td>HBSS (+ Ca/Mg)</td>
<td>0.512</td>
<td>0.011</td>
</tr>
<tr>
<td>20 mM HEPES</td>
<td>2.187</td>
<td>0.254</td>
</tr>
<tr>
<td>HBSS (+ Ca/Mg) + 20 mM HEPES</td>
<td>2.514</td>
<td>0.109</td>
</tr>
</tbody>
</table>

Table 2.4. The summary of the absorbance values of buffer systems at different wavelength.

Copper sulphate at the concentration of 6.25 mM and higher was not fully dissolved in the buffer systems including HBSS (+ Ca/Mg), 20 mM HEPES and HBSS (+ Ca/Mg) containing 20 mM HEPES. Furthermore, it precipitated once left in the reaction tube or cuvette.

2.5.4. SOD-LIKE AND CATALASE ACTIVITIES ASSOCIATED WITH COPPER-TOBRAMYCIN

As mentioned earlier in this chapter (Section 2.1.3), copper-drug complexes have SOD or SOD and catalase activity. It was therefore crucial to examine the SOD-like activity of copper-tobramycin.

Superoxide anions have a very short half life and, accordingly, they must be produced continuously. In a cell- and enzyme-free colorimetric assay, superoxide ions are generated via the ability of an aerobic mixture of NADH and PMS to produce superoxide anions, which converts NBT to formazan, a coloured product absorbing light at 560 nm. SOD activity reduces the superoxide ion concentration and thereby
lowers the rate of formazan formation. In the SOD-like activity test, the copper-tobramycin and other compounds compete with nitro NBT for reaction with the generated superoxide ions. The more efficient the complex, the lower complex concentration that corresponds to 50% inhibition of NBT reduction (IC₅₀).

Nitro blue tetrazolium reduction was inhibited dose dependently by copper-tobramycin. Figure 2.13 represents the percentage inhibition of NBT reduction with an increasing concentration of copper-tobramycin (0.001 – 500 µM) with the best fit of r=0.999. IC₅₀ was calculated to be 200 nm for this complex. EC SOD served as a positive control. A single concentration of 200 U/ml SOD inhibited NBT reduction by 60%. However, tobramycin alone had no inhibitory effect at concentrations up to 500 µM. This result clearly indicates SOD-like activity of copper-tobramycin and no such activity of tobramycin itself.

![Graph showing inhibition of rate of NBT reduction](image)

Figure 2.13. Superoxide dismutative activity of copper-tobramycin measured as inhibition of the rate of nitro blue tetrazolium reduction. Copper-tobramycin potently inhibited NBT reduction with an IC₅₀ of 200 nM, n=3.

SOD-mimetics are often divided into distinct classes, compounds with SOD-like activity only and compounds with additional activity of catalase that allows them to dismutate hydrogen peroxide generated by SOD. Therefore, it was of interest to examine the catalase activity of the copper-tobramycin complex. The Amplex Red Neuraminidase assay utilizes Amplex Red to detect H₂O₂, which, in the presence of horseradish peroxidase (HRP), reacts with a 1:1 stoichiometry with Amplex Red reagent to
generate the red-fluorescent oxidation product, resorufin. Resorufin can be detected by measuring absorbance at 550 nm or fluorescence. The above described reaction is shown in Figure 2.14.

Figure 2.14. Principle of coupled enzymatic assays using Amplex Red reagent. Oxidation of glucose by glucose oxidase results in generation of H$_2$O$_2$, which is coupled to conversion of the Amplex Red reagent to fluorescent resorufin by HRP.

Figure 2.15 illustrates no ability of copper-tobramycin to dismutate hydrogen peroxide in the concentration range between 0.01 to 0.5 mM after 15 minutes pre-incubation. For comparison, the same concentrations of copper chloride and tobramycin were tested. Those results clearly show that copper chloride and tobramycin do not have catalase activity in the range of 0.01 – 0.5 mM, concentrations that were used in most experimental procedures.

Figure 2.15. Hydrogen peroxide dismutation by tobramycin, copper-tobramycin and copper chloride in the range of 0.01 – 2 mM at the wavelength 550 nm. The experiment was performed in triplicate and the data points show the mean ± SEM.
To confirm that copper-tobramycin had no catalase activity at the concentrations used in cell based assays, a rapid kinetic assay for catalase activity was performed by measuring absorbance of H$_2$O$_2$ at $\lambda=240$.

To evaluate the assay sensitivity and accuracy, decomposition of H$_2$O$_2$ was measured over time with 1 – 10 U/ml catalase (Figure 2.16). Hydrogen peroxide was shown to be stable and it was decomposed by catalase dose dependently. As predicted, higher concentrations of catalase lowered the absorbance values.

![Figure 2.16. Decomposition of hydrogen peroxide (10 mM) over time by varying concentrations of catalase. The change of concentration of hydrogen peroxide is proportional to the decrease in absorbance at $\lambda=240$ nm.](image)

Different compounds were then examined for their catalase activity (Figure 2.17, over). Tobramycin clearly did not possess catalase activity by showing no difference from the hydrogen peroxide baseline. Copper-tobramycin and copper sulphate also did not dissociate hydrogen peroxide. However, copper maximal absorption at 250 nm effectively interferes in the assay, resulting in a high absorbance values, ranging from 0.74 to 0.77 for copper-tobramycin complex and 0.82 for copper sulphate. To double check the copper interference in the assay 10 mM hydrogen peroxide and 0.5 mM copper-tobramycin absorbance were measured at $\lambda=240$ separately, followed by the absorbance reading of hydrogen peroxide mixed with copper-tobramycin. There were 0.15, 0.52 and 0.68, respectively and the results were additive.
Finally, MnTBAP, which was reported to possess both superoxide dismutase and catalase activity (Ferret et al, 2001), was examined for its ability to decompose hydrogen peroxide. MnTBAP had a negligible effect up to the concentration of 50 µM.

2.6. SUMMARY OF RESULTS

Copper is present in cell culture medium and buffers as well as neutrophils, platelets and HLMVEC, cells used in the model of neutrophil transendothelial migration, described in Chapter 5. Tobramycin in the presence of copper immediately and spontaneously becomes a copper-tobramycin complex, which is stable over time. Copper-tobramycin was shown to have SOD-like activity but no catalase activity in the concentration range up to 0.5 mM.

2.7. DISCUSSION

Cell culture medium and buffers used in the experimental procedures in this investigation were shown to contain copper. Percival et al (1993) had reported that cell culture media (RPMI containing HEPES buffer, 10 % (v/v) fetal bovine serum, 2 mmol/l glutamine, antibiotics and the insulin-transferrin-selenium supplement) are relatively low in copper, giving the value of 0.5 µM, which corresponds to 31.5 µg/l copper concentration. Here, RPMI 1640 supplemented with 2.5 % (v/v) FBS, HBSS (-Ca/Mg) and PBS (-Ca/Mg) contain 23.11 ± 1.98 µg/l, 24.85 ± 1.84 µg/l and 29.30 ±
6.00 µg/l copper concentration, respectively. Full growth medium for HLMVEC contains almost 1.5 times more copper compared to the other medium and buffers, presumable because it contains 5 % (v/v) fetal bovine serum. Moreover, in plasma or serum, most of the copper is firmly bound to the ceruloplasmin (Gubler et al, 1953) and this protein contains 8 atoms of copper per molecule (Holmberg & Laurell, 1948). Copper ions are essential for proliferation of human endothelial cells under culture conditions (Hu, 1998). Ceruloplasmin maintains enzymes activity during cell differentiation and if not supplied in the medium, the cells may acquire copper from an intracellular source, namely Cu/Zn-SOD. Moreover, copper supplementation resulted in an increase in intracellular Cu/Zn-SOD activity, but had no effect on the level of Cu/Zn-SOD protein (Percival et al, 1993).

Cellular copper uptake in mammals is accomplished primarily by the copper importer, CTR1 (Wang et al, 2011) previously described in Section 1.8.2. CTR1 was suggested to be expressed in multiple tissues in selected cell types, including the endothelial cells of the capillary small blood vessel in the brain (Kuo et al, 2006). HLMVEC were found to contain the highest copper concentration compared to other cells used in the current study, namely 23.37 ± 1.11 ng/10^6 cells. In the cell culture conditions, HLMVEC could take up copper from FBS present in the culture medium presumably via CTR1.

Polymorphonuclear cells were previously reported to contain 1.65 ± 0.22 ng copper/mg cell protein (Percival et al, 1999). However, copper was not detected in neutrophils in the study reported here. Similarly, another study by Schmitt (1997) also reported the level of copper in neutrophils to be below the detection limit. The results of White et al (2009) suggest that the inflammatory response of macrophages stimulates CTR1-mediated copper uptake. Moreover, marginal copper deficiency results in impaired function of rat neutrophils with 60 % decrease in superoxide anion production in comparison to control (Percivall et al, 1998). It suggests that copper is stored in unactivated neutrophils mainly as superoxide dismutase, which is also true for hepatocytes (Osterberg, 1980).

Platelets also contain micromolar concentrations of copper (Lind et al, 1993). Milne & Nielsen (1996) found 24.4 ± 10.7 ng copper/10^9 cells. In the current study, platelets were found to contain 14.33 ± 2.49 ng copper/10^9 cells. Schmitt (1997) detected
304.29 ± 240 ng copper/10^9 cells, although these results are marked by a large variance (standard deviation). The studies of O’Sullivan et al (2005) suggested a link between platelet activation and progressive impairment of CF lung function. Platelets were therefore suspected to release copper under thrombin stimulation. However, release of copper from thrombin-activated platelets was below the limit of detection in the current study. A more sensitive method of analysis, inductively coupled plasma mass spectrometry (ICP-MS) could be applied in future experiments to detect release of copper. It was however possible to calculate the amount of copper in one platelet to be approximately 14 x 10^{-18} g copper/cell.

Platelet size range from 1 – 2 µm diameter (Dopheide et al, 2002). Activated platelets could be considered as a sphere and it is possible to calculate platelet volume, based on a mathematical formula:

\[ V = \frac{4}{3} \times \pi \times R^3 \], where R is a radius of a sphere.

MPV (mean platelet volume) was reported to be 7.9 – 11.7 µm³ (Demirin et al, 2011; Tuncell et al, 2011; Vagdatli et al, 2010; Bansal et al, 2002). Interestingly, the study of Uysal et al (2011) shown lower MPV values of pediatric CF patients compared to healthy donors and suggests that the platelet volume is associated with exacerbations. This study indicates that MPV might be used as a diagnostic, predictive indicator for platelet activation in pediatric CF patients related to chronic inflammation, which might be helpful to discriminate or estimate exacerbation.

Normal plasma copper level was determined previously to be approximately 790 – 1090 µg/l as measured by AAS (Khorasani et al, 2008; Percival et al, 1999; Schmitt, 1997; Milne & Nielsen (1996); Hatano et al, 1982 and Inutsuka & Araki, 1978). The copper concentration in plasma of healthy volunteers in this study was similar, 991.08 ± 229.01 µg/l, which validates the analytical procedure used in the current study. Normal serum copper level, measured at 1262.78 ± 211.16 µg/l, was also comparable to previously published values, determined by AAS, in the range of 977 – 1203 µg/l (Strecker, 2005; Gonzales et al, 1999; Carpentieri et al, 1986).

In general, plasma values were lower than serum concentrations, but these differences were not statistically significant. Frank and colleagues (2001) found the same
dependence without statistically significant difference. Rosenthal & Blackburn (1974) also found serum copper levels to be higher compared to plasma. Foley et al (1968) had similar observations for zinc and he attributed this difference to a possibility that copper might be released from platelets, leukocytes, or erythrocytes during coagulation and clot retraction. However, the release of copper from platelets during their isolation could decrease cell copper and make the released copper hard to detect.

Moreover, blood collection, processing and storage before analysis are critical for accurate trace element analysis. Anticoagulants and blood collection containers may affect albumin, copper and zinc levels. Frank et al (2001) reported that copper concentrations measured in EDTA plasma were lower than copper measured in any other collection container. Based on their experience and recommendation, here, plasma samples were collected into lithium-heparin tubes and serum samples were collected into serum clot activator tubes for trace element testing. However, chelation of copper in the specimen by EDTA should not substantially affect the copper concentration measured by ICP-MS (Frank et al, 2001). It could be explained by the greater tolerance of ICP-MS over GF-AAS to the sample matrix.

ICP-MS has many advantages over other elemental analysis techniques, including high speed, precision and sensitivity. ICP-MS is capable of the determination of a range of metals at concentrations below one part per trillion \((10^{12})\), whereas the limit of GF-AAS used in the current study is one part per billion \((10^9)\). ICP-MS could therefore be helpful in detecting copper release by thrombin-activated platelets.

The role of transition metal ions in the biological activity of aminoglycoside antibiotics has not been previously reported (Jezowska-Bojczuk et al, 1998). Among aminoglycoside complexes with metal, the properties of copper-tobramycin complex has been investigated, since aminoglycosides form the most stable complexes with Cu(II) ions compared to both toxic and basic metal ions, such as iron(III), nickel(II), cobalt(II) and zinc(II) (Szczepanik et al, 2004a). Indeed, tobramycin in the presence of copper sulphate forms a stable copper-tobramycin complex. In vitro, this complex was formed immediately and presented the same spectrum over time, suggesting its stability. Additionally, the blue colour of the copper complex remains the same even
after months of keeping the solution at 4°C. Moreover, Szczepanik et al (2004a) also suggest that copper(II) complexes of aminoglycosides can easily be formed in vivo, i.e. in blood plasma by withdrawing of the metal bound to human serum albumin.

Cu$^{2+}$ coordination by aminoglycosides is pH dependent and CuL (copper-ligand) species dominates at the physiological pH (Jezowska-Bojczuk et al, 1998) (Figure 2.18).

Even over a wide pH range, copper(II)-aminoglycoside complexes are often isolated as monomeric species. The coordination complex is usually formed in 1:1 metal:ligand stoichiometry (Kozlowski et al, 2005). Also, in the present study, it is assumed that copper binding to tobramycin resulted in 1:1 metal ligand complex formation at mM concentration in water. This ratio was suggested in the method for synthesis of copper-aminoglycoside complexes by Sreedhara, Freed & Cowan (2000) and further confirmed for kanamycin A (Szczepanik et al, 2003) vancomycin (Swiatek et al, 2005) and tobramycin (Patwardhan & Cowan, 2011; Jezowska-Bojczuk et al, 1998). Moreover, complexes of 1:2 metal:ligand stichometry are rare and are typically formed at higher pH (Kozlowski et al, 2005).
An extinction coefficient was calculated for copper-tobramycin complex only at the wavelength $\lambda=250$. The complex at the wavelength $\lambda=706$ nm showed very low absorbance values, that resulted in a poor fit of 0.8650. The molar extinction coefficient equalled $1229\, \text{M}^{-1}\text{cm}^{-1}$. In general, all ionic compounds, including complexes dissociate, when dissolved in water into smaller particles or ions. Indeed, aqueous solutions of strong electrolytes are characterised by non ideal properties, due to their partial dissociation and hydration (Heyrovská, 1996). Patwardhan & Cowan (2011) calculated the value of $\varepsilon$ of copper-tobramycin to be slightly higher, $1453\, \text{M}^{-1}\text{cm}^{-1}$. Moreover, $K_D$ of copper-tobramycin, prepared by a titration of a range of tobramycin concentrations ($0.0244 - 0.7813$ mM) to a constant, $0.7813$ mM copper sulphate, was calculated to be $0.05$ mM ($pK_D 4.301$). In comparison, Patwardhan & Cowan (2011) calculated $K_D$ of their copper-tobramycin complex to be $0.1$ mM ($pK_D 5.000$). Additionally, they titrated a range of copper sulphate to a constant concentration of tobramycin, prepared the samples in $100$ mM HEPES buffer, pH 7.4 and performed UV-VIS spectroscopy at $25^\circ\text{C}$. There are however two possible explanations for different values obtained for the same complex. The first one could be related to the different solutes used in the assay, HEPES and water. HEPES is one of the zwitterionic N-substituted aminosulphonic buffers first introduced by Good and coworkers (1966). Hegetschweiler & Saltman (1986) reported a weak but significant interaction between HEPES and Cu(II). Their conclusion was based on indirect evidence gained from the increased reduction rates of Cu$^{2+}$ to Cu$^{+}$ in a series of alcohols and heme proteins in the presence of HEPES. Also, Vascenocelos et al (1996) observed that HEPES at pH 8.0 had the ability to complex copper(II). Vascenocelos et al (1998) noted the aggregation between buffer molecules that decrease the value of the stability constant with increased buffer concentrations. Moreover, the level of contamination in Good’s buffers with metal binding ligands, such as 4-(2-hydroxyethyl)piperazine-1-propanesulphonic acid (HEPPS) and N-(2-hydroxyethyl)piperazine-N’-(2-hydroxy-propanesulphonic acid) (HEPPSO) appears to be highly variable with the greatest amount in HEPES (Mash et al, 2003). In the current work, the preparation of copper sulphate in HEPES, especially in high concentrations, indicated aggregation. Additionally, Vascenocelos et al (1996) suggested that Cu-HEPES complexes might be responsible for the rise of copper toxicity in cell culture experiments. However, the presence in cell culture media, of a high concentration of
HEPES buffer, enhances the rate of copper(II) binding to ligands on the algal surfaces, thus increasing metal bioavailability (Vascenocelos et al., 1996). However, HEPES does not form ternary complexes with copper and therefore it can still be recommended for copper studies, if metal binding characteristic of tested substances is determined prior to use (Sokolowska & Bal, 2005; Mash et al., 2003).

Another difference could be related to the solution pH. Patwardhan & Cowan (2011) conducted their experiments at pH 7.4, whereas the pH in the current study was 6.0. Lower pH and hyperacidification has been reported in CF airway surface fluid and organelles of CF respiratory epithelial cells, such as trans-Golgi and endosomes, as a result of CFTR defect (Poschet et al., 2002). Therefore, pH 6.0, not physiological pH 7.4, better reflects the CF lung environment. Moreover, kanamycin B, amikacin and tobramycin complexes with Cu(II) are more stable in lower pH, demonstrating lower pK$_D$ (Gokhale et al., 2007). Copper-tobramycin complex in the current study (K$_D$ = 0.05 mM) was therefore twice as stable as the complex in the study of Patwardhan & Cowan (2011) (K$_D$ = 0.1 mM).

The difference in the assay temperature, 25°C (Patwardhan & Cowan, 2011) versus 37°C, here, should not influence the results.

Many SOD mimetics reported are copper(II) complexes (Laplyue, 1990). Indeed, the copper(II) ion is the most active SOD catalyst known. In the pH range, excluding hydrolysis of copper(II) complex, copper(II) ion activity is four times higher than that of the Cu/Zn SOD at identical copper concentrations. Moreover, hydrated Cu(II) ions are easily complexable and possess a short life-time in biological media containing many potential soluble or immobilised ligands. The complexes formed may or may not possess the SOD activity (Laplyue, 1990).

Copper-tobramycin was shown to have SOD-like activity, unlike tobramycin and therefore, this activity is due to the presence of copper. Many other copper complexes have been previously reported to have SOD-like activity. Mixed-ligand bipyridyl Cu(II) complexes demonstrated high SOD-like activity in a chemical superoxide anion-generating system (NBT system), with IC$_{50}$ values in the low micromolar range (0.8 - 1.7 µM). These bipyridyl complexes were also effective scavengers of ROS generated
by phagocytes ex vivo. Thus, they represent a promising class of SOD mimetics for future development (Potapov et al, 2009).

Copper-tobramycin was demonstrated to be more efficient as a SOD-like complex than mixed-ligand bipyridyl Cu(II) complexes. Copper-tobramycin potently inhibited NBT reduction with a lower IC₅₀ of 200 nM. Copper-tobramycin was also shown not to have a catalase activity up to 0.5 mM, a top concentration used in most experimental procedures. This is therefore crucial to further investigate the role of copper-tobramycin complex in relation to its SOD-like activity in a model of neutrophil transendothelial migration described.
CHAPTER 3
THE ROLE OF HEPARAN SULPHATE IN THE UPTAKE
OF TOBRAMYCIN AND COPPER-TOBRAMYCIN BY
HLMVEC

3.1. INTRODUCTION

3.1.1. MIMICKING CF ENDOTHELium

Human lung microvascular endothelial cells (HLMVEC) are a useful tool for studying various aspects of the pathology and biology of the pulmonary microvasculature in vitro. HLMVEC form tight junctions and are known to express adhesion molecules including ELAM, P-selectin, ICAM and VCAM. They stain positive for vWF antigen and PECAM and negative for smooth muscle α-actin (Hewett & Murray, 1993b). This cell line was therefore chosen to study pulmonary inflammation present in respiratory disorders, such as CF.

Additionally, the lack of a CF endothelial cell line, entailed the use of a specific inhibitor of CFTR, CFTRinh-172 [3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4thiazolidinone] to model CF endothelium. Human endothelial cells also express CFTR (Tousson et al, 1998). As CFTR is a low-conductance cyclic, nucleotide-regulated Cl⁻ channel, its mutation may affect ion transport and pH regulation, ATP transport, vascular tone and signalling pathways within EC. In the effect, CFTR inhibition is expected to mimic CF inflammatory profile (Perez et al, 2007).

3.1.2. GLYcosaminoglycANS AND PROTEOGlycANS IN NORMAL AND CF Lung

A glycosaminoglycan (GAG) is a linear and negatively charged heteropolysaccharide possessing a characteristic disaccharide repeat sequence. One monosaccharide of the disaccharide repeat is an aminosugar with D-glucosamine or galactosamine and the
other unit is typically an uronic acid residue of either D-glucuronic acid or L-iduronic acid. Both units are variably N- and O-sulphated, which adds to the heterogeneity of these complex macromolecules (Jackson et al, 1991). The chain lengths can vary hugely and the molar weight of roughly 10 – 100 kDa allows these chains to have a great variance in structure and size. GAGs are divided into two principal types. Non-sulphated GAGs consist of hyaluronic acid (HA), whereas sulphated GAGs include chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS) and heparin (Ghanghi & Mancera, 2008) (Figure 3.1). With the exceptions of heparin and HA, GAGs chains are covalently attached at their reducing end through an O-glycosidic linkage to a serine residue or N-linked to asparagine in the core protein; the resulting molecule is termed proteoglycan. Proteoglycans contain one of the major GAGs thus are termed chondroitin sulphate proteoglycan (CSPG), heparan sulphate proteoglycan (HSPG), keratan sulphate proteoglycan (KSPG) and dermatan sulphate proteoglycan (DSPG) (Jackson et al, 1991).

Proteoglycans are present in the intracellular granules, extracellular spaces and occur as integral components of BMs in probably all mammalian tissues (Jackson et al, 1991). However, Proteoglycans are not only structural components, but are now recognized to play an important role in controlling the inflammatory response (Wight, 2008;
Parish, 2006; Day & de la Motte, 2005; Gotte, 2003). Studies of Jiang et al (2005), Schaefer et al (2005) and Taylor et al (2004) show that fragments of GAGs and soluble proteoglycans initiate the inflammatory process through activation of TLRs. Proteoglycans in the extracellular matrix (ECM) also interact and modify the function of cytokines, chemokines, adhesion molecules and proteases to influence immune cell phenotype. Proteoglycans and GAGs play a crucial role in leukocyte adhesion and subsequent migration into tissues (Parish, 2006, Taylor & Gallo, 2006; Gotte, 2003). Migrating leukocytes also enzymatically modify the ECM microenvironment to drive further the inflammatory response (Schor et al, 2000). Moreover, proteoglycans may interact with matrix metalloproteinases (MMPs) and increase their activity (McCawley & Matrisian, 2001).

In the lung, GAGs are distributed in the ECM, including the interstitial space between the capillary endothelium and alveolar epithelium, the subepithelial tissue, bronchial walls, and airway secretions (Reeves et al, 2011). All classes of GAGs are found in normal lungs, where HS is the predominant one (40 – 60 %), followed by CS/DS (31 %), HA (14 %) and heparin (5 %) (Sampson et al, 1984).

HS is the most ubiquitous GAG, which is expressed on practically every cell in the body and comprises 50 – 90 % of the total endothelial proteoglycans (Ihrcke et al, 1993). Although HS is synthesized in a cell-bound form, it can also be shed in a soluble form (Ihrcke & Plat, 1996). HS proteoglycans expressed in the ECM include perlecan, agrin and collagen type XVIII, which are found in normal lungs. The transmembrane syndecans and the glycosylophosphoinositide (GPI)-linked glypicans are two HSPG families localised to cell surfaces (Bernfield et al, 1999) (Figure 3.2, over). In the lungs, syndecan-1 is expressed primarily on the basolateral surface of airway epithelial cells and localised to specific cells in the alveolar septa. Syndecan-2 is expressed on endothelial cells and human lung fibroblasts. Syndecan-3 is not found in normal lung tissue, while syndecan-4 is ubiquitously expressed in a number of tissues, including the lungs, and found on both stromal cells and leukocytes (Kim et al, 1994).
HS by binding to laminin, supports BM structure (Battaglia et al, 1992) and by binding to collagen V plays an important role in cell substrate adhesion (LeBaron et al, 1989). A strong charge of HS, influences its interaction non-covalently with basic proteins and involvement in cell-matrix interactions, activation of cytokines, enzymes and growth factors (Whitelock & Iozzo, 2005). HS reversibly interacts with the following cytokines, IL-5, IL-6, IL-8, IL-10, TNF-α and PF-4 (Menart et al, 2002; Mummery & Rider, 2000; Salek-Adrakani et al, 2000; Lipscombe et al, 1998; Spillmann et al, 1998, Stringer & Gallagher, 1997). Binding of IL-8 to HSPGs, including syndecan-1, renders IL-8 resistant to proteolysis, thus increasing the half-life and activity of the chemokine of the site of inflammation within the airways (Solic et al, 2005, Marshall et al, 2003; Kuschert et al, 1999). In CF, increased expression of HS and subsequent binding to IL-8 has been related to the sustained inflammatory response and continued recruitment of neutrophils in CF bronchial tissue. Intact HS was significantly more abundant in epithelial and endothelial BMs in CF than COPD patients. These authors concluded that sustained neutrophil recruitment in the CF airway may therefore arise from not only increased IL-8 expression, but also from the increased stability, prolonged activity and retention of IL-8 bound to elevated levels of HS in CF bronchial tissue (Solic et al,
2005). Studies by Reeves et al (2010) identified the potential of IL-8 to determine the fate of other inflammatory molecules, such as IL-18, within the inflammatory milieu of the CF lung. Moreover, Plotkowsky et al (2001) suggested that HS PGs are potential receptors for Pseudomonas aeruginosa adherence to epithelial respiratory cells.

3.1.3. HEPARANASES

Heparanase is an endoglycosidase cleaving cell surface HS and therefore participating in degradation and remodeling of the ECM (Nakajima et al, 1983). It has been directly implicated in the promotion of tumor invasiveness and angiogenesis (Vlodavsky et al, 1988). Heparanase may regulate growth factor action by releasing HS-bound growth factors, such as basic fibroblast growth factor (bFGF) from cell surfaces or from the ECM (Ishai-Michaeli et al, 1990).

Human heparanase has an important housekeeping function within the late endosome and lysosomal organelles in cancer and inflammatory cells. This location determinates its role in the processing and recycling of HS from internalized HS PGs. However, the heparanase protein is also present in the nucleus, perinuclear regions and plasma membrane (Goldshmidt et al, 2002a; Goldshmidt et al, 2002b). Activation of macrophages by PMA induced HS degradation of endothelium-derived ECM and redistribution of heparanase to the cell surface (Sasaki et al, 2004). Additionally, HS secretion was augmented by treatment of human microvascular endothelial cells with TNF-α and IL-1β as well as human peripheral T cells with TNF-α (Chen et al, 2004, Sotnikov et al, 2004).

Heparanase activity has been detected in activated leukocytes, such as neutrophils, monocytes and T cells mediating extravasation and traffic to inflammatory sites (Vaday & Lider, 2000). Platelets are also recognised as a very rich source of heparanase and their aggregation with tumor cells is believed to facilitate tumor cell metastases and ECM disassembly following platelet degranulation (Freeman & Parish, 1998). Indeed, platelet heparanase activity derives from CTAP III and other amino terminal deletion isoforms, isolated from human platelets and characterized. Interestingly, highly
purified native CTAP III from platelets was shown to be completely devoid of heparanase activity (Castor et al, 2002).

Based on biochemical studies, an occurrence of several distinct heparanases has been reported (Bame et al, 2002; Bame et al, 1998). However, cloning the enzyme from different human tissues revealed a single gene (Hulett et al, 1999; Toyoshima & Nakajima, 1999; Vlodavsky et al, 1999), suggesting that mammalian cells express primarily one heparanase (Vlodavsky et al, 2002). Heparanase is synthesized as a 65 kDa inactive precursor, which is proteolytically cleavage to 8 and 50 kDa protein subunits that form an active enzyme by heterodimerization (Levy-Adam et al, 2003).

Three forms of heparinase (heparitinase) enzymes or heparin lyase from Flavobacterium heparinum, involved in degradation of heparin have been isolated and characterised (Figure 3.3) (Silva & Dietrich, 1974). Heparinase I cleaves heparin and HS at the linkages between hexosamines and O-sulfated iduronic acids. Additionally, heparinase I showed activity against the saccharide sequence of antithrombin binding region in heparin. Heparinase II cleaves HS, and to a lesser extent heparin, at the 1–4 linkages between hexosamines and uronic acid residues (both glucuronic and iduronic). Heparinase III cleaves at the 1–4 linkages between hexosamine and glucuronic acid residues in HS. The enzyme is not active towards heparin or low molecular weight heparins (Desai et al, 1993; Linhardt et al, 1990; Nader et al, 1990).

Figure 3.3. Substrate specificities of the heparin lyases (Yamada et al, 1998).
3.1.4. GAG – Cu(II) INTERACTIONS

Heparin complexes with transition metal ions have been studied previously (Singh & Bhattacharya, 1965). The ability of heparin to form complexes with cobalt ions and a high cobalt affinity was associated with a high biological activity of heparin (Mathews, 1964). Also, the presence of zinc greatly augmented the binding of histamine molecules by heparin (Kerp, 1963; Kerp & Steinhaeuser, 1961). Liberti and Stivala (1967) reported a positive correlation between copper binding of heparin and heparin biological activity. The sites for this cooperative binding may be the ionized carboxyl groups and additional binding of a smaller number of copper ions occurs initially, probably with the N-sulphate groups (Lages & Stivala, 1973). Mukherjee et al (1978) demonstrated that all GAGs, except KS, were capable of binding copper(II), affording a complex of an absorbance maxima near 237 nm. They hence suggested that this absorbance was a result of a charge transfer complex between copper(II) ions and carboxylate groups in the GAG.

Previous reports have shown that ROS are able to degrade proteoglycan core proteins, and GAGs, including HS and heparin or can inhibit de novo synthesis of proteoglycans (Moseley et al, 1995; Liu & Perlin, 1994; Panasyuk et al, 1994; Klebanoff et al, 1993). The results of Moseley et al (1995) suggest that the presence of sulphate on the GAG chain may protect the molecule against ROS attack. Heparin affects leukocyte function by inhibiting the generation of ROS (Dadona et al, 1999). Moreover, heparin enhances the activity of SOD, in addition to releasing it from human umbilical veins, which suggests that heparin itself may have some superoxide dismutase activity in vitro (Gill et al, 1999). Also, glycosaminoglycans were reported to reduce oxidative damage induced by copper (Cu^{2+}), iron (Fe^{3+}) and hydrogen peroxide (H_{2}O_{2}) in human fibroblast cultures (Campo et al, 2004).

3.1.5. THE UPTAKE OF AMINOGLYCOSIDES

Aminoglycoside antibiotics are indicated in the treatment of acute exacerbations of CF, to control chronic infection, and to eradicate *Pseudomonas aeruginosa* after recent
acquisition (reviewed by Prayle & Smyth, 2010). Aminoglycosides have been associated with high clinical effectiveness and their uptake by bacteria has been well characterised. However, the action of aminoglycoside on human cells results in nephro- and oto-toxicity (Section 1.9.2). There are at least two characterized mechanisms for aminoglycoside entry into human cells.

A receptor-mediated endocytosis at the apical membrane of sensory hair cells of the inner ear determines one mechanism of aminoglycoside uptake (Hashino & Shero, 1995). Internalized aminoglycosides are transported via vesicular traffic into lysosomes, where they accumulate over time and eventually disrupt lysosomes (Hashino et al., 1997). In kidney cells, aminoglycosides are also transported through the endoplasmic reticulum (ER) and the Golgi complex in a retrograde manner, and are subsequently released into the cytosol from the ER (Sandoval & Molitoris, 2004).

Another mechanism of aminoglycoside entry is ion channel permeation into cells. In cochlear sensory hair cells, aminoglycosides penetrate through the mechanosensitive transduction channels, which are located at the tips of individual stereocilia comprising the hair bundle (Marcotti et al., 2005). In cultured kidney cells, chemical agonists of the transient receptor potential vanilloid type 1, low extracellular Ca\(^{2+}\) levels, negative cellular potentials or acidic extracellular pH, increase the cellular uptake of aminoglycosides, further suggesting that they permeate through cation channels (Myrdal & Steyger, 2005; Meyers et al., 2003).

In spite of the fact that aminoglycoside toxicity occurs via multiple pathways in a both a drug- and time course-specific manner, divalent cations, such as Ca\(^{2+}\) and Mg\(^{2+}\), have been shown to provide protection from aminoglycoside antibiotics (Coffin et al., 2009; Humes et al., 1984; Ramirez-Ronda et al., 1975).

Also, acidic phospholipids have been implicated as integral binding components for the energy-dependent aminoglycoside transporter of bacterial membranes (Dickie et al., 1978). The interaction between aminoglycosides and phosphatidylinositol could be an alternative mechanism of the otoxicity of this class of drugs (Orsulakowa et al., 1976).
3.1.6. THE ROLE OF PROTEOGLYCANS IN AMINOGLYCOSIDE UPTAKE

Fluorescent aminoglycosides (1 and 3, Figure 3.4, over) were reported to exhibit poor cellular uptake (Luedtke et al, 2003), unlike to their derivatives, in which the amino groups of naturally occurring aminoglycoside antibiotics were converted to guanidinum groups, as described by Luedtke et al (2000). Indeed, upon guanidinylation, the cellular uptake of tobramycin is enhanced by approximately 10-fold and the enhancement of neomycin B is approximately 20-fold (Luedtke et al, 2003). To examine how the cellular uptake of guanoglycosides is affected by guanidinylation, Luedtke et al (2003) have synthesized a series of boron dipyrromethene (BODIPY)-tagged aminoglycosides and guanidinoglycosides based upon tobramycin and neomycin B. BODIPY is a fluorescent probe, claimed to be insensitive to the changes in local environment. Guanidino-tobra-BODIPY (2) has four fewer guanidinum groups than BODIPY-Cys(Arg)$_9$ (5), but shows approximately the same transport efficiency. Importantly, guanidine-neo-BODIPY (4) consistently has a better cellular uptake as compared to the poly-Arg peptide BODIPY-Cys(Arg)$_9$ (5). Moreover, guanidino-neomycin B (6) effectively inhibits the transport of BODIPY-Cys(Arg)$_9$ into cells, suggesting a common pathway responsible for the uptake of both compounds. Microscopy experiments carried out by the same authors indicates that approximately one-half of HeLa cells incubated with guanidinoglycosides or fluorescein-labelled conjugates exhibit a highly diffuse, cytoplasmic, and nuclear distribution of these compounds, while the other one-half exhibit more localized nucleolar staining, similar to that reported for poly-Arg peptides (Luedtke et al, 2003). Moreover, Elson-Schwab et al (2007) demonstrated that a derivative of the aminoglycoside antibiotic neomycin, in which all of the ammonium groups have been converted into guanidinum groups (guanidinoneomycin), can carry large (over 300 kDa) bioactive molecules across cell membranes. Delivery occurs at nanomolar transporter concentrations and under these conditions depends entirely on cell surface HSPGs. In contrast, arginine-rich peptides show both HS-dependent and HS-independent cellular pathway (Elson-Schwab et al, 2007; Suzuki et al, 2002).
Figure 3.4. Aminoglycosides and guanidinoglycosides evaluated for cellular uptake (Luedtke et al., 2003).

3.2. HYPOTHESIS, AIMS AND OBJECTIVES

The hypothesis of the study was that tobramycin and copper-tobramycin are taken up by human endothelial cells.

The aim was to investigate tobramycin and copper-tobramycin complex uptake by the host cells, such as HLMVEC and neutrophils in order to analyse the role of HS and CFTR in tobramycin uptake by endothelial cells using immunocytochemistry.
3.3. MATERIALS

Lab-Tek™ II Chamber Slide™ System was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fisher Scientific (Loughborough, Leicestershire, UK) provided methanol, ethanol, Tween-20 and TRIS base.

Collagen IV from human placenta, heparitinase II from Flavobacterium heparinum, bovine serum albumin (BSA), inactivated fetal bovine serum (FBS), Hoechst blue and Poly-L-Lysine solution 0.1 % (w/v) in water were from Sigma-Aldrich Inc. (Poole, Dorset, UK). Streptavidin-HRP conjugate and normal rabbit serum was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). Complete, mini, protease inhibitor cocktail tablets were from Roche Ltd. (Wewlyn Garden City, Hertfordshire, UK).

Peprotech EC (London, UK) provided recombinant human TNF-α and recombinant human IL-8. Invitrogen Ltd. (Paisley, UK) supplied Phosphate Buffered Saline (PBS), donkey anti-sheep Alexa Fluor 594, Alexa Fluor 488® F(ab’)2 fragment of rabbit anti-mouse IgG (H+L) and Alexa Fluor 555® F(ab’) fragment of goat anti-rabbit IgG (H+L). Anti-HS Hepss-1 mouse was from Seikagaku Corporation (Tokyo, Japan). A polyclonal antibody raised against NBD1, with three prominent epitopes in NBD1 rabbit (Mr. Pink) and 1086.2 Mab mouse CFTR antibody was purchased from Sorscher Lab (Birmingham, Alabama, USA). Goat, anti-mouse polyclonal biotinylated antibody was from Dako UK Ltd. (Ely, Cambridgeshire, UK).

BDH/Merck (Poole, Dorset, UK) supplied paraformaldehyde. FluorPreserve™ Reagent, unfractioned heparin and CFTR inhibitor-172, 3-[(3-Trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone were from Calbiochem/Merck (Nottingham, Nottinghamshire, UK).

Randox Laboratories Ltd. (Crumlin, Co. Antrim, UK) provided competitive ELISA kit for tobramycin evaluation, which also included sheep anti-tobramycin.

Cyto Tox-Glo™ Cytotoxicity Assay was from Promega Inc. (Rocky Hill, NJ).
3.4. METHODS

3.4.1. COLLAGEN IV COATING OF CELL CULTURE DISHES

The method was adapted from Sigma, as supplied with product. Lyophilised collagen IV (Sigma Aldrich Inc., Poole, Dorset, UK) was reconstituted to 0.1 mg/ml in 3 % (v/v) acetic acid and left to dissolve for half an hour at room temperature on the roller mixer. Different volumes of collagen solution were added into appropriate cell culture dishes: 100 µl/well of a 96-well plate or 250 µl/well of a chamber slide for 1 to 2 hours incubation at room temperature. Then, the excess of collagen IV was removed and wells allowed to dry for 1 hour at room temperature. Wells of cell culture dishes were washed with HBSS (- Ca/Mg) containing phenol red and this was repeated at least 3 times. To ensure all acid was removed, the wells were also washed with RPMI 1640, followed by a final rinsing with 70 % (v/v) ethanol to sterilize. The dishes were allowed to air dry and they were stored at 2 – 8°C before use, maintaining sterility.

3.4.2. SUBCULTURING OF HLMVEC INTO 8-WELL CHAMBER SLIDES

250 µl fresh and warmed growth medium was added per well of a collagen IV coated 8-well chamber slide to allow better cell distribution. It was followed by an addition of 250 µl of cell suspension at 0.7 x 10^5/ml (0.175 x 10^5/well).

3.4.3. TREATMENT OF HLMVEC IN 8-WELL CHAMBER SLIDES

50 mM tobramycin/copper-tobramycin stock and appropriate dilutions were prepared in water. The drugs were added to cell cultures (0.01 – 0.5 mM final concentrations) and incubated for 3.5 hours or 24 hours at 37°C and 5 % CO₂.

To investigate the role of HS, the HLMVEC cultures were treated with 5 U/ml heparitinase II from Flavobacterium heparinum (Sigma Aldrich) 1/20 dilution from 100 U/ml stock) for 4 hours, followed by incubation with tobramycin and copper-tobramycin (0.01 – 0.5 mM) for 3.5 hours at 37°C and 5 % CO₂.

In order to model CF endothelium, confluent HLMVEC were treated with 20 µM CFTRinh-172 for 30 hours, 6 hours before the 24-hour incubation with 10 ng/ml TNF-α.
at final concentrations. The CFTRinh-172 stock of 20 mM was prepared in DMSO and the TNF-α stock of 100 µg/ml was prepared in 1 % (w/v) BSA/PBS. Both, CFTRinh-172 and TNF-α dilutions were carried out in full growth medium. CFTRinh-172 was also added to unactivated cells for 24 hours or in the presence of 5 U/ml heparitinase II for 4 hours. Tobramycin and copper-tobramycin were added at the concentration of 0.01 – 0.5 mM to cell cultures for last 3.5 hours incubation after heparitinase II treatment. Also, NAC (20 mM from 500 mM stock) and CuSO₄ (0.5 mM from 50 mM stock) were tested on CFTRinh-172-treated endothelium in the absence and presence of TNF-α (10 ng/ml) for 3.5 hours.

All experiments, including 8-well chamber slides, were carried out in full growth medium, which was added to every well in the total volume of 250 µl.

3.4.4. IMMUNOCYTOCHEMICAL STAINING OF HLMVEC FOR GAGs

HLMVEC at were cultured at 0.7 x 10⁵/ml (0.175 x 10⁵/well, 0.25 ml) on collagen IV coated Microtek 8-well chamber slides (Thermo Fisher) in full EGM and challenged with drugs when confluent (1 – 2 days). The supernatants were removed and the cells were washed using 500 µl/well warm PBS (- Ca/Mg). The cells were then fixed by adding 500 µl/well of 1 % (w/v) PFA dissolved in 15 % (v/v) picric acid for 30 minutes at room temperature. The cells were washed 3 x by adding 500 µl/well of PBS (- Ca/Mg). The cells were permeabilised by adding 500 µl/well ice-cold methanol (100 %) for 5 minutes at -20°C or this step was omitted. The cells were washed 3 x by adding 500 µl/well of PBS (- Ca/Mg) and incubated with 500 µl/well of 2 % (w/v) BSA/PBS plus 10 % (v/v) rabbit serum at 4°C overnight (16 hours) to block non-specific binding sites. The primary antibody, anti HS-mouse IgM at 1 mg/ml was diluted 1/1000 in 1 % (w/v) BSA/PBS, resulting in 1 µg/ml final concentration and 100 µl added per well for 1 hour incubation at room temperature. The cells were washed 5 x using 0.02 % (v/v) Tween-20/PBS for 5 minutes. The secondary antibody, rabbit anti-mouse Alexa Fluor 488, F(ab')₂ 2 mg/ml was diluted 1/800 in 1 % (w/v) BSA/PBS, resulting in 5 µg/ml final concentration. The cells were washed 5 x with 0.02 % (v/v) Tween-20/PBS for 5 minutes. The chamber scaffold and the gasket were removed and the slide was
mounted in Fluor Preserve Reagent (Calbiochem). The cells were viewed using a Zeiss LSM 710 confocal microscope (ex. 488 nm, em. 540 nm).

3.4.5. IMMUNOCYTOCHEMICAL STAINING OF HLMVEC FOR TOBRAMYCIN

HLMVEC were subcultured for Microtek 8-well chamber slides (Thermo Fisher) at 0.7 x 10^5/ml (0.175 x 10^5/well, 0.25 ml). The cells were cultured until confluency (at least 48 hours). The supernatants were removed and the cells washed with 500 µl of PBS (-Ca/Mg) per well. The cells were fixed by adding 500 µl/well 4 % (w/v) PFA/PBS for 30 minutes at room temperature. The slides were washed 3 x by adding 500 µl PBS (-Ca/Mg) per well. The cells were permeabilised by adding 500 µl per well of ice-cold methanol (100 %) for 5 minutes at -20°C. The slides were washed 3 x as before. The cells were incubated with in PBS containing 1 % (w/v) BSA at 4°C for 16 hours to block non specific binding sites. The primary antibody, sheep anti-tobramycin (Randox) was diluted from the stock concentration of 5.84 mg/ml to the final concentration of 10 µg/ml in 1 % (w/v) BSA/PBS. 100 µl/well primary antibody was incubated for 1 hour at room temperature. The cells were washed 5 x 5 minutes with 500 µl of 0.02 % (v/v) Tween-20/PBS (-Ca/Mg) per well. The stock concentration of 2 mg/ml of the secondary antibody, donkey anti-sheep Alexa Fluor 594 (Molecular Probes) was diluted 1/400 in 1 % (w/v) BSA/PBS (-Ca/Mg) and 100 µl of diluted secondary antibody was added per well for 1 hour incubation at room temperature. Each well of the slide was washed 5 x 5 minutes using 500 µl/well 0.02 % (v/v) Tween-20/PBS (-Ca/Mg). The chamber scaffold and the gasket were removed and the slide was mounted using Fluor Preserve Reagent (Calbiochem). The cells were viewed on a Zeiss LSM 710 confocal microscope (ex. 591 nm, em. 618 nm).

3.4.6. IMMUNOHISTOCHEMICAL STAINING OF HLMVEC FOR CFTR

HLMVEC were cultured on collagen IV coated Microtek 8-well chamber slides chamber slides (Thermo Fisher) in full EGM until confluent (1 – 2 days) and challenged with drugs. After drug treatment, the cells were washed 2 x 5 minutes using PBS (+ Ca/Mg), 500 µl/well. The cells were fixed for 30 minutes using 1 % (w/v) PFA dissolved in 15 %
(v/v) picric acid in PBS (+ Ca/Mg) and washed again. They were then permeabilised by incubating for 10 minutes in 0.1% (v/v) Triton X-100/PBS (+ Ca/Mg). The cells were washed 3 x 5 minutes using PBS (+ Ca/Mg) and incubated in 2% (w/v) BSA/PBS (+ Ca/Mg) overnight at 4°C. The primary antibody, Mr Pink (against purified NBD1 domain of human CFTR in rabbit) was diluted from 1 mg/ml stock 1/500 dilution in 0.2% (w/v) BSA/PBS (+ Ca/Mg) and incubated for 1 hour at room temperature (100 µl/well). The cells were washed 3 x 5 minutes using PBS (+ Ca/Mg). The secondary antibody, goat anti-rabbit-555 was diluted 1/400 in 0.2% (w/v) BSA/PBS (+ Ca/Mg) and incubated for 40 minutes at room temperature (100 µl/well). The cells were washed 3 x 5 minutes using PBS (+ Ca/Mg), followed by removal of the chamber scaffold and the gasket. The slide was mounted using Fluor Preserve Reagent (Calbiochem) and the cells examined using a Zeiss LSM 710 confocal microscope (ex. 544, em. 590 nm).

3.4.7. TOBRAMYCIN UPTAKE BY NEUTROPHILS

Neutrophils were isolated according to the procedure described previously (Section 2.4.1), re-suspended in RPMI 1640 + 2.5% (v/v) FBS and diluted to 1 x 10^7 cells/ml. The cells were pre-incubated with tobramycin or copper-tobramycin (0.5 mM) for 30 minutes at 37°C, followed by treatment with IL-8 (6.25 x 10^-8 M) for 3 hours. The final concentration of IL-8, 6.25 x 10^-8 M, was obtained from 1.25 x 10^-5 M IL-8 stock by 1/200 dilution in RPMI 1640 + 2.5% (v/v) FBS. The experiment was performed in duplicate in the reaction volume of 100 µl. After 3.5 hours incubation time, the reaction was stopped on ice and cytospins on neutrophil suspensions were prepared by centrifugation at 450 x g for 5 minutes. The glass slides used for cytospins were coated with poly-L-lysine solution (Sigma) for 5 minutes and stored dry before use.

Other experiments included 5, 15 and 30 minutes incubation of neutrophils at 1 x 10^7 cells/ml at 37°C without further activation, followed by cytospins.

3.4.8. IMMUNOCYTOCHEMICAL STAINING OF NEUTROPHILS FOR TOBRAMYCIN

Dried slides were fixed by adding 500 µl/slide of 4% (w/v) PFA/PBS for 30 minutes at room temperature, followed by 3 washes with 500 µl PBS (- Ca/Mg) per slide. The cells
were permeabilised by adding 500 µl per slide of ice-cold methanol (100 %) for 5 minutes at -20\(^{\circ}\)C and washed 3 x by adding 500 µl PBS (- Ca/Mg) per slide. They were then blocked overnight with 1 % (w/v) BSA/PBS (- Ca/Mg) at 4\(^{\circ}\)C. The slides were washed with PBS (- Ca/Mg). The primary antibody, sheep anti-tobramycin (Randox) was diluted from the stock concentration of 5.84 mg/ml to 10 µg/ml final concentration in 1 % (w/v) BSA/PBS and incubated on slides for 1 hour at room temperature (100 µl/slide). The slides were washed 5 x 5 minutes with 500 µl of 0.02 % (v/v) Tween-20/PBS (- Ca/Mg) per slide. The stock concentration of 2 mg/ml of the secondary antibody, donkey anti-sheep Alexa Fluor 594 (Molecular Probes) was diluted 1/400 in 1 % (w/v) BSA/PBS (- Ca/Mg) and added for 1 hour incubation at room temperature (100 µl/well). Each slide was washed 5 x 5 minutes using 500 µl/well 0.02 % (v/v) Tween-20/PBS (- Ca/Mg) and eventually mounted using Fluor Preserve Reagent (Calbiochem) before proceeding for confocal microscopy.

3.4.9. SUBCULTURING OF HLMVEC INTO COLLAGEN IV COATED 96-WELL PLATES FOR A VIABILITY ASSAY

Collagen IV coated 96-well plates were incubated for several minutes at 37\(^{\circ}\)C and 5 % CO\(_2\) before using for subculturing. An aliquot of 100 µl growth medium was dispensed per well for better cell distribution. HLMVEC were diluted to 2 x 10\(^5\)/ml in growth medium and 100 µl cell suspension was dispensed per well of each 96-well plate. The plates were incubated for at least 48 hours at 37\(^{\circ}\)C and 5 % CO\(_2\) until confluent. The cells were fed with 100 µl of fresh and warmed medium.

3.4.10. TREATMENT OF HLMVEC IN COLLAGEN IV COATED 96-WELL PLATES FOR A VIABILITY ASSAY

Confluent HLMVEC grown on collagen IV coated 96-well plates were treated with the following compounds: tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM), TNF-α (10 ng/ml) and CFTRinh-172 (20 µM) for 24 hours at 37\(^{\circ}\)C and 5 % CO\(_2\).
3.4.11. ENDOTHELIAL CELL VIABILITY ASSESSMENT

The endothelial cell viability was assessed by using the CytoTox-Glo™ assay (Promega). This assay measures a distinct protease activity, released from cells that have lost membrane integrity and therefore associated with cytotoxicity. A luminogenic peptide substrate (alanyl-alanylphenylalanyl-aminoluciferin; AAF-Glo™ Substrate) is here cleaved by a dead-cell protease activity. Following cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase-mediated production of light.

The CytoTox-Glo™ Cytotoxicity Assay Reagent was prepared by transferring the contents of one bottle of Assay Buffer to the AAF-Glo™ Substrate bottle. The Lysis Reagent was prepared by transferring Digitonin to the Assay Buffer and mixed well to ensure homogeneity.

Confluent HLMVEC set up on collagen IV coated 96-well plate were treated with test drugs for 24 hours in a total volume of 100 μl. 50 μl of CytoTox-Glo™ Cytotoxicity Assay Reagent was added to all wells of a plate, mixed briefly by orbital shaking and incubated for 15 minutes at room temperature. The total luminescence was measured on a Wallac Victor² plate reader (Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland). Then, 50 μl of Lysis Reagent was added to all wells of a plate, mixed and incubated at room temperature for 15 minutes. The dead luminescence was measured. The luminescent contribution of previously viable cells (after lysis) was calculated by subtracting the luminescent signal resulting from experimental cell death from total luminescence death according to the following formula:

Viable cell luminescence = Total luminescence – Experimental dead cell luminescence

The experiment was performed once in duplicate.

3.4.12. STATISTICAL ANALYSIS

The results were analysed by using Graph Pad Prism 4.0. Two-tailed unpaired t-test and one-way ANOVA with Dunnett’s multiple comparison test were performed on the data. The results were expressed as average plus/minus SEM and p<0.05 were considered as significant.
3.5. RESULTS

3.5.1. THE PRESENCE OF HS ON ENDOTHELIAL CELL SURFACE AND THE EFFECT OF TNF-α, CFTRinh-172 AND HEPARITINASE II ON GAGs EXPRESSION

The presence of HS on the surface of endothelial cells was examined by using two methods of slide preparation, including both fixation and permeabilization (Figure 3.5, page 104) or fixation step only (Figure 3.6, page 105). Fixation should immobilize the antigens, while retaining authentic cellular and subcellular structure. Fixation can be done using crosslinking reagents, such as paraformaldehyde. It forms intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components and require the addition of a permeabilization step, to allow access of the antibody to the specimen. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Methanol or acetone could also be used for permeabilization. Permeabilization should only be required for intracellular epitopes when the antibody required has access to the inside of the cell to detect the protein. However, it will also be required for detection of transmembrane proteins if the epitope is in the cytoplasmic region. As shown here (Figure 3.5 and Figure 3.6), fixed and permeabilized cells ensured free access of the antibody to the antigen, resulting in better surface staining, compared to cell fixation only. Moreover, the overall quality of staining is better, when the cells were both fixed and permeabilized.

The surface of endothelial cells is decorated with a wide variety of membrane-bound macromolecules, including glycoproteins, proteoglycans, and their associated glycosaminoglycan (GAG) side chains that constitute the glycocalyx. Heparan sulfates are the most common endothelial cell-surface GAG, comprising 50% to 90% of the GAG pool (Ihrcke et al, 1993). Therefore, a specific HS monoclonal antibody was used to detect HS on HLMVEC surface and intracellularly in both fixed and permeabilized cells. Untreated HLMVEC (Figure 3.5, panel 1AB) expressed low level of HS, which was located mainly in the cell nucleus and the cytoplasm. Membrane staining was negligible. TNF-α (panel 2AB), CFTRinh-172 (panel 3AB) and the combination of TNF-α
and CFTRinh-172 (panel 4AB) treatment increased HS expression on the endothelial cell surface compared to untreated control (panel 1AB). TNF-α induced low, whereas CFTRinh-172, especially in the presence of TNF-α-induced, high endothelial HS expression. Additionally, membrane staining was observed in the presence of CFTRinh-172 plus TNF-α.

Figure 3.5. HS on HLMVEC, fixed and permeabilised. Cells were stained with anti-HS Hepss-1 antibody. 1AB) control cells, no stimulus; 2AB) 10 ng/ml TNF-α; 2CD) 10 ng/ml TNF-α plus heparitinase II; 3AB) 20 µM CFTRinh-172; 3CD) 20 µM CFTRinh-172 plus heparitinase II; 4AB) TNF-α + CFTRinh-172; 4CD) TNF-α + CFTRinh-172 plus heparitinase II. Scale bar 100 µm. These represent typical results from two independent experiments.

Heparitinase II from Flavobacterium heparinum was used to demonstrate degradation of HS on the cell surface. Indeed, HLMVEC following heparitinase treatment (panel 2CD, 3CD, 4CD) expressed less HS compared to treatment with TNF-α, CFTRinh-172 or
TNF-α plus CFTRinh-172. However, heparitinase reduced the amount of HS to the level similar as presented by untreated cells.

Figure 3.6. HS on HLMVEC, fixed, not permeabilised. Cells were stained with anti-HS Hepss-1 antibody. 1AB) no treatment; 2AB) 10 ng/ml TNF-α; 2CD) 10 ng/ml TNF-α plus heparitinase II; 3AB) 20 µM CFTRinh-172; 3CD) 20 µM CFTRinh-172 plus heparitinase II; 4AB) TNF-α + CFTRinh-172; 4CD) TNF-α + CFTRinh-172 plus heparitinase II. Scale bar 100 µm, n=1.

3.5.2. TOBRAMYCIN UPTAKE BY ENDOTHELium

The aminoglycoside tobramycin and the copper-tobramycin complex were incubated with HLMVEC at 37°C and 5 % CO₂ in different time frames in order to examine whether these drugs are taken up by endothelial cell cultures. Figure 3.7 and 3.8 (over) show tobramycin and copper-tobramycin uptake by HLMVEC for 3.5 hours, respectively. Tobramycin and copper-tobramycin uptake by HLMVEC for 24 hours is
presented on Figure 3.9 and 3.10 (page 107), respectively. All images presented at each panel were captured at the same confocal microscopy settings and therefore could be directly compared.

Figure 3.7. Tobramycin uptake of HLMVEC for 3.5 hours. 1 – 4) no primary anti-tobramycin antibody; 1 and 5) untreated HLMVEC; 2 and 6) 0.01 mM tobramycin; 3 and 7) 0.1 mM tobramycin; 4 and 8) 0.5 mM tobramycin, Scale bar 100 µm. Microscope magnification 200x. These represent typical results from at least seven independent experiments.

Figure 3.8. Copper-tobramycin uptake of HLMVEC for 3.5 hours. 1 – 4) no primary anti-tobramycin antibody; 1 and 5) untreated HLMVEC; 2 and 6) 0.01 mM tobramycin; 3 and 7) 0.1 mM tobramycin; 4 and 8) 0.5 mM tobramycin, Scale 100 µm. Microscope magnification 200x. These represent typical results from at least four independent experiments.
Figure 3.9. Tobramycin uptake of HLMVEC for 24 hours. 1 – 4) no primary anti-tobramycin antibody; 1 and 5) untreated HLMVEC; 2 and 6) 0.01 mM tobramycin; 3 and 7) 0.1 mM tobramycin; 4 and 8) 0.5 mM tobramycin, Scale 100 µm. Microscope magnification 200x. These represent typical results from at least two independent experiments.

Figure 3.10. Copper-tobramycin uptake of HLMVEC for 24 hours. 1 – 4) no primary anti-tobramycin antibody; 1 and 5) untreated HLMVEC; 2 and 6) 0.01 mM tobramycin; 3 and 7) 0.1 mM tobramycin; 4 and 8) 0.5 mM tobramycin, Scale 100 µm. Microscope magnification 200x. These represent typical results from at least four independent experiments.

There was no cell staining observed in the absence of primary antibody (panel 1 – 4) and very little background staining of untreated cells (panel 5). Both tobramycin and copper-tobramycin were taken up by unactivated human endothelium and this uptake was dose dependent, increasing with increasing concentration of the drugs (panel 6 – 8).
It was also of interest to find out in which cell compartment tobramycin and copper-tobramycin are located following the uptake by human endothelium. For this reason, 400 x microscope magnification was used accompanied by a z-stack with the slice thickness of 1 µm. Figure 3.11 showed localisation of tobramycin within HLMVEC. Tobramycin is located mostly in the cell cytoplasm and in perinuclear area of the cells. There is little nuclear staining, as the nucleus remained dark on the scan. This is also true for copper-tobramycin.

HLMVEC were treated with 0.5 mM of both tobramycin or copper-tobramycin and as an additional control, HLMVEC were counterstained with Hoechst blue to visualise cell
nuclei (Figure 3.12). The split and merged channels of tobramycin and Hoechst blue are shown.

Figure 3.12. HLMVEC stained for tobramycin (anti-tobramycin antibody) with counterstained nucleus (Hoechst blue) under tobramycin and copper-tobramycin. 1) no primary antibody; 2) no treatment; 3) 0.5 mM tobramycin; 4) 0.5 mM copper-tobramycin. A) nucleus stain; B) tobramycin stain, C) both. Scale bar 25 µm.
Although confocal fluorescence allows demonstration of the precise location of fluorophores, confocal images are not suitable for quantification because they have high levels of background noise. In order to quantify tobramycin and copper-tobramycin uptake by HLMVEC for 3.5 hours incubation, a background correction was applied in a manual mode, as described by Zinchuk & Zinchuk (2008) and an online guide: http://www.macbiophotonics.ca/imagej/image_intensity_proce.htm by using ImageJ 1.42q software. Manual mode is much more sensitive and customizable than auto mode and more importantly, it allows performance of background correction using the unique pixel profile of analysed images.

The background correction is exemplified by Figure 3.13.

Figure 3.13. Background correction. A) Original immunocytochemical image of HLMVEC stained for tobramycin. B) The same image after applying threshold, which removes the black background. Note, the edges of the cells must be seen. Scale bar 100 µm. C) Histogram. The highest peak on the left hand side representing the background was removed by applying a threshold at the intensity 8 that is showed by a vertical blue line.
The background was relatively even across the image (panel A) and it was corrected using a threshold value, which was moved from the minimum 0 value until most of the background displayed blue, however, with visible cell edges (panel B). The background removal was controlled by the image histogram (panel C), which displays both the number and intensity of pixels. The low intensity peak accounted for the background, whereas the other peak represented the red fluorescent endothelial stain. The threshold at the value 8 (blue vertical line) successfully removed the background peak from the total intensity of the image area by changing the mean intensity from 42.825 to 45.188.

The mean intensity, calculated as the relationship between the total intensity and the pixel count, was used to express each tobramycin and copper-tobramycin stain. Figure 3.14 confirmed that HLMVEC uptake of both tobramycin and copper-tobramycin is concentration dependent.

![Figure 3.14](image)

Figure 3.14. Immunocytochemical quantitative analysis of tobramycin and copper-tobramycin uptake by HLMVEC for 3.5 hours, following subtracting of baseline. The data is expressed by mean ± SEM of n=3 or 4 separate experiments and analysed using unpaired two-tailed t-test. **<0.01, ***p<0.001.

3.5.3. THE ROLE OF HS IN TOBRAMYCIN AND COPPER-TOBRAMYCIN UPTAKE

The next step was to examine the mechanism of tobramycin and copper-tobramycin uptake by HLMVEC over 3.5 hours. Heparitinase II was used to degrade heparan
sulphate present on the surfaces of HLMVEC, as described in Section 3.4.3. and in Figure 3.5. The effect of heparitinase II on the uptake of tobramycin and its complex with copper was evaluated (Figure 3.15). Tobramycin and copper-tobramycin (0.01 – 0.5 mM) (panels 2 – 4 and 6 – 8, respectively) were taken up in a dose dependent way as shown previously (Figure 3.7 & 3.8). Heparinase treatment (5 U/ml) reduced the uptake of the 0.5 mM concentration of both tobramycin (panel 5) and copper tobramycin (panel 9) by HLMVEC compared to appropriate drug treatment alone (panel 4 and 8, respectively). These results suggest that binding of positively charged aminoglycosides to negatively charged heparan sulphate could be a mechanism of tobramycin and copper-tobramycin uptake by HLMVEC. However, the fluorescence reduction introduced by heparitinase II was not complete, as the cell stain did not return to the baseline (panel 1).

![Figure 3.15](image_url)

Figure 3.15. The effect of heparitinase II on tobramycin and copper-tobramycin uptake by HLMVEC. The cells were stained with anti-tobramycin antibody. 1) negative control; 2) 0.01 mM tobramycin; 3) 0.1 mM tobramycin; 4) 0.5 mM tobramycin; 5) 0.5 mM tobramycin plus 5 U/ml heparitinase II; 6) 0.01 mM copper-tobramycin; 7) 0.1 mM copper-tobramycin; 8) 0.5 mM copper-tobramycin; 9) 0.5 mM copper-tobramycin plus 5 U/ml heparitinase II. Scale bar 100 µm. These results are typical from at least two independent experiments.
3.5.4. TOBRAMYCIN UPTAKE BY NEUTROPHILS

Neutrophils were also examined for tobramycin and copper-tobramycin uptake. Figure 3.16 showed tobramycin and copper-tobramycin treatment of both resting and IL-8-activated normal neutrophils. In respect of the negative controls used in this experiment, which were the absence of primary antibody (panel 1) and no drug treatment (panel 2), tobramycin and copper-tobramycin (0.5 mM) were not taken up by unactivated (panel 3 and 4, respectively) or IL-8 (5 x 10⁸ M)-activated neutrophils (panel 5 and 6, respectively).

Figure 3.16. Tobramycin and copper-tobramycin is not taken up by resting or IL-8 activated neutrophils. The cells were stained with anti-tobramycin antibody. Neutrophils (1 x 10⁷/ml) untreated or treated with tobramycin and copper-tobramycin (0.5 mM) for 3.5 hours and stained for tobramycin, 200x. 1) Control PMN, no primary antibody; 2) Untreated PMN; 3) PMN + 0.5 mM Tob; 4) PMN + 0.5 mM Cu-Tob; 5) PMN + 0.5 mM Tob + IL-8; 6) PMN + 0.5 mM Cu-Tob + IL-8. Bar scale 100 µm, n=1.

3.5.5. THE EFFECT OF CFTRinh-172 ON TOBRAMYCIN UPTAKE BY ENDOTHELIUM

HLMVEC pre-treated for at least 24 hours with 20 µM CFTRinh-172 was used to model CF endothelium. Normal and CF endothelium were then tested with a range of
tobramycin and copper-tobramycin concentrations (0.01 mM – 0.5 mM) for 3.5 hours at 37°C and 5% CO₂. Figure 3.17 showed tobramycin uptake by HLMVEC (panels 2 – 4) compared to untreated endothelium (panel 5). No primary antibody control was also presented (panel 1). CFTR-inh172 did not increase or decrease tobramycin uptake by HLMVEC (panel 6 – 8).

Figure 3.17. The effect of CFTRinh-172 on tobramycin uptake. HLMVEC were incubated for 24 hours with CFTRinh-172 and treated with tobramycin (0.01 - 0.5 mM) for 3.5 hours. 1) no anti-tobramycin primary antibody; 2) 0.01 mM tobramycin; 3) 0.1 mM tobramycin; 4) 0.5 mM tobramycin; 5) no treatment; 6) 0.01 mM tobramycin plus 20 µM CFTRinh-172; 7) 0.1 mM tobramycin plus 20 µM CFTRinh-172; 8) 0.5 mM tobramycin plus 20 µM CFTRinh-172. Scale bar 100 µM. These represent typical results from at least two independent experiments.

Figure 3.18 (over) shows an effect of CFTRinh-172 on copper-tobramycin uptake of endothelium compared to normal (panels 6 – 8 versus 2 – 4) with respect to the appropriate controls (panels 1 and 5). Copper-tobramycin, similarly to tobramycin was taken up by both normal and CF endothelium without visible differences seen in confocal microscopy due to CFTRinh-172.
Figure 3.18. The effect of CFTRinh-172 on copper-tobramycin uptake. HLMVEC were incubated for 24 hours with CFTRinh-172 and treated with copper-tobramycin (0.01 - 0.5 mM) for 3.5 hours. 1) no anti-tobramycin primary antibody; 2) 0.01 mM copper-tobramycin; 3) 0.1 mM copper-tobramycin; 4) 0.5 mM copper-tobramycin; 5) no treatment; 6) 0.01 mM copper-tobramycin plus 20 µM CFTRinh-172; 7) 0.1 mM copper-tobramycin plus 20 µM CFTRinh-172; 8) 0.5 mM copper-tobramycin plus 20 µM CFTRinh-172. Scale bar 100 µM. These represent typical results from at least two independent experiments.

As shown in Section 3.5.1, CFTRinh-172, especially in the presence of TNF-α, increased HS expression on the surface of human endothelium. It was therefore interesting to check whether this effect was dependent on the surface heparan sulphate. For this reason CF endothelium stimulated with TNF-α, was incubated with heparitinase II for 4 hours prior the addition of tobramycin at 0.01 mM - 0.5 mM final concentration for 3.5 hours (Figure 3.19, over). CFTRinh-172 in the presence of TNF-α could be considered as a better model of CF because, beside inactive CFTR, it presents a pro-inflammatory profile, generated by TNF-α. It would be also expected that the uptake of tobramycin is greater in this condition compared to normal EC, hence, it was impossible to make a direct comparison of different experiments. CFTRinh-172 plus TNF-α in the absence of tobramycin (panel 5) did not affect HLMVEC in comparison to untreated control (panel 1). EC endothelium presented a dose dependent pattern of tobramycin uptake (panel 2 – 4). In presence of heparitinase II, tobramycin uptake was slightly reduced also dose dependently (panel 6 – 8). This results indicate that heparitinase II might affect tobramycin and copper-tobramycin uptake by CF endothelium and suggest again a role of HS in this uptake.
Figure 3.19. Tobramycin stain showing the effect of heparitinase II on HLMVEC incubated with tobramycin in the presence of CFTRinh-172 and TNF-α. The cells were stained with anti-tobramycin antibody. 1) no drug; 2 – 8) 10 ng/ml TNF-α plus 20 μM CFTRinh-172; 2) 0.01 mM tobramycin; 3) 0.1 mM tobramycin; 4) 0.5 mM tobramycin; 5) no drug; 6) 0.01 mM tobramycin plus 5 U/ml heparitinase II; 7) 0.1 mM tobramycin plus 5 U/ml heparitinase II; 8) 0.5 mM tobramycin plus 5 U/ml heparitinase II. Scale bar 25 µm.

3.5.6. THE EFFECT OF TOBRAMYCIN, COPPER-TOBRAMYCIN, COPPER-SULPHATE AND N-ACETYLCYSTEINE ON ENDOTHELIAL CFTR

CFTR protein is localized on the plasma membrane of CFTR-expressing cells. However, it could also be inserted into the membrane of intracellular organelles, including the ER (Pasyk & Fosket et al, 1997). The presence of CFTR within HLMVEC has previously been indicated and verified in HLMVEC by immunocytochemistry (Tousson et al, 1998). The effect of tobramycin, copper-tobramycin, N-acetylcysteine (NAC) and copper sulphate on endothelial CFTR was examined. Figure 3.20 (over) clearly shows that CFTR protein is present and expressed in endothelial cell cultured in vitro. Without any additional treatment, CFTR levels are low in HLMVEC and CFTR is mostly located in the perinuclear area and cytoplasm, possibly ER (panel 2). Both tobramycin and copper-tobramycin increased the expression of intracellular CFTR (panels 3 and 4, respectively). NAC, a known mucolytic and antioxidant drug and indirect precursor of glutathione, increases CFTR expression (Varelogianni et al, 2010). Indeed, NAC increased intracellular CFTR expression in HLMVEC (panel 5). However, no visible membrane expression of CFTR protein might suggest that this protein is not functional.
Copper sulphate had no effect of endothelial CFTR (panel 6) compared to the untreated control (panel 2). Additionally, HLMVEC was incubated with the absence of primary antibody and displayed no background detection (panel 1).

Figure 3.20. The effect of tobramycin, copper-tobramycin, NAC and copper sulphate on CFTR expression in HLMVEC. 1AB) No primary antibody (Mr. Pink); 2AB) No treatment; 3AB) 0.5 mM tobramycin; 4AB) 0.5 mM copper-tobramycin; 5AB) 20 mM NAC; 6AB) 0.5 mM CuSO₄. Scale bar 25 µm, n=3

HLMVEC were also treated with tobramycin, copper-tobramycin, NAC and CuSO₄ in the presence of CFTRinh-172 alone or the pro-inflammatory cytokine, TNF-α (Figure 3.21, over). Appropriate controls of no primary antibody (panel 1) and no drug treatment (2) were included. CFTRinh-172 (panel 3) and TNF-α (panel 4) had an equal effect on endothelial CFTR, they increased mostly perinuclear and cytoplasmic CFTR protein expression. The combination of CFTRinh-172 and TNF-α, which better reflects the CF condition, had additive effect on intracellular CFTR level (panel 5). Tobramycin in the presence of CFTR-inh172 increased not only intracellular but also membrane CFTR expression (panel 6). These results suggest that tobramycin might increase the amount of functional membrane CFTR, especially since NAC had a similar effect (panel 8).
Figure 3.2. The effect of tobramycin, copper-tobramycin, NAC and copper sulphate on CFTR expression in HLMVEC activated with TNF-α in the presence or absence of CFTRinh-172. 1AB) No primary antibody (Mr. Pink); 2AB) No drug; 3AB, 6AB – 9AB) 20 µM CFTR-inh172; 4AB) 10 ng/ml TNF-α; 5AB, 10AB – 12AB) 20 µM CFTRinh-172 plus 10 ng/ml TNF-α; 6AB and 10AB) 0.5 mM tobramycin; 7AB and 11AB) 0.5 mM copper-tobramycin; 8AB and 12 AB) 20 mM NAC; 9AB) 0.5 mM CuSO₄. Scale bar 25 um, n=2.
Copper-tobramycin increased perinuclear and cytoplasmic levels of detectable CFTR protein (panel 7), however membrane CFTR was increased to a lesser extent compared to tobramycin and NAC. Copper sulphate in the presence of CFTR-inh172 (panel 9) had no effect on CFTR expression, alike to copper sulphate alone. Both tobramycin and copper-tobramycin in the presence of CFTRinh-172 and TNF-α induced increased intracellular expression of endothelial CFTR. The membrane was stained weakly (panels 10 and 11). NAC highly up-regulated the CFTR expression only inside the cell (panel 12).

To confirm the exact location of CFTR within HLMVEC, a z-stack with 1 µm interval was performed on untreated control (Figure 3.22).

![Figure 3.22. Z-stack of untreated HLMVEC control stained for CFTR. 1 µm intervals. Scale bar 50 µm.](image-url)
CFTR protein was located in the central part of the cell. It was mainly associated with the perinuclear membrane and the perinuclear area corresponding to the location of the ER. Moreover, CFTR protein was abundantly present in the cell cytoplasm. No CFTR was seen on the cell membrane.

Figure 3.23 presents the effect of tobramycin in CFTRinh-172-treated HLMVEC. Tobramycin or CFTR-inh172 were already shown to increase the intracellular expression of CFTR protein (Figure 3.20 and 3.21, respectively). Tobramycin in the presence of CFTRinh-172 increased the intracellular expression of CFTR, especially in the area close to the nucleus. Interestingly, tobramycin in the presence of CFTRinh-172 caused the appearance of CFTR on the cell membrane.

![Figure 3.23. Z-stack of tobramycin-treated HLMVEC in the presence of CFTRinh-172 and stained for CFTR. 1 µm intervals. Scale bar 50 µm.](image)

120
It can be noted not only on the central image of Figure 3.23, but also on the first and last slides, indicating the apical and basal cell membrane.

3.5.7. ENDOTHELIAL VIABILITY

The viability of HLMVEC during 24 hour experiments in the presence of tobramycin, copper-tobramycin, copper sulphate, TNF-α and CFTRinh-172 was assessed luminometrically by measuring the percentage of cell viability. None of the drugs used were toxic to HLMVEC in full growth medium (Figure 3.24A and 3.24B).

Figure 3.24. HLMVEC viability in full growth medium during 24 hours incubation. A) HLMVEC were incubated with tobramycin, copper-tobramycin (0.01 – 0.5 mM) and copper sulphate (0.5 mM); B) HLMVEC were incubated with 10 ng/ml TNF-α and 20 µM CFTRinh-172. The cell viability was assessed by using CytoTox-Glo™ Cytotoxicity Assay (Promega), n=1, in duplicate.
3.6. SUMMARY OF RESULTS

CFTR-inh172, especially in the presence of TNF-α, increased HS expression in HLMVEC. Tobramycin and copper-tobramycin were taken up by normal and CF-induced EC in the culture and accumulated in the cell cytoplasm and in the perinuclear area. This uptake is at least, partly dependent on an interaction between positively charged aminoglycosides and negatively charged HS on the cell surface.

There was a low level of CFTR expression in untreated HLMVEC, expressed mainly in perinuclear membrane, ER and cytoplasm with no plasma membrane stain, suggesting non functional protein. However, upon tobramycin, copper-tobramycin and NAC treatment CFTR expression was increased and in presence of CFTR-inh172 appeared on the endothelial cell surface.

3.7. DISCUSSION

Under non-inflamed conditions, the endothelial surface is covered with a relatively thick sheath of glycosylated molecules, referred to as the glycocalyx. HS is the most abundant GAG within the glycocalyx, although CS/DSPGs and HA are also present, but their levels vary between different endothelial cells preparations. HSPGs are partly responsible for the negative charge to the glycocalyx (Reitsma et al, 2007; Weinbaum et al, 2007). Here, untreated HLMVEC were also shown to express HS. They were detected with HepSS-1, which specifically recognizes an epitope present in HS-GAG, closely related to O-sulfated and N-acetylated glucosamine, but not other types of GAG, such as hyaluronic acid, heparin, chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and keratan sulfate (Kure & Yoshie, 1986). In the current study, HS in HLMVEC was mostly located in the nucleus and the cell cytoplasm. Indeed, endothelial HSPGs may be found at the intracellular level, associated with the plasma membrane and ECM, or present in a soluble form. Syndecan 1 is the major HSPG in microvascular endothelial cells and it is mainly stored inside the cell, while a small portion is located at the basal surface (Rusnati et al, 2002).
Significant changes occur in the concentration and composition of GAGs at sites of inflammation. Increased concentrations of GAGs have been found in BALF from children with CF (Hilliard et al, 2010) and the secretion of heparan sulphate, chondroitin sulphate and hyaluronic acid is markedly increased in bronchial cells and CF tissues (Solic et al, 2005; Wyatt et al, 2002; Rahmoune et al, 1991). Moreover, Solic et al (2005) reported endothelial activation in CF patients, beside increased HS expression. This might not be due to defective CFTR, but to the inflammatory response (Solic et al, 2005), which is characterised by increased number of pro-inflammatory cytokines, including TNF-α. However, lung proteoglycans are susceptible to degradation by TNF-α-stimulated neutrophils (Shum et al, 2000). TNF-α reduced the amount of syndecan-1 protein in EA.hy 926 endothelial cells (Kainulainen et al, 1996) and increased release of HSPGs in HUVEC to the medium (Reine et al, 2012). On the contrary, mouse and human glomerular endothelial cells activated by TNF-α or IL-1β increased expression of inflammatory N- and 6-O-sulfated HS domains (Rops et al, 2008), recognised by HepSS-1, used in the discussed study. CFTRinh-172 was here employed as a pharmacological tool to create a model of the CF endothelium. This approach was previously used by Perez et al (2007) in lung epithelial cells. CFTRinh-172 treatment of HLMVEC increased the level of HS on the cell surface. This expression was hence further increased in the presence of TNF-α, in the agreement to the studies mentioned above.

Injection of heparitinase I, which specifically degrades HS, into mouse cremaster muscle venules increased the number of adherent leukocytes, indicating that HS in non-inflammed venules is anti-adhesive (Constantinescu et al, 2003). Heparitinase II was also shown to degrade HS. Indeed, it cleaves heparan sulphate and heparin at the 1 - 4 linkages between hexosamines and both glucuronic and iduronic acid residues (Desai et al, 1993; Linhardt et al, 1990; Nader et al, 1990). Here, heparitinase II treatment of HLMVEC inhibited tobramycin and copper-tobramycin complex uptake, confirming the involvement of HS and more importantly proposing the role of HS in aminoglycoside uptake by human endothelial cells. However, uptake was not completely inhibited following heparitinase II treatment which could be due to either
incomplete removal of surface HS or the presence of other mechanisms of aminoglycoside uptake, which will now be considered here.

Charged molecules with a molecular weight over 500 typically exhibit poor availability (Lipinski et al., 1997). Also, aminoglycosides cannot diffuse passively through the eukaryotic cell membrane because of their large size and positive charge (Tulkens, 1990). Tulkens (1990) suggested that cell uptake of aminoglycosides corresponds to an active mechanism of pinocytosis by the eukaryotic cell, which explains the slow intracellular accumulation of these drugs and is only detectable after 48 to 72 hours of antibiotic exposure. In the present study, the uptake and subsequent accumulation of aminoglycosides within human endothelium appeared within 3.5 hours, but after 24 hours of incubation, more staining was observed, suggesting an alternative mechanism of uptake other than pinocytosis.

Schmitz et al. (2001) proposed that renal epithelial cell accumulation of gentamicin is due to endocytosis via a giant endocytic complex formed by megalin and cubilin, which is restricted to the proximal tubule. Aminoglycosides then traffic through the endosomal compartment and accumulate mostly in lysosomes, the Golgi and endoplasmic reticulum (Silverblatt & Kuechn, 1979). Gentamicin binds to membrane phospholipids, however this binding is also a requirement for gentamicin endocytosis (Frommer et al., 1983).

Phospholipid mediated aminoglycoside uptake may occur in addition to the HS binding mechanism. Previous reports have suggested that acidic phospholipids may be binding sites for aminoglycosides and that this binding was due to a charged interaction. The polyphosphoinositides, a group of acidic phospholipids, have been shown to bind neomycin with high affinity (Schacht, 1978). Indeed, Sastrasinh et al. (1982) identified acidic phospholipids as the renal brush border membrane binding site for aminoglycosides with the order of affinity being neomycin > netilmicin ≥ tobramycin ≥ gentamycin > amikacin ≥ kanamycin. The competitive binding studies demonstrated that kanamycin and amikacin, each with four ionisable groups, had relatively similar affinities for the brush border membranes binding site, whereas netlimicin, tobramycin
and gentamycin, each with five ionisable amino groups, were more avid than kanamycin and amikacin. Neomycin, with six ionisable amino groups, was the most avid aminoglycoside in the tested group. These results, therefore, demonstrate that the ability of aminoglycosides to interact with the anionic components of biological membranes is importantly influenced by the number of ionisable primary and secondary amino groups present within the molecule, that contribute to the overall molecule charge.

Lipsky and co-workers (1980) observed that gentamycin uptake was biphasic, with an initial rapid uptake followed by a prolonged slower phase. Approximately half of the total uptake represented binding and the other half represented transport into an intravesicular space. Presumably, aminoglycoside binding to phospholipids was responsible for the faster phase of aminoglycoside uptake, followed by a slower endocytosis.

There is a limited number of techniques aiming to quantify tobramycin concentrations. Gupta (1988) developed a calorimetric method for the determination of tobramycin in parenteral solutions. Kimura & Mukaida (1996) proposed a method for the quantitation of tobramycin concentration by laser microscopy in epithelial cells of the renal tubule, which was based on the reaction between tobramycin and colloidal gold. However, an ELISA based method for tobramycin determination was thought to be the most accurate way for quantitative analysis and it could be used in further experiments.

BODIPY-tagged aminoglycosides, such as tobramycin and neomycin B, introduced by Luedtke et al (2003) were shown to display poor cellular uptake. This research group claimed that BODIPY was an excellent fluorescent probe for cellular uptake studies, because its fluorescence is relatively insensitive to changes in the local environment. However, their results are opposite to the ones presented here. It is possible that BODIPY inhibits aminoglycoside uptake into HeLa cells. Alternatively aminoglycosides are not taken up by HeLa (human epithelial carcinoma) cells, as they are by HLMVEC.
Bonventre et al (1967) first reported that streptomycin was unable to penetrate mouse peritoneal macrophages. However, a prolonged incubation of these macrophages with streptomycin up to one week resulted in a linear accumulation of the drug within cells (Bonventre & Imhorff, 1970). These observations were confirmed by Prokes & Hand (1982) and Johnson et al (1980), who respectively demonstrated that aminoglycosides did not accumulate within human PMNs and rabbit alveolar macrophages when incubated for 2 hours. In agreement, here, resting and IL-8-activated human neutrophils did not take up tobramycin or copper-tobramycin up to 3.5 hours incubation.

In CF patients the pharmacokinetics of antibiotics are known to be abnormal. Lower blood concentrations and higher clearance rates have been found for many antibiotics, including the aminoglycosides: gentamycin, tobramycin and amikacin (Kearns et al, 1982; Kelly et al, 1982; Vogelstein et al, 1977). In vitro data suggests that gentamycin accumulation in non-CF cells (human nasal polyp tissue) is under regulation by effectors and inhibitors of the CFTR channel. In CF cells with ΔF508 mutations, this regulation is lost with an inhibition of exocytosis since the CFTR channel is inoperable. As a result, aminoglycosides accumulate within the affected cells. For instance, CF cells accumulate over twice as much gentamycin as non-CF cells (Quesnel et al, 1998). Santos et al (2007) suggests an adverse drug reaction of a CF patient after administration of tobramycin solution for inhalation or tobramycin intravenously, which results from a significant tissue accumulation of tobramycin. Additionally, Al-Aloul and coworkers (2005) hypothesized that long-term use of intravenous nephrotoxic antibiotics (aminoglycosides and colistin sulphomethate) may contribute to renal disease in CF patients. In the present study, tobramycin and copper-tobramycin uptake by HLMVEC was higher after 24 hours than 3.5 hours. There was no analysis done comparing normal with CF uptake of tobramycin and copper-tobramycin. Anyway, this uptake was not associated with cytotoxicity.

CFTR protein as integral membrane glycoprotein is mainly expressed at the apical membrane of epithelial cells (Collins, 1992). HUVEC and HLMVEC were also demonstrated to express CFTR protein as determined via RT-PCR (real time polymerase chain reaction) and immunohistochemical and immunoprecipitation
analyses (Tousson et al, 1998). CFTR is considered to be held on the plasma membrane and can also be inserted into the membrane of intracellular organelles, including the ER (Pasyk & Fosket et al, 1997). Moreover, Bertrand & Frizzell (2003) suggested that CFTR could be distributed in the Golgi apparatus or secretory vesicles in normal epithelial cells. Therefore, the CFTR endocytosis and exocytosis are based on CFTR interactions with proteins involved in membrane trafficking.

There are a number of available monoclonal and polyclonal antibodies to CFTR (Kartner & Riordan, 1998). Tousson et al (1998) stained endothelial cells (HUVEC and HLMVEC) with antibodies directed against different domains of human CFTR, including the R-domain, COOH terminus, nucleotide binding domain (NBD)-1 or the first extracellular loop and obtained positive results. Cells analyzed with antibodies directed against the R-domain or the first extracellular loop displayed a punctate staining pattern around the nucleus and along cellular processes. In addition, the antibody against the first extracellular loop of CFTR stained the plasma membrane positively and exhibited intense perinuclear staining. Endothelial cells analyzed with antibodies that recognized NBD-1 or the COOH terminus of CFTR yielded a diffuse staining pattern throughout the cytoplasm and also stained the plasma membrane. Here, an anti-CFTR antibody that recognises NBD-1 was used and CFTR was mainly found in the nuclear membrane, perinuclear area, thought to be ER, and in the cytoplasm on resting HLMVEC. There was no CFTR protein detected on the plasma membrane. The differences could be a result of distinct staining methods. For NBD-1 detection Tousson et al (1998) fixed the cells with absolute methanol only and postfixed with formaldehyde. Here, 1 % (w/v) PFA dissolved in 15 % (v/v) picric acid in PBS preceded the 0.1 % (v/v) Triton X-100/PBS permeabilization step.

In the present study, TNF-α and/or CFTRinh-172 increased the level of intracellular CFTR protein over a 24 hour incubation. TNF-α was shown to decrease CFTR mRNA in human colon epithelial cell lines (HT-29), but not in airway epithelial cell lines (Calu-3). Additionally, CFTR mRNA is increased in mast cells at TNF-α treatment (Baudouin-Legros et al, 2004). However, TNF-α had no effect on CFTR-mediated chloride
transport activity (Estell et al, 2003). The main function of CFTRinh-172 and other CFTR inhibitors such as glycine hydrazide GlyH-101 is to cause inhibition of Cl⁻ conductance. Both inhibitors, CFTRinh-172 and GlyH-101, are additionally able to induce a rapid increase in ROS levels and depolarize mitochondria, suggesting that these effects are independent of CFTR inhibition (Kelly et al, 2010). It was previously shown that CFTRinh-172 also significantly elevated the total protein content of gland fluid secretions, which may also be due to ROS (Thiagarajah et al, 2004).

It was reported that defective CFTR induces upregulation of ROS (Luciani et al, 2010). Moreover, NO and reactive oxygen nitrogen species (RONS) decrease wild type CFTR protein levels in airway epithelial cell monolayers, which is related to the loss of CFTR function. Decreased CFTR expression may results partly from nitration of nascent CFTR, and its subsequent degradation by the proteasome (Bebok et al, 2002).

Indeed, several lines of evidence suggest that misfolded CFTR chains are substrates for the ubiquitin-proteasome pathway. For instance, treatment of cells expressing CFTR or ΔF508 with proteasome inhibitors, such as lactacystin, results in the accumulation of multiubiquitinated forms of CFTR, pointing out proteasome degradation of CFTR and ΔF508. Also, in proteasome-inhibited cells, CFTR antibodies immunoprecipitate is recognized with antibodies to ubiquitin or to an epitope tag on ubiquitin, suggesting that CFTR is modified by ubiquitin (reviewed by Kopito, 1999).

Tobramycin, copper-tobramycin and NAC in the presence of CFTRinh-172 restored CFTR expression on the HLMVEC surface. Tobramycin and copper-tobramycin may elicit antioxidant activity that allows the stabilisation of the CFTR protein via protection from ROS, similar to the effect elicited by NAC.

Indeed, NAC has been used for many years as a mucolytic and antioxidant agent in biomedical research (Atkuri et al, 2007) and in clinical practice (Millea, 2009). NAC is deacetylated to cysteine on the cell surface or inside the cell and therefore promote the formation of glutathione, which provides antioxidant activity in the cytoplasm. NAC can also have a reducing and antioxidant role by directly scavenging free radicals, including the hydroxyl radical, superoxide ion and hydrogen peroxide (Aruoma et al,
1989). It was also shown to protect against oxygen toxicity in the lung (Erzurum et al, 1993). Finally, NAC was demonstrated to increase CFTR expression by stimulating Cl− efflux in CF airway epithelial cells (Varelogianni et al, 2010). NAC was also shown to prevent the cadmium-induced suppression of CFTR (Rennolds et al, 2010).

Alternatively, tobramycin and copper-tobramycin may act in the similar manner to COMMD1 (Copper Metabolism gene MURR1 domains). COMMD1, which regulates hepatic copper excretion and sodium uptake via ENaC, has the ability to interact with CFTR. It was suggested that COMMD1 may function as a scaffold for protein ubiquitination and therefore regulate intracellular protein trafficking (Drevillon et al, 2011).

Additionally, the CFTR form in HLMVEC at tobramycin and copper-tobramycin treatment may be deciphered by further Western blotting experiments.
CHAPTER 4
THE EFFECT OF TOBRAMYCIN AND COPPER-
TOBRAMYCIN ON OXIDATIVE STRESS IN
NEUTROPHILS AND ENDOTHELIUM

4.1. INTRODUCTION

4.1.1. OXIDATIVE STRESS IN THE LUNG

Although all cells are exposed to oxidative stress during normal metabolism (Foronjy RF et al, 2006), the lungs are directly exposed to high oxygen tensions. Indeed, the local oxygen partial pressure at the alveolar level is much higher than in other vital organs including heart, liver and brain. Oxygen exposure in inhaled air, 150 mmHg, equals 100 mmHg at the alveolus, decreasing to 45 mmHg in venous blood, whereas in some sites within other organs it may be only 1 mmHg. Lung cells experience enhanced oxidant stress by environmental irritants and air pollutants including oxidants, such as cigarette smoke, ozone and free radical-generating environmental carcinogens. In addition, inflammation and activation of inflammatory cells with consequent free radical generation is characteristic of most lung disorders and lung infections (Kinnula & Crapo, 2003).

Most living cells, including lung cells, generate free radicals under normal conditions nonenzymatically via autooxidation (Kinnula & Crapo, 2003). During homeostasis, ROS are released in noncytotoxic quantities and act as mediators and regulators of cell metabolism (Valko et al, 2007). ROS initiate cell differentiation and apoptosis, influence synthesis, release or inactivation of NO and induce transport of glucose into the cells. ROS affect the inflammatory process by enhancing permeability of capillary vessels. One of the most important roles of ROS is regulation of cell signalling (Droge, 2002). Superoxide and hydrogen peroxide are considered to be secondary signal transducers, because of their low reactivity and high selectivity and cell abundance (Valko et al, 2007; Droge, 2002). Most thiol proteins are inactivated via ROS; however the activity of some proteins increases in the presence of ROS. These proteins include
guanyl cyclase and particular transport proteins, such as 5-lipoxygenase, which is a source of T-cell generated ROS. 5-lipoxygenase oxidises polyunsaturated fatty acids and derived metabolites maintain intracellular redox balance by activating signalling pathways and gene expression (Bonzzi et al, 2000; Los et al, 1995b). Phagocytes, such as granulocytes, monocytes or macrophages, use ROS to eliminate pathogens in the process called respiratory burst, which involves generation of superoxide (Hampton et al, 1998). ROS were also shown to upregulate T cell activity and induce adhesion of leukocytes to endothelium, which determines their migration to the site of inflammation (Los et al, 1995a). In addition, a regulatory activity of low H$_2$O$_2$ concentrations manifests in the activation of NF-$\kappa$B, which induces expression of genes that encode cytokines (IL-1$\beta$ or IL-6), immunity proteins, thioredoxin or SOD (Li & Karin, 1999). Finally, the activation of transcription factors via low levels of ROS induces cell differentiation and enables their adjustment to different conditions, while the exposure to higher ROS levels results in cell apoptosis, which is crucial in the elimination of damaged and potentially dangerous cells (Valko et al, 2007).

In contrast, overproduction of ROS, arising either from the mitochondrial electron transport chain or excessive stimulation of NADPH oxidase, results in oxidative stress, which could be responsible for damage to cell structures and tissue destruction. The negative impact of ROS depends on their concentration and time of exposure (Valko et al, 2007). The harmful effects of ROS manifest by their ability to oxidise proteins with the generation of ROS via xanthine oxidase, mitochondrial respiratory chain or activated phagocytes. Proteins that undergo irreversible changes are selectively degraded by proteases (Du & Gebicki, 2004; Stadtman & Lewine, 2003; Stadtman & Lewine, 2000). Protein oxidation could lead to polypeptide chain disruption, appearance of modified aminoacid residues and formation of dimers or protein aggregates. In consequence, these changes cause loss of functional activity of enzymes, regulatory proteins and membrane transporters (Lushchak, 2007; Hensley et al, 2000). The hydroxyl radical, a toxic metabolite of the superoxide anion, is usually the mediator of protein breakdown. Superoxide anion and hydrogen peroxide could in particular elicit oxidation of $\sim$SH groups. Peroxynitrite, which if formed from a reaction of NO and O$_2^-$, is responsible for the oxidative damage of amino acids. It is highly reactive and able to form its derivative, 3-nitrotyrosine (Valko et al, 2007; Alvarez &
Radi, 2003). Moreover, peroxinitrite could react with free intracellular sulphydryl groups. It has been demonstrated that oxidative stress resulting from the action of peroxynitrite inhibits the activity of fibrinogen and tissue factor (Ischiropoulos, 2003; Gow et al, 1996).

The reactions of hydroxyl radical and singlet oxygen with nucleic acids may lead to the breakdown of purine and pyrimidine bases, sugar residues or the breakdown of phosphodiester bonds that connect nucleotides (Nunomura et al, 2004; Higuchi, 2003). This further leads to the break of nucleic acid strands. Some of the products of the oxidative modification of nucleic acids are 8-hydroxy-2-deoxyguanine and 8-hydroxyguanine, compounds which are recognized as markers of intracellular nucleic acid oxidation. Additionally, mitochondrial DNA is more susceptible to oxidative damage, regarding its proximity to the mitochondrial respiratory chain (Higuchi, 2004; Cookie et al, 2003; Mecocci et al, 1994).

ROS are also involved in the process of lipid peroxidation, which could be divided into three stages: initiation, propagation and termination. Initiation of lipid peroxidation relies on detachment of hydrogen from the unsaturated fatty acid, a compartment of phospholipids that are the main compounds of the cell membrane and it is started by the following radicals: hydroxyl (OH•), superoxide (ROO•), alkyl (RO•) as well as nitric oxide and nitric dioxide. In propagation, alkyl radicals react with oxygen resulting in peroxide radicals (ROO•) and eventually in free fatty acids. This reaction could be repeated many times until it is terminated with a result of modified and damaged lipid molecules. Free radicals formed during lipid peroxidation could react with proteins giving free protein radicals. Such products of lipid peroxidation change the physical properties of cell membranes, leading to the inhibition of the activity of membrane enzymes and transport proteins (Gutteridge, 1995). Moreover, they could induce the expression of cyclooxygenase-2 (COX-2) in macrophages and activate a pro-inflammatory potential of these cells (Kumagai et al, 2004).
4.1.2. MAIN CONTRIBUTORS TO OXIDATIVE STRESS

4.1.2.1. NADPH OXIDASE

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was first described and characterized in professional phagocytes, such as neutrophils, monocytes, macrophages and eosinophils. It is however not restricted to leukocytes, because similar NADPH oxidases are present in a wide variety of non-phagocytic cells and tissues (Quinn & Gauss, 2004).

The phagocytic NADPH oxidase is a multiprotein enzyme complex composed of an integral membrane protein as well as cytosolic proteins. Two essential membrane-associated proteins, gp91phox and p22phox, form a non-covalent heterodimer, known as flavocytochrome b558, which is responsible for conducting electron transport across the membrane. The cytosolic NADPH oxidase proteins include p40phox, p47phox, p67phox and Rac2, a member of Rho family of small GTPases, associated with GDP-dissociation inhibitor, RhoGDI (Quinn & Gauss, 2004). Upon activation, p47phox undergoes stimulation on multiple serine residues and translocates, along with p67phox and p40phox, to the membrane. Rac2 undergoes GDP-GTP exchange, with subsequent translocation of prenylated Rac2 to the membrane. At the membrane, the assembled oxidase complex shuttles electrons from cytosolic NADPH, across the stacked heme groups in gp91phox to molecular oxygen, thereby generating superoxide anion (Figure 4.1, over) (Nauseef, 2007).

NADPH is activated upon phagocytosis by most microorganisms to generate superoxide radical, O₂⁻. O₂⁻ can further dismutate to form H₂O₂ and both can form other ROS, which are bactericidal (Figure 1.5) (Root & Cohen, 1981, Badwey & Karnowsky, 1980, Babior, 1978). In addition, Klebanoff (2005) demonstrated that microbicidal activity of NADPH-oxidide-derived ROS is significantly augmented by MPO.
Figure 4.1. Model of NADPH oxidase activation. Cytosolic phox proteins and Rac translocate to the phagosomal or plasma membrane, where they assemble with flavocytochrome b 558 to form the active $O_2^-$-generating complex (Quinn et al., 2006).

4.1.2.2. MYELOPEROXIDASE

Myeloperoxidase (MPO) is a haem peroxidase extensively present in azurophilic granules of neutrophils and monocytes and released upon cell activation into the phagolysosome or into the extracellular space (Schultz et al., 1965). Myeloperoxidase uses hydrogen peroxide to oxidize a variety of aromatic compounds by a 1-electron mechanism and produce reactive intermediates (Hampton et al., 1998). It oxidizes chloride ions to hypochlorous acid (HOCl), the major product of MPO and the most potent bactericidal oxidant to be produced by the neutrophil (Klebanoff, 1968). MPO has a wide range of substrates leading to a wide variety of by-products. Chloramines, long-lived oxidants, which are generated indirectly through the reaction of HOCl with amines, are bactericidal (Forman & Thomas, 1986). The myeloperoxidase/H$_2$O$_2$/chloride system forms tyrosyl radical and chlorination products, generates tyrosine peroxide, reactive aldehydes (Hazen et al., 1998) and contributes to the oxidation of serum proteins (Leeuwenburgh et al., 1997) and lipoproteins (Daugherty et al., 1994). MPO can utilize nitrite and hydrogen peroxide as substrates to catalyze tyrosine nitration in proteins (Sampson et al., 1998) and can react with peroxynitrite (Floris et al., 1993).
Moreover, the myeloperoxidase/H$_2$O$_2$/chloride system contributes to the termination of NADPH oxidase activity (Jandl et al., 1978).

4.1.2.3. NEUTROPHIL ELASTASE

Neutrophil elastase (NE), together with other neutrophil-derived serine proteases, such as cathepsin G (Salvesen & Enghild, 1990), proteinase 3 (Bories et al., 1989) and enzymatically inactive azurocidin or CAP-37 (Morgan et al., 1991), is a highly cationic glycoprotein. Approximately 67,000 NE molecules are stored in the primary granules of neutrophils at a mean concentration of 5.33 mM (Liou & Campbell, 1995).

NE is capable of cleaving insoluble elastin and other ECM proteins, including laminin, fibronectin, vitronectin and collagens (Janoff & Scherer, 1968). The degradation of elastin generates elastin-derived fragments, which exhibit potent chemotactic activity for other leukocytes, stimulate fibroblast and smooth muscle cell proliferation, and display proangiogenic activity as potent as VEGF (Antonicelli et al., 2007).

NE could interact in a variety of ways with cytokines, including: the release of active cytokines from their inactive precursors (IL-1β, TNF-α, TGF-β, IL-8); proteolytic cleavage and inactivation of active cytokines (TNF-α, IL-6, IL-2); cleavage of cell-surface bound cytokine receptors (TNF-α, IL-6, IL-2); proteolysis of the cytokine binding proteins (TGF-β, IGF); and activation of specific cell surface receptors (PARs, TLR4) (Bank & Ansorge, 2001). In particular, NE is capable of degrading various proinflammatory cytokines such as TNF-α (van Kessel et al., 1991), IL-2 (Ariel et al., 1998), IL-6 (Bank et al., 1999) and IL-8 (Witherden et al., 2004). NE upregulates IL-8 expression and secretion in bronchial epithelium via Toll-like receptor-4 (TLR-4) (Devaney et al., 2003). Moreover, neutrophil proteinases released in inflamed tissues convert IL-8 to more potent, N-terminally truncated forms, which tend to enhance IL-8 activity (Padrines et al., 1994).

In addition, NE can cleave coagulation factors (fibrinogen and factors V, VII, XII and XIII) (Samis et al., 2004), digest platelet IIb/IIIa receptor (Bykowska et al., 1990), cleave complement receptors (C3 and C5) (Berger et al., 1989) and plasminogen in the process of converting it into active plasmin (Gombas et al., 2004). NE inactivates endothelial
thrombomodulin (Abe et al., 1994), degrades immunoglobulin G (IgG), IgA and IgM (Doring et al., 1986) and lung surfactant proteins (Liau et al., 1996).

NE was shown to have a role in neutrophil adhesion, as it binds to MAC-1 (Cai & Wright, 1996) and cleaves ICAM-1 (Champagne et al., 1998) and endothelial cadherins (Carden et al., 1998).

NE contributes to chronic inflammatory airway diseases by inducing mucin production in airway epithelial cells (Fisher & Voynow, 2000). NE can also induce apoptosis of human lung epithelial cells mediated via PAR-1 (Suzuki et al., 2005). Additionally, NE upregulates LTB4 secretion from activated macrophages (Hubbard et al., 1991).

However, neutrophils also store α1-AT, the major inhibitor of NE, which is secreted following stimulation concordantly with NE. Moreover, some of the secreted α1-AT is capable of forming complexes with NE in order to maintain tissue homeostasis (Paako et al., 1996; du Bois et al., 1991).

4.1.3. OXIDATIVE STRESS IN CYSTIC FIBROSIS

Cystic fibrosis lung disease is characterised by a substantial oxidative stress, since the airway infection and inflammatory processes in the CF lung are potential sources of oxidants. An oxidative environment influences multiple intracellular signalling events, leading to increased synthesis and secretion of mucins (Takeyama et al., 2000), defective apoptosis (Yalcin et al., 2009), and alterations in ion transport, including chloride secretion (Cowley & Lindsell, 2002). Moreover, oxidant stress could also suppress CFTR expression (Qu et al., 2008; Cantin et al., 2006).

It is well established that activated neutrophils are the major generator of free radicals due to their NADPH oxidase involvement in bacterial phagocytosis (Babior, 1984). Neutrophils represent 70% of the ELF (epithelial lining fluid) inflammatory cells (Birrer et al., 1994). BALF from children with CF has an increased concentration of neutrophil-derived protease, myeloperoxidase and 3-chlorotyrosine, a biomarker of the potent oxidant hypochlorous acid, which is formed by myeloperoxidase, compared to the control subjects (Kettle et al., 2004). The neutrophil in individuals with CF is considered
by some researchers to be abnormal and secretes more NE than non-CF neutrophils in response to IL-8, TNF and LPS, all of which are found in abundance in the CF lung. This further brings more neutrophils onto the epithelial surface and therefore creates an NE-induced inflammation cycle (Taggard et al, 2000).

Although CF patients have normal or high serum and ELF levels of anti-NE compounds, such as α1-AT, SLPI and elafin, they are completely inactive as they are complexed with or cleaved by NE (Birrer et al, 1994; McEvaney et al, 1992). Also, they are inactivated by oxidative stress. Moreover, CF patients have decreased levels of GSH to 5 – 10 % of normal in ELF and to 50 % of normal in plasma (Roum et al, 1993). Poor nutritional status, vitamin E deficiency (Farrell et al, 1997) and decreased levels of carotenoid and retinol (Homnick et al, 1993) may contribute to low GSH levels in CF.

Total elemental zinc and iron concentrations are elevated in sputum from subjects with CF and non-CF bronchiectasis compared with healthy control subjects (Gray et al, 2000). Individuals with CF have also altered copper distribution compared to control individuals (Percival et al, 1999). Indeed, transition metals may contribute to oxidative stress in CF via Fenton chemistry.

The increased oxidative stress in CF patients could be detected by markers of oxidative damage to lipids (malondialdehyde-like substances and lipid hydroperoxides) and proteins (protein carbonyls) (Brown & Kelly, 1994).

4.1.4. ANTIOXIDANT DEFENCE OF THE LUNG

Lung cells contain different types of antioxidants with a specific localization and expression (Figure 4.2, over). Among them, superoxide dismutases, previously described in Section 2.1.1, are hypothesized to play a significant role against oxidative stress especially in the lung (Kinnula et al, 1995; Crapo, 1975). Extracellular SOD3 (Cu/Zn-SOD) is the major lung SOD and accounts for approximately 80 % of SOD activity. This enzyme is located primarily in the bronchial epithelium, alveolar epithelium, mesenchymal cells, arterioles and capillary endothelial cells of the lung (Foronjy et al, 2006, Kinnula & Crapo, 2003; Foronjy et al, 2006).
Figure 4.2. Major antioxidant pathways in the lung. Antioxidant pathways in scavenging superoxide and hydrogen peroxide in the intracellular and extracellular spaces. CAT - catalase; ECSOD - extracellular superoxide dismutase; GPXc - classic (intracellular) glutathione peroxidase; GPXe - extracellular glutathione peroxidase; GR - glutathione reductase; GRXs - glutaredoxins; GSH - reduced glutathione; GSSG - oxidized glutathione; PRXs- peroxiredoxins (thiortoedoxin peroxidase); TRXs - thioredoxins (Kinnula & Crapo, 2003).

H$_2$O$_2$, a product of the dismutation of superoxide, can be further destroyed by catalase and glutathione peroxidase, a selenium-containing enzyme requiring glutathione (GSH). Interestingly, the total concentration of GSH in normal ELF is 140-fold higher than in plasma and moreover, these concentrations are sufficient to protect lung cells against the burden of H$_2$O$_2$ released by alveolar macrophages removed from the lower respiratory tract (Cantin et al., 1987). Other redox-regulating proteins able to decompose hydrogen peroxide have been described as thioredoxins, glutaredoxins (Holmgren, 2000) and peroxiredoxins (Rhee et al., 2001), localized in both cytoplasmic and nuclear compartments of lung cells (Kinnula et al., 2002; Soini et al., 2001).
Additionally, storage and transport proteins, including ceruloplasmin, ferritin, transferrin, can sequester transition metals and further prevent the formation of highly reactive radicals (Valko et al, 2005). Plasma contains a variety of redox active low molecular weight antioxidant molecules, such as vitamin E, uric acid, bilirubin, ascorbic acid, and thiol groups (Frei et al, 1988).

4.2. HYPOTHESIS, AIMS AND OBJECTIVES

The hypothesis of the study is that tobramycin and copper-tobramycin have antioxidant properties.

Neutrophils, platelets and endothelium actively contribute to the inflammatory response. An important aspect was to determine the role of each cell type in the oxidative stress. The predominant aim was to investigate the antioxidant activity of tobramycin and copper-tobramycin on neutrophil respiratory burst by measuring superoxide and hydrogen peroxide release and on neutrophil elastase activity in the absence or presence of α1-antitrypsin. This antioxidant activity was further examined on intracellular EC ROS generation by immunocytochemistry.

4.3. MATERIALS

Zymosan A from Saccharomyces cerevisiae, PMA (phorbol 12-myristate 13-acetate), cytochrome C from equine heart, hydrogen peroxide 30 %, sodium azide (SA), NE substrate, N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide, protamine sulphate salt from salmon and DCF-DA (2′,7′-dichlorofluorescein diacetate) were purchased from Sigma-Aldrich Inc. (Poole, Dorset, UK).

Zemaira α1-AT was a gift from CSL Behring Ltd. (Melbourne, Australia).

4.4. METHODS

4.4.1. OPSONIZATION OF ZYMO SAN

The method was adapted from Petreccia, Nauseaf & Clark (1987) and the preparation of OPZ was made fresh daily. Serum was prepared from normal venous blood collected
into Vacuette clot activator tubes (Greiner-Bio-One) and allowed to clot for 10 minutes at room temperature and centrifuged at 1500 x g for 10 minutes.

Zymosan A from *Saccharomyces cerevisae* (Sigma) was prepared at 10 mg in 1 ml of water. Particles were centrifuged at 900 x g for 10 minutes at 4°C, washed in 10 ml of water and centrifuged again. Zymosan A was opsonised in fresh human serum (2 ml) at 5 mg/ml for 60 minutes at 37°C with gentle shaking. The reaction was stopped by placing on ice. Opsonised zymosan (OPZ) was washed twice with ice-cold water and centrifuged at 1500 x g for 10 minutes at 4°C. A final re-suspension was made in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 at 10 mg/ml.

4.4.2. SUPEROXIDE ASSAY

Superoxide ion release from neutrophils was measured by the reduction of cytochrome C. The experiment was carried out in assay buffer, HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 in triplicate. Neutrophils were isolated according to the procedure in Section 2.4.1 and diluted in assay buffer. Neutrophils (1 x 10⁷ cells/ml, 200 µl) were incubated with tobramycin, copper-tobramycin, copper sulphate (0.001 – 0.5 mM) and SOD (3.125 – 100 U/ml) in the presence of cytochrome C (40 µM) and with OPZ-activation (1 mg/ml final concentration) at 37°C for 15 minutes with gentle shaking in a final volume of 250 µl. The reactions were stopped on ice and the samples were centrifuged at 1100 x g for 5 min at 4°C. 200 µl of cleared supernatant was transferred into a 96-well plate and the absorbance measured at 550 nm.

4.4.3. HYDROGEN PEROXIDE RELEASE OF NEUTROPHILS

Hydrogen peroxide release from neutrophils was detected using the Amplex Red Neuraminidase (Sialidase) Assay (Molecular Probes) as previously described in Section 2.4.9. Hydrogen peroxide consumption by neutrophil myeloperoxidase was inhibited by using 1 mM sodium azide (Sigma). The experiment was carried out in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 in duplicate. Neutrophils (1 x 10⁷ cells/ml, 100 µl) were incubated with tobramycin, copper-tobramycin (0.01 – 0.5 mM) and SOD (100 U/ml) upon OPZ-activation (1 mg/ml) or PMA (1 µg/ml) at 37°C for 15 minutes with gentle shaking. The reactions were stopped on ice and the samples were cleared
by centrifugation at 1100 x g for 5 minutes at 4°C. 100 µM Amplex Red reagent containing 0.2 U/ml HRP in 50 µl was added to each 50 µl supernatant sample in a 96-well plate. The plate was incubated at 37°C for 30 minutes, whilst protected from light. The absorbance was measured at 550 nm and the hydrogen peroxide concentration of samples was calculated from a hydrogen peroxide standard curve, carried out in the range 0 - 100 µM H₂O₂.

4.4.4. NEUTROPHIL ELASTASE ACTIVITY

Neutrophil elastase activity was measured using the specific NE substrate, N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma). The experiment was carried out in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 in duplicate. Neutrophils (1 x 10⁷ cells/ml, 200 µl) were pre-incubated with tobramycin, copper-tobramycin (0.01 – 0.5 mM) and α1-AT (2 mg/ml) for 15 minutes in a final volume of 250 µl, followed by OPZ-activation (1 mg/ml) for 60 minutes at 37°C with gentle shaking. The reactions were stopped on ice and the samples were centrifuged at 1100 x g for 5 min at 4°C. The cell pellets were lysed in an equal volume of 1 % (v/v) Triton X-100 in water. Both, supernatants and cell pellets were incubated for 1 minute at 37°C with gentle shaking and the absorbance at 405 nm was read over time in the presence of 0.555 mM NE substrate.

4.4.5. ASSESSMENT OF INTERFERENCE IN THE IL-8 ELISA WITH DIFFERENT COMPONENTS

To assess the interference of components in neutrophil incubations in the IL-8 ELISA, IL-8 (500 pg/ml) was incubated with tobramycin, copper-tobramycin, copper sulphate (0.5 mM), copper chloride (0.4 mM) or protamine sulphate (0.1 mg/ml). The incubation was carried out in 0.1 % (w/v) BSA/PBS (- Ca/Mg) containing 0.05 % (v/v) Tween-20 in a reaction volume of 200 µl for 90 minutes at 37°C. Additionally, these compounds were incubated in the absence of IL-8. The experiment was performed once in duplicate or triplicate.
4.4.6. IL-8 ELISA

IL-8 samples from the experiments described in Section 4.4.4 and 4.4.5 were subsequently analysed by ELISA (PeproTech) according to the manufacturer’s instructions (PeproTech).

4.4.7. PMN VIABILITY ASSESSMENT

TACS™ annexin V-biotin apoptosis detection kit (Trevigen) was used to assess the cytotoxicity of tested drugs on PMN. The assay was performed on a FACS Calibur Immunocytometry System.

Neutrophils were isolated as indicated in Section 2.4.1, re-suspended in 1 x HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 and diluted to 5 x 10⁶ cells/ml. Neutrophils were then challenged with the following drugs: tobramycin, copper-tobramycin, copper sulphate (0.01 – 0.5 mM), SOD (3.125 – 100 U/ml) in a total volume of 250 µl for 0.5 – 3 hours at 37°C. According to the flow cytometry instruction manual provided by TACS™ Annexin V-Biotin kit (Trevigen), after the incubation time the samples were placed on ice and collected by centrifugation at 300 x g for 10 minutes at room temperature. The cells were washed by re-suspending in 500 µl of cold PBS (- Ca/Mg) and collected again. 100 µl Annexin V Incubation Reagent was prepared per sample by mixing: 10 µl 10 x Binding Buffer, 10 µl propidium iodide, 1 µl Annexin V-biotin and 79 µl distilled water. The cells were gently re-suspended in the Annexin V Incubation Reagent and incubated in the dark for 15 minutes at room temperature. The cells were collected by centrifugation at 300 x g for 10 minutes at room temperature. 1 x Binding Buffer was prepared from a 10 x stock by 1/10 dilution. The cells were re-suspended in 100 µl 1 x Binding Buffer containing AlexaFluor 488 streptavidin conjugate (1 mg/ml diluted 1/200) and incubated for further 15 minutes in the dark at room temperature. 400 µl 1 x Binding Buffer was added per 100 µl reaction and the fluorescence was measured using flow cytometry.

Before proceeding, 3 control samples were used to calibrate the instrument: PMNs re-suspended in Binding Buffer to evaluate the level of autofluorescence and PMNs stained separately with Annexin V and Propidium Iodide to define the boundaries of
each population. Additionally, a negative (PMNs with no drug treatment) and positive
(PMNs treated for 24 hours with 1 µM staurosporine from *Streptomyces sp.*) apoptosis
controls were used.

4.4.8. IMMUNOCYTOCHEMICAL STAINING OF HLMVEC FOR ROS

HLMVEC were cultured in collagen IV-coated Microtek 8-well chamber slide (Thermo
Fisher) at 0.7 x 10⁵/ml (250 µl/well) and grown to confluency for at least 48 hours. On
the day of the experiment, 50 mM tobramycin and copper-tobramycin stock solution
were prepared and diluted to obtain 10 mM and 1 mM working concentrations. 100 x
concentrated drug stocks were added to the cell culture (0.01, 0.1 and 0.5 mM final
concentration) and incubated for 3 hours at 37°C and 5 % CO₂. Fresh, 100 mM DCF-DA
stock solution in DMSO was prepared and diluted to final concentration of 10 µM.
Supernatants were removed and the cells washed with 500 µl/well of 1 x HBSS (-
Ca/Mg). The experiment was carried out in full growth medium. The cells were
incubated with or without DCF-DA (Sigma) for 30 minutes at 37°C and 5 % CO₂,
protected from light, then washed again and post-incubated for further 10 minutes in
the absence of the dye to remove its excess. The cells were washed with 500 µl/well 1
x HBSS (- Ca/Mg). A TNF-α working concentration of 1 µg/ml was prepared and used to
activate the cells in full medium (10 ng/ml final concentration) for 15 minutes at 37°C
and 5 % CO₂. CFTRinh-172 was also prepared fro 2 mM stock to the final concentration
of 20 µM and added to the cells for 30 minutes incubation. Tobramycin and copper-
tobramycin were present throughout. The cells underwent fixation, permeabilisation
and the blocking step described in Section 3.4.5 for the tobramycin and copper-
tobramycin uptake experiment. The slide was finally mounted in FluorPreserve™
Reagent (Calbiochem) and viewed on a Zeiss LSM 710 Confocal Microscope (green
fluorescent stain ex. 488 nm, em. 540 nm).

ROS generation was quantified by counting total fluorescence units in 3 – 5 fields of
view from two independent experiments.
4.4.9. HLMVEC VIABILITY ASSESSMENT

HLMVEC viability was assessed using Caspase-Glo® 3/7 Assay (Promega). This assay measures caspase-3 and -7 activities. The addition of Caspase-Glo® 3/7 Reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present in the sample.

HLMVEC were grown in collagen IV coated 96-well plates until confluency. They were then treated with drugs of interest for either 3 or 24 hours at 37°C and 5% CO₂. Caspase-Glo® Reagent was prepared by transferring the content of Caspase-Glo® Buffer to Caspase-Glo® Substrate. 100 μl of Caspase-Glo 3/7 ®Reagent was added to each well of a 96-well plate containing 100 μl of cells treated with appropriate drugs in a culture medium. The content of wells was mixed gently using a plate shaker (Mini orbital shaker SSM1, Bibby Scientific Ltd., Stone, Staffordshire, UK) at 300 – 500 rpm for 30 seconds. The plate was incubated for 30 minutes at room temperature and eventually the luminescence of each sample was measured on Wallac Victor² (Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland).

Another luminometric assay assessing the viability of HLMVEC was previously described in Section 3.4.11.

4.4.10. STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism 4 version 4. The results were expressed as mean ± SEM. Two-tailed paired or unpaired Student’s t-test and one-way ANOVA with Dunnett’s multiple comparison post hoc test were performed as required. The p values less than 0.05 were considered as statistically significant.
4.5. RESULTS

4.5.1. SUPEROXIDE ANION DETECTION FROM NEUTROPHILS AND THE EFFECT OF TOBRAMYCIN, COPPER-TOBRAMYCIN, SOD AND COPPER SUPLHATE

The superoxide radical is the major ROS released by activated neutrophils. Indeed, neutrophils were demonstrated to undergo a significant (p<0.05) spontaneous and further significant (p<0.001) OPZ-stimulated respiratory burst compared to a reagent control (Figure 4.3). The values, expressed by optical density (OD) at 550 nm, were 0.259 ± 0.002 for the reagent control, 0.308 ± 0.010, when cells were present and 0.596 ± 0.013, when cells were activated, respectively. Moreover, significantly (p<0.001) more superoxide was released from activated than unactivated neutrophils.

![Figure 4.3](image.png)

Figure 4.3. OPZ-stimulated neutrophil respiratory burst in comparison to a spontaneous respiratory burst and reagent control. The data is expressed as mean ± SEM, n=3, in triplicates. One-way ANOVA with Tukey’s post hoc test was used to analyse the data, * p<0.05, *** p<0.001.

Other stimuli, such as fMLP and PMA were also used. However, OPZ was the strongest stimulus and it was used to test the effect of tobramycin, copper-tobramycin, SOD and copper sulphate on the level of superoxide detected from neutrophils.

Tobramycin unlike copper-tobramycin did not significantly influence superoxide anion detection from OPZ-stimulated neutrophils (Figure 4.4, over). However, copper-tobramycin significantly (p<0.01) reduced the OPZ-stimulated respiratory burst of
neutrophils in the concentration range 0.005 – 0.5 mM. Interestingly, 0.05 – 0.5 mM copper-tobramycin concentration reduced the detectable level of superoxide from OPZ-stimulated neutrophils below the level of spontaneous superoxide release. It could mean that copper-tobramycin inhibits release or scavenging of activated and spontaneous superoxide release. Indeed, the decrease in the amount of $O_2^-$ detected in the presence of copper-tobramycin potentially reflects inhibition of the NADPH oxidase, scavenging of $O_2^-$ ions or dismutation of $O_2^-$ to $H_2O_2$.

Figure 4.4. The effect of tobramycin and copper-tobramycin on OPZ-stimulated neutrophil respiratory burst. Tobramycin (A) and copper-tobramycin (B) effect on neutrophil respiratory burst stimulated by OPZ. The data is expressed as mean ± SEM, n=3, each experiment carried in triplicates. One-way ANOVA with Dunnett’s post hoc test was used to analyse the data, ** p<0.01.
As copper-tobramycin was shown to possess SOD-like activity, SOD was tested in the same experimental settings (Figure 4.5). As low as 3.125 U/ml of SOD potently and significantly (p<0.01) inhibited or dismutated the O$_2^-$ that was detected from OPZ-stimulated neutrophils. SOD was tested at concentrations up to 100 U/ml, but this top concentration of SOD did not reduce detectable superoxide below the untreated cell control and did not inhibit spontaneous detection.

![Figure 4.5. The effect of SOD on OPZ-stimulated neutrophil respiratory burst. The data is expressed as mean ± SEM, n=3, in triplicates. One-way ANOVA with Dunnett’s post hoc test was used to analyse the data, ** p<0.01.](image)

Copper sulphate was also tested for its effect on the neutrophil respiratory burst (Figure 4.6, over). Copper sulphate in the range of 0.001 – 0.5 mM significantly (p<0.05) inhibited OPZ-stimulated neutrophil detection of superoxide ion, which suggests that the effect of copper-tobramycin was due to the presence of copper in the copper-tobramycin complex. The detection of stimulated O$_2^-$ was completely inhibited by 0.005 mM CuSO$_4$ and high concentrations inhibited detection of spontaneous O$_2^-$ release, as seen for copper-tobramycin (Figure 4.4B).

In addition, preliminary data demonstrated that platelets or HLMVEC stimulated with thrombin (2 U/ml), PAF (10$^{-5}$ M), PMA (1 µg/ml) and OPZ (1 mg/ml) on normal and collagen I- or collagen IV-coated 96-well plates did not undergo a significant respiratory burst.
4.5.2. HYDROGEN PEROXIDE RELEASE BY NEUTROPHILS AND THE EFFECT OF TOBRAMYCIN, COPPER-TOBRAMYCIN AND SOD

Hydrogen peroxide release was measured from OPZ- and PMA-stimulated neutrophils in the presence of 1 mM of sodium azide (SA) (Figure 4.7, over). The use of SA was crucial to inhibit myeloperoxidase, which is released during cell activation and which consumes H$_2$O$_2$.

OPZ in the presence of SA stimulated a significant (p<0.05) amount of H$_2$O$_2$, 57.90 ± 5.81 µM, measured from neutrophils, compared to 2.42 ± 1.58 µM, in the absence of SA (Figure 4.7A, over). Tobramycin in the range of 0.01 – 0.5 mM did not significantly affect the detection of H$_2$O$_2$ from OPZ-activated neutrophils in the presence of SA. However, copper-tobramycin at 0.1 mM and 0.5 mM significantly (p<0.05 and p<0.01) reduced the H$_2$O$_2$ level detected in PMN supernatants from 57.90 ± 5.81 µM to 19.31 ± 7.60 and 1.80 ± 0.36 µM, respectively. Copper-tobramycin at 0.01 mM, similarly to SOD at 100 U/ml was not effective.

There was no statistically significant difference between the H$_2$O$_2$ level detected in OPZ- and PMA-stimulated PMN supernatants in the presence of SA. The amount of hydrogen peroxide released from PMA-stimulated neutrophils in the presence of SA
was significantly (p<0.05) higher, 42.77 ± 6.89 µM in comparison to the H₂O₂ level in supernatants from PMNs stimulated by PMA in the absence of SA (Figure 4.7B). Again, tobramycin (0.01 - 0.5 mM), SOD (100 U/ml) and copper-tobramycin at 0.01 mM concentration did not affect the H₂O₂ level. Copper-tobramycin at 0.01 mM and 0.5 mM significantly (p<0.01) reduced the amount of H₂O₂ detected from PMA-stimulated PMN in presence of SA to 8.69 ± 3.21 and 1.37 ± 0.44 µM compared to OPZ plus SA control.

These results suggest that copper-tobramycin is not acting as SOD to produce H₂O₂, but may be a scavenger or an inhibitor of NADPH oxidase.

Figure 4.7. The effect of tobramycin, copper-tobramycin and extracellular SOD on hydrogen peroxide release from OPZ- and PMA-activated neutrophils at 1 x 10⁷ cells/ml. A) Hydrogen peroxide release from OPZ-stimulated neutrophils. B) Hydrogen peroxide release from PMA-stimulated neutrophils, n=3, in duplicates. One-way ANOVA with Dunnett’s post hoc test was used to analyse the data expressed as mean ± SEM, * p<0.05, ** p<0.01.
4.5.3. VIABILITY OF NEUTROPHILS DURING THE RESPIRATORY BURST

The viability of neutrophils incubated with tobramycin, copper-tobramycin, copper-sulphate (0.001 – 0.5 mM) and SOD (3.125 – 100 U/ml) in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes at 37°C was assessed by annexin V binding and measured by flow cytometry. In the untreated control cells, 88.74 ± 3.60 % were live, 5.11 ± 1.80 % apoptotic and 6.15 ± 1.98 % necrotic neutrophils.

Tobramycin in the range of 0.001 – 0.5 mM was not toxic to neutrophils in the conditions of the respiratory burst experiment (Figure 4.8).

Copper-tobramycin in the range of 0.001 – 0.01 mM was also not toxic to neutrophils during 30 minutes incubation in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 (Figure 4.9, over), however at 0.05, 0.1 and 0.5 mM, the pool of live neutrophils significantly (p<0.01) decreased in favour of apoptotic cells, 27.26 ± 8.27, 59.51 ± 2.54 and 98.96 ± 0.15 %, respectively. The effect of copper sulphate was very similar (Figure 4.10, over), suggesting a role of copper in inducing neutrophil apoptosis in these settings.

Figure 4.8. The effect of tobramycin on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C. The data expressed as mean ± SEM, n=3 independent experiments. The apoptosis data was analysed using one-way ANOVA and Dunnett’s post hoc test.
Figure 4.9. The effect of copper-tobramycin on neutrophil viability at HBSS (Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C. The data expressed as mean ± SEM, n=3 independent experiments. The apoptosis data was analysed using one-way ANOVA and Dunnett’s post hoc test.

Figure 4.10. The effect of copper sulphate on neutrophil viability at HBSS (Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C. The data expressed as mean ± SEM, n=3 independent experiments. The apoptosis data was analysed using one-way ANOVA and Dunnett’s post hoc test.

SOD did not induce toxicity of neutrophils in the range of concentration of 3.125 – 100 U/ml in the same conditions (Figure 4.11, over).
Figure 4.11. The effect of SOD on neutrophil viability at HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 for 30 minutes incubation at 37°C, n=3 independent experiments. The apoptosis data was analysed using one-way ANOVA and Dunnett’s post hoc test.

There was no time-dependent effect of tobramycin, copper-tobramycin, copper sulphate (0.001 – 0.5 mM) and SOD (3.125 – 100 U/ml) on neutrophil viability, when they were incubated over 0.5 – 3 hours in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4, n=3 independent experiments (data not shown).

4.5.4. NEUTROPHIL ELASTASE ACTIVITY AND THE EFFECT OF α1-ANTITRYPsin

Upon activation, neutrophil elastase (NE) is abundantly released from granules of normal neutrophils to the neutrophil cell surface, where it is active and protected from natural inhibitors. However, in the CF airways NE is also released by necrotic neutrophils into the extracellular space and therefore plays a central role in chronic inflammation. The effect of tobramycin, copper-tobramycin and copper sulphate in the concentration range 0.01 – 0.5 mM was tested on NE activity in cells and supernatants of neutrophils stimulated with OPZ. Also, the effect of protamine sulphate, which is positively charged and may therefore have a similar effect to copper-tobramycin, was used at 10 – 100 µg/ml.

Release of NE activity was induced by OPZ (1 mg/ml) and compared to the spontaneously released NE activity and measured as the rate of cleavage of N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (mOD/min). NE activity was not
released spontaneously (Spont) and the rate was not statistically different to the spontaneous hydrolysis of the substrate in the reagent control (Reag). However, OPZ induced significantly (p<0.05) higher NE activity in supernatants (2.17 ± 0.44 mOD/min) compared to both reagent control (0.50 ± 0.29 mOD/min) and spontaneously released NE activity (0.83 ± 1.67 mOD/min) (Figure 4.12 – 4.15).

Tobramycin at 0.5 mM significantly (p<0.01) increased NE activity in supernatants to 5.17 ± 0.73 mOD/min compared to the OPZ-stimulated neutrophil control (Figure 4.12).

![Figure 4.12. The effect of tobramycin on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of tobramycin or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control; Spont – spontaneously released NE activity; 0 – OPZ-stimulated NE activity.]

Copper-tobramycin at 0.1 and 0.5 mM significantly (p<0.01) increased NE activity in supernatants to 18.00 ± 4.31 and 53.00 ± 6.51 mOD/min, respectively in comparison to the OPZ-stimulated neutrophil control (Figure 4.13, over). Importantly, NE activity in the presence of copper-tobramycin was approximately 10 times higher than in the presence of tobramycin. While at first glance this appears to be a pro-inflammatory effect, subsequent experiments (Figure 4.20) shows that this renders NE susceptible to inhibition by α1-antitrypsin.
Also CuSO₄ at 0.1 and 0.5 mM significantly (p<0.01) increased NE activity to 15.33 ± 4.87 and 16.50 ± 5.62 mOD/min, respectively compared to the OPZ-stimulated PMN (Figure 4.14, over). NE activity released by copper sulphate was higher than NE activity in the presence of tobramycin and lower than in the presence of copper-tobramycin.

Figure 4.13. The effect of copper-tobramycin on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of copper-tobramycin or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control; Spont – spontaneously released NE activity; 0 – OPZ-stimulated NE activity.

Figure 4.14. The effect of copper sulphate on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of copper sulphate or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control; Spont – spontaneously released NE activity; 0 – OPZ-stimulated NE activity.
Figure 4.15 shows that NE activity was significantly (p<0.05 and p<0.01) increased upon protamine sulphate incubation at 50 and 100 µg/ml to 19.50 ± 7.42 and 24.83 ± 4.57 OD/min, respectively.

Figure 4.15. The effect of protamine sulphate on NE activity in supernatants of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of protamine sulphate or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control; Spont – spontaneously released NE activity; 0 – OPZ-stimulated NE activity.

Analysis of NE activity in cell pellets showed that most is mostly associated with cell pellets, 96.79 ± 4.92 %, while released NE activity is minimal. OPZ-stimulated NE activity in the cell pellet, 55.50 ± 1.53 mOD/min, was not significantly lower than the NE activity in unstimulated cells, 65.50 ± 3.04 mOD/min (Figure 4.16 – 4.19), tobramycin (0.01 – 0.5 mM) did not influence cell-bound NE activity stimulated by OPZ (Figure 4.16, over).

However, in parallel with the significantly increased release of NE activity, copper-tobramycin in the concentration range 0.01 – 0.5 mM significantly (p<0.01) decreased the cell pellet-associated NE activity to 20.50 ± 0.87 mOD/min at 0.5 mM copper-tobramycin concentration (Figure 4.17, over).
Figure 4.16. The effect of tobramycin on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of tobramycin or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control equals 0; Spont – NE activity in unstimulated cells; 0 – OPZ-stimulated NE activity.

Only 0.025 mM copper sulphate significantly (p<0.05) reduced the cell pellet-associated NE activity, 45.00 ± 3.50 mOD/min, but other concentrations did not elicit any significant effect (Figure 4.18, over).
Figure 4.18. The effect of copper sulphate on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of copper sulphate or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control equals 0; Spont – NE activity in unstimulated cells; 0 – OPZ-stimulated NE activity.

Protamine sulphate in any concentration tested, similarly to tobramycin, was ineffective in changing the cell-associated NE activity (Figure 4.19).

Figure 4.19. The effect of protamine sulphate on NE activity in cell pellets of OPZ-stimulated PMNs measured over 30 minutes at λ=405 nm and expressed as NE rate mOD/min. Mean ± SEM results were calculated using one-way ANOVA and Dunnett’s post hoc test to compare the effect of protamine sulphate or Tukey’s post hoc test to compare controls, n=3, in duplicate. Reag – reagent control equals 0; Spont – NE activity in unstimulated cells; 0 – OPZ-stimulated NE activity.
Additionally, NE activity was measured in both the supernatant and cell pellet of OPZ-stimulated neutrophils treated with 0.5 mM tobramycin and copper-tobramycin, in the presence and absence of α1-AT (Figure 4.20).

As before, tobramycin and copper-tobramycin significantly (p<0.01 and p<0.05) increased NE activity in the supernatants. Released NE was inhibited by α1-AT at physiological plasma concentration (2 mg/ml). This inhibition was significant (p<0.05) in the presence of tobramycin, copper-tobramycin and OPZ-stimulated control.
Moreover, copper-tobramycin, but not tobramycin significantly (p<0.001) reduced cell-associated NE activity, which was further significantly (p<0.01) inhibited by α1-AT. NE activity in the cell pellet was not significantly inhibited by α1-AT in the presence of tobramycin or the OPZ-stimulated control.

Since both NE and IL-8 have previously been reported to bind to HS or CS, supernatants and cell pellets from the above described experiments were also assessed in relation to the level of IL-8.

Unstimulated neutrophils released a small amount of IL-8 (8.49 ± 3.88 pg/ml) and IL-8 release from OPZ-stimulated neutrophils was not significantly higher at 29.91 ± 13.77 pg/ml (Figure 4.21 – 4.24).

Only 0.05 mM tobramycin elicited significantly (p<0.01) more IL-8 release, 71.53 ± 22.39 pg/ml, from OPZ-stimulated neutrophils than in the absence of tobramycin (Figure 4.21). IL-8 release at any other tobramycin concentration was not different to the OPZ-stimulated neutrophil control IL-8 release.

Figure 4.21. IL-8 level released from OPZ-stimulated neutrophils treated with tobramycin. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s multiple comparison test to compare drug treated and OPZ-stimulated IL-8 release. Additionally, two-tailed, paired t-test was applied to analyse OPZ-stimulated versus unstimulated IL-8 release, n=3, ns – not significant, **p<0.01.
Copper-tobramycin at 0.05 and 0.1 mM significantly (p<0.01 and p<0.05) reduced the concentration of IL-8 released from OPZ-stimulated neutrophils to 6.20 ± 3.37 and 10.93 ± 4.76 pg/ml, respectively, compared to the IL-8 level released from OPZ-stimulated neutrophils without copper-tobramycin treatment (Figure 4.22).

![Graph showing IL-8 concentration in response to copper-tobramycin](image)

Figure 4.22. The level of IL-8 released from OPZ-stimulated neutrophils incubated with copper-tobramycin. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s post hoc test regarding to IL-8 of released of OPZ-stimulated PMNs. Two-tailed, paired t-test was used to analyse OPZ-stimulated versus unstimulated IL-8 release, n=3, ns – not significant, * p<0.05, **p<0.01.

Also, both copper sulphate and protamine sulphate had similar effects to copper-tobramycin on IL-8 released from OPZ-stimulated PMNs. Copper sulphate significantly (p<0.05) decreased IL-8 released to the supernatant in the concentration 0.025 – 0.1 mM; however 0.5 mM of copper sulphate was the most effective and significantly (p<0.01) decreased IL-8 level to 5.59 ± 3.65 pg/ml compared to the IL-8 concentration in the absence of the copper sulphate (Figure 4.23, over). IL-8 released from OPZ-stimulated neutrophils was significantly (p<0.05) diminished by incubation with protamine sulphate at 25 – 100 µg/ml (Figure 4.24, over).
Figure 4.23. The effect of copper-sulphate on IL-8 level released from OPZ-stimulated neutrophils. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s post hoc test to compare drug treated and OPZ-stimulated IL-8 release. Two-tailed, paired t-test was used to analyse OPZ-stimulated versus unstimulated IL-8 release, n=3, ns – not significant, * p< 0.05, **p<0.01.

Figure 4.24. The effect of protamine sulphate on IL-8 level released from OPZ-stimulated neutrophils. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s multiple comparison test to compare drug treated and OPZ-stimulated IL-8 release. Two-tailed, paired t-test was applied to analyse OPZ-stimulated versus unstimulated IL-8 release, n=3, ns – not significant, *p<0.05.

Cell pellet-associated IL-8 was approximately 30 times higher than IL-8 released to the supernatant. IL-8 detected in the cell pellet of OPZ-stimulated neutrophils was not significantly lower than unstimulated IL-8, 850.4 ± 225.6 versus 395.9 ± 121.4 pg/ml (Figure 4.25 – 4.28).
After tobramycin (0.01 – 0.5 mM) treatment, IL-8 was unchanged compared to cell-associated IL-8 from stimulated PMNs (Figure 4.25).

![Graph showing cell pellet-associated IL-8 of OPZ-stimulated neutrophils treated with tobramycin. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s multiple comparison test in comparison to untreated OPZ-stimulated control. Also, two-tailed, paired t-test was applied to analyse cell-associated OPZ-stimulated versus unstimulated IL-8, n=3, ns – not significant.]

The incubation of OPZ-stimulated neutrophils with copper-tobramycin at 0.05, 0.1 and 0.5 µM concentration significantly (p<0.01) decreased IL-8 concentration measured in the cell pellet compared to the untreated OPZ-stimulated neutrophil control to 116.7 ± 54.26, 61.82 ± 7.75 and 50.68 ± 20.76 pg/ml, respectively (Figure 4.26, over).

The IL-8 level from copper-sulphate treated OPZ-stimulated neutrophils was also significantly (p<0.01) decreased in the range of 0.025 – 0.5 mM from 29.91 ± 13.77 pg/ml to 46.41 ± 5.40 to 22.16 ± 13.42 pg/ml in comparison to OPZ-stimulated neutrophils in the absence of the drug (Figure 4.27, over).
Figure 4.26. The effect of copper-tobramycin on IL-8 levels in the cell pellet of OPZ-stimulated PMNs. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s multiple comparison test in comparison to cell-associated IL-8 from OPZ-stimulated control. Two-tailed, paired t-test was used to analyse OPZ-stimulated versus unstimulated IL-8 level in the cell pellet, n=3, ns – not significant, **p<0.01.

Figure 4.27. IL-8 level from OPZ-stimulated neutrophils incubated with copper sulphate in the cell pellet. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s post hoc test to compare drug treated and OPZ-stimulated cell-associated IL-8 level. Also, two-tailed, paired t-test was applied to analyse OPZ-stimulated versus unstimulated IL-8 in the cell pellet, n=3, ns – not significant, **p<0.01.

Additionally, protamine sulphate at 100 µg/ml significantly (p<0.05) diminished IL-8 concentration measured in the cell pellet from 850.4 ± 225.6 to 61.19 ± 21.54 pg/ml (Figure 4.28, over).
Figure 4.28. The effect of protamine sulphate in OPZ-stimulated PMN on IL-8 level in the cell pellet. The data expressed as mean ± SEM was analysed using one-way ANOVA and Dunnett’s post hoc test in comparison to IL-8 level in OPZ-stimulated PMNs in the cell pellet. Two-tailed, paired t-test was used to analyse OPZ-stimulated versus unstimulated cell pellet-associated IL-8, n=3, ns – not significant, *p<0.05.

Figure 4.29 (over) shows the effect of α1-AT on the IL-8 level in both supernatants and cell pellets of OPZ-stimulated PMNs incubated with tobramycin and copper-tobramycin (0.5 mM). Tobramycin or copper-tobramycin at 0.5 mM concentration did not change the IL-8 released to the supernatant in comparison to the untreated control. However, tobramycin in the presence of α1-AT significantly (p<0.05) increased this level from 43.73 ± 18.56 to 124.1 ± 34.52 pg/ml, while copper-tobramycin in the presence of α1-AT was ineffective compared to the appropriate control. The presence of α1-AT significantly (p<0.05) increased the concentration of IL-8 measured in the supernatant only in the presence of tobramycin (Figure 4.29A). Moreover, tobramycin or copper-tobramycin (0.5 mM) did not change significantly cell pellet-associated IL-8 level compared to the OPZ-stimulated PMNs control in the absence or the presence of α1-AT. α1-AT was also ineffective on IL-8 concentrations measured in the cell pellet of PMN-stimulated with OPZ and treated or untreated with tobramycin and copper-tobramycin (Figure 4.29B).
Figure 4.29. The effect of α1-AT on the level of IL-8 in supernatants (A) and cell pellets (B) of OPZ-stimulated neutrophils treated with tobramycin and copper-tobramycin (0.5 mM). The data expressed as mean ± SEM was analysed using two-tailed, paired t-test, n=3 independent experiments, ns – not significant, *p<0.05.

4.5.5. VIABILITY OF NEUTROPHILS DURING NEUTROPHIL ELASTASE ACTIVITY MEASUREMENT

α1-AT at 2 mg/ml was not toxic to PMNs over 1 hour incubation in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 compared to the untreated control, which contained 85.70 ± 0.20 % live, 0.07 ± 0.02 % apoptotic and 14.24 ± 0.20 % necrotic cells (Figure 4.30, over). However, protamine sulphate diminished the population of live
neutrophils concentration-dependently and 100 mg/ml protamine sulphate decreased the live cell pool by 15.66 ± 2.84 % (Figure 4.31).

Figure 4.30. The effect of α1-AT (2 mg/ml) on neutrophil viability during 1 hour incubation in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4, n=1, in triplicate.

Figure 4.31. Neutrophil viability during 1 hour incubation with protamine sulphate in HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4, n=1, in triplicate.
The effect of tobramycin, copper-tobramycin and copper sulphate on neutrophil viability during neutrophil elastase activity measurements was not different to the effect elicited after 3 hour incubation with the assay buffer, HBSS (+ Ca/Mg) containing 20 mM HEPES, pH 7.4 (results not shown).

4.5.6. THE EFFECT OF DIFFERENT COMPOUNDS ON IL-8 LEVEL DETECTED BY ELISA

In the view of the apparent loss of IL-8 from both cell supernatants and pellets on treatment with copper-tobramycin, copper sulphate and protamine sulphate, the compounds were tested for their interference in the ELISA.

Tobramycin, copper-tobramycin, copper sulphate (0.5 mM), copper chloride (0.4 mM) and protamine sulphate (0.1 mg/ml) were tested either alone for a false positive effect (Table 4.1) or in the presence of a constant IL-8 concentration (500 pg/ml) for IL-8 degradation (Table 4.2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-8 detected in compound [pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM Tob</td>
<td>7.84 ± 0.11</td>
</tr>
<tr>
<td>0.5 mM Cu-Tob</td>
<td>Non detected</td>
</tr>
<tr>
<td>0.5 mM CuSO₄</td>
<td>35.66 ± 3.83</td>
</tr>
<tr>
<td>0.4 mM CuCl₂</td>
<td>42.25 ± 20.40</td>
</tr>
<tr>
<td>0.1 mM Protamine sulphate</td>
<td>13.587 ± 0.200</td>
</tr>
</tbody>
</table>

Table 4.1. IL-8 detected in different compounds measured by ELISA. The data showing the effect of compound alone on IL-8 level is subtracted from the buffer control [pg/ml] and shown as mean ± SEM, n=1 in triplicate. Tob – tobramycin, Cu-Tob – copper-tobramycin.

<table>
<thead>
<tr>
<th>Compound + IL-8 (500 pg/ml)</th>
<th>% of IL-8 control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM Tob</td>
<td>101.53 ± 17.34</td>
</tr>
<tr>
<td>0.5 mM Cu-Tob</td>
<td>69.90 ± 7.34</td>
</tr>
<tr>
<td>0.5 mM CuSO₄</td>
<td>73.33 ± 8.16</td>
</tr>
<tr>
<td>0.4 mM CuCl₂</td>
<td>91.78 ± 14.38</td>
</tr>
<tr>
<td>0.1 mM Protamine sulphate</td>
<td>78.54 ± 24.50</td>
</tr>
</tbody>
</table>

Table 4.2. The effect of different compounds on IL-8 level detected by ELISA. The data showing the effect of compound in the presence of IL-8 (500 pg/ml) is expressed as a percentage of the control. The data is shown as mean ± SEM, n=1 in triplicate. Tob – tobramycin, Cu-Tob – copper-tobramycin.
IL-8 detected in samples containing tobramycin, copper-sulphate, copper chloride or protamine sulphate alone may indicate the assay limitation or sample contamination.

Tobramycin and copper chloride did not reduce the level of IL-8 detected in the sample, however other compounds did. Among them, copper-tobramycin had the strongest effect and decreased IL-8 by 30.10 ± 7.34 %, while copper sulphate and protamine sulphate decreased IL-8 by 26.66 ± 8.16 % and 21.46 ± 24.50 %, respectively.

4.5.7. OXIDATIVE STRESS IS NOT INDUCED IN HLMVEC BY COPPER AND/OR HYDROGEN PEROXIDE

2′,7′-Dichlorodihydrofluorescein diacetate (H₂DCF-DA) at 10 µM was used as an indicator of intracellular ROS generation in HLMVEC. It is a cell permeable probe that enters the cell and is deacetylated to a nonfluorescent product, 2′,7′-dichlorodihydrofluorescein (H₂-DCF) by cellular esterases and oxidised by ROS to a fluorescent product, 2′,7′-dichlorofluorescein (DCF).

In order to find a stimulus to induce intracellular ROS generation (Figure 5.32, B, over) in a layer of confluent HLMVEC, they were treated with CuCl₂ (400 µM) alone, H₂O₂ (50 µM) alone or with a combination of CuCl₂ (400 µM) and H₂O₂ (50 µM) for 30 minutes in full growth medium. Then tobramycin and copper-tobramycin (0.5 mM) in the presence of these stimuli were tested to check whether they have any anti-oxidant properties. A nuclear stain (Hoechst) was used as an additional control to visualize the cell nucleus (Figure 5.32, A, over). CuCl₂ alone (panel 2), H₂O₂ alone (panel 5) as well as CuCl₂ plus H₂O₂ (panel 6) induce a minimal level of ROS generation in HLMVEC compared to a negative control (panel 1). This induced ROS level was not high enough to observe an effect of tobramycin (panel 3 and 7) or copper-tobramycin (panel 4 and 8).
4.5.8. TNF-α-INDUCED ROS GENERATION IN HLMVEC AND THE PROTECTIVE ROLE OF TOBRAMYCIN AND COPPER-TOBRAMYCIN

TNF-α was used to generate oxidative stress in the same cells. TNF-α in a concentration range of 1 – 10 ng/ml was tested on the oxidative stress generated in HLMVEC for 15 minutes (Figure 4.33, over). TNF-α at 10 ng/ml (panel 8) induced more ROS than 1 and 5 ng/ml (panel 6 and 7) compared to the absence of TNF-α (panel 5) or DCF-DA (panel 1 – 4). The effect of 10 ng/ml TNF-α was then tested in the time-course experiment.
Figure 4.33. TNF-α concentration curve of HLMVEC stained for ROS using DCF-DA. 1. no TNF-α, no DCF-DA; 2. 1 ng/ml TNF-α, no DCF-DA; 3. 5 ng/ml TNF-α, no DCF-DA; 4. 10 ng/ml TNF-α, no DCF-DA; 5. no TNF-α, 10 uM DCF-DA; 6. 1 ng/ml TNF-α, 10 uM DCF-DA; 7. 5 ng/ml TNF-α, 10 uM DCF-DA, 8. 10 ng/ml TNF-α, 10 uM DCF-DA. Scale bar 100 µm.

TNF-α-treatment (10 ng/ml) of HLMVEC for 15 minutes triggered the most intense ROS generation (Figure 4.34, panel 7) compared to the same treatment for 5 (panel 6) and 30 minutes (panel 8). ROS were generated at a minimal level in the absence of TNF-α (panel 5) and not detected in the absence of DCF-DA (panel 1 – 4), respectively.

Figure 4.34. TNF-α time course of ROS generation in HLMVEC. 1) no TNF-α; no DCF-DA; 2) 10 ng/ml TNF-α for 5 minutes, no DCF-DA; 3) 10 ng/ml TNF-α for 15 minutes, no DCF-DA; 4) 10 ng/ml TNF-α for 30 minutes, no DCF-DA; 5) no TNF-α, 10 µM DCF-DA; 6) 10 ng/ml TNF-α for 5 minutes, 10 µM DCF-DA; 7) 10 ng/ml TNF-α for 15 minutes, 10 µM DCF-DA, 8) 10 ng/ml TNF-α for 30 minutes, 10 µM DCF-DA. Scale bar 100 µm.
Figure 4.35 confirms that TNF-α at 10 ng/ml incubated on HLMVEC for 15 minutes at 37°C was the best stimulus to induce oxidative stress in EC. ROS were most likely generated predominantly in mitochondria and cytoplasmic organelles, rather than the nucleus.

Having established optimum conditions for intracellular ROS generation in HLMVEC, the anti-oxidative capacity of tobramycin and copper-tobramycin in a concentration range of 0.01 – 0.5 mM was tested (Figure 4.36, over). Copper-tobramycin at 0.5 mM concentration completely reduced oxidative stress induced by TNF-α treatment in HLMVEC. Tobramycin also reduced ROS generation, but was less effective than copper-
tobramycin. The effect of both copper-tobramycin and tobramycin was concentration dependent.

Figure 4.36. The effect of tobramycin and copper-tobramycin (0.01 – 0.5 mM) on TNF-α-induced ROS generation in HLMVEC. HLMVEC were incubated with tobramycin and copper-tobramycin for 3.5 hours, treated with 10 μM DCF-DA for 30’, activated with TNF-α for 15’ and fixed with 4 % (w/v) PFA/PBS. 1. Negative control (no drug, no DCF-DA); 2. Positive control (no drug, TNF-α activation); 3-8 TNF-α activation, DCF-DA; 3. 0.01 mM Tob; 4. 0.1 mM Tob; 5. 0.5 mM Tob; 6. 0.01 mM Cu-Tob; 7. 0.1 mM Cu-Tob; 8. 0.5 mM Cu-Tob. These represent typical results from three independent experiments.

The anti-oxidant effect of tobramycin and copper-tobramycin was analysed quantitatively (Figure 4.37, over). TNF-α induced a significant (p<0.001) oxidative stress in EC compared to the untreated control, 20.64 ± 1.66 versus 7.54 ± 0.75 mean intensity units. Tobramycin reduced ROS level generated by TNF-α, however only 0.01 and 0.5 mM tobramycin elicited a significant (p<0.01) effect. In comparison, ROS level was significantly (p<0.01) reduced concentration-dependently by copper-tobramycin over the whole concentration range, 0.01 – 0.5 mM. There was no statistically significant difference between the effect of 0.01 mM tobramycin and 0.01 mM copper-tobramycin concentration, 14.45 ± 1.43 versus 11.96 ± 1.06 mean intensity units. However, the effect of copper-tobramycin was significantly greater than that of tobramycin at 0.1 mM (8.98 ± 0.39 versus 15.97 ± 1.38 mean intensity units, p<0.001) and 0.5 mM (7.77 ± 0.53 versus 13.28 ± 1.59 mean intensity units, p<0.01), respectively.
4.5.8. THE EFFECT OF CFTRinh-172 ON ROS GENERATION IN HLMVEC AND THE ROLE OF TOBRAMYCIN AND COPPER-TOBRAMYCIN

The CFTR inhibitor, CFTRinh-172 was reported to induce oxidative stress in epithelial cell lines (Kelly et al, 2010). The pro-oxidant ability of CFTRinh-172 on endothelial cells was investigated in the absence and presence of TNF-α (Figure 4.38, over). TNF-α (10 ng/ml) incubated on HLMVEC for 15 minutes was shown to induce oxidative stress (panel 7) and 30 minutes-treatment of HLMVEC with CFTRinh-172 (20 µM) induced a similar level of ROS generation in those cells (panel 5), while the presence of CFTRinh-172 for 5, 15 or 60 minutes had a smaller effect (panel 3, 4 and 6). The highest degree of ROS was generated by a combination of TNF-α and CFTRinh-172, when TNF-α was present on HLMVEC for 15 minutes and CFTRinh-172 for 30 minutes (panel 9). Shorter (15 minutes, panel 8) or longer (60 minutes, panel 10) treatment of EC with CFTRinh-172 in the presence of TNF-α was less effective. Moreover, ROS were not detected in the presence of TNF-α or CFTRinh-172 alone without DCF-DA (panel 1 and 2, respectively).
The contribution of CFTRinh-172 to endothelial ROS generation was previously reported, therefore CFTRinh-172 alone was used as an oxidative stress inducer in HLMVEC. Furthermore, the anti-oxidative effect of tobramycin and copper-tobramycin in the concentration range of 0.01 – 0.5 mM and NAC (1 – 20 mM) on CFTRinh-172-treated endothelium was investigated (Figure 4.39, over). Tobramycin and copper-tobramycin reduced oxidative stress concentration-dependently (panel 3 – 5 and 6 – 8, respectively), in regard to the positive effect of CFTRinh alone (panel 2). However copper-tobramycin was more effective in terms of maximal effect, than tobramycin. NAC also reduced ROS level, but its effect was not dose dependent (panel 9 – 11).
4.6. SUMMARY OF RESULTS

Copper-tobramycin was demonstrated to have anti-oxidant properties, as it significantly decreased the detection of both superoxide anion and hydrogen peroxide (extracellular oxidative stress) from OPZ- and PMA-activated neutrophils at concentrations which did not induce apoptosis, while tobramycin was without any effect. Also, copper-tobramycin reduced ROS generated in HLMVEC (intracellular oxidative stress) by TNF-α or CFTRinh-172. Tobramycin had the same qualitative inhibitory effect on endothelial ROS as copper-tobramycin, but it was less efficacious.
Moreover, both tobramycin and copper-tobramycin increased released NE activity, which was inhibitable by α1-AT, while cell-associated NE activity was reduced by copper-tobramycin and was inhibited by α1-AT.

4.7. DISCUSSION

PMNs can participate in host defence through their ability to metabolize oxygen to ROS through NADPH oxidase (Lambeth, 2004). The importance of this process is exemplified in patients suffering from chronic granulomatous disease, a disorder of non functioning oxidase due to a deficiency of gp91 phox, resulting in recurrent bacterial and fungal infections (Segal et al, 1978). The stimulation of leukocyte oxidative metabolism takes place during direct contact with the pathogen and subsequent phagocytosis. Indeed, the respiratory burst can be induced by a variety of stimuli, including chemoattractants (fMLP, C5a and C3a complement fragments), cytokines (TNF-α, TNF-β, GM-CSF, G-CSF), nonchemotactic stimuli (IgG, PMA, ionophore A23187), lectins (concanavalin A) or opsonised crystals (monosodium urate crystals, calcium pyrophosphate dehydrate, zymosan) (Burt & Jackson, 1993; Hoogewerf et al, 1990; Nathan et al, 1989; Balsinde & Mollinedo, 1988; McPhail & Snyderman, 1983; Simchowitz et al, 1982).

Unopsonised zymosan was shown to be a poor activator of PMN respiratory burst, but human PMN that express CR3 (CD11/CD18) bind and ingest zymosan after exogenous opsonization (Ezekowitz et al, 1985). In the current study, zymosan particles were opsonised with normal human serum. Moreover, the OPZ-stimulated respiratory burst of neutrophils was significantly higher compared to the spontaneous production of O$_2^-$. Also, OPZ elicited a stronger effect compared to other stimuli, such as PMA or fMLP. Others also reported OPZ to be the strongest and the most physiological activator of the neutrophil respiratory burst (Ramasamy et al, 2010; Azuma et al, 1993). Importantly, the activation of the respiratory burst in neutrophils could be elicited in independent ways. Both, OPZ and fMLP activates the respiratory burst through a receptor-dependent pathway, whereas PMA is a direct activator of protein kinase C (Azuma et al, 1993, Ezekowitz et al, 1985).
Hydrogen peroxide release was only detectable in the presence of the MPO and catalase inhibitor, sodium azide (Nakagawara et al, 1981), in agreement with the current study. Also, Nahum et al (1990) reported using sodium azide to allow detection of H$_2$O$_2$ from OPZ-activated PMNs and to improve sensitivity and reproducibility of H$_2$O$_2$ measurement. No H$_2$O$_2$ was detected in the absence of SA.

Hydrogen peroxide is an unusual oxidant. It reacts slowly with organic substrates and thus can diffuse large distances in biological systems. Its small size and lack of charge facilitate its movement across plasma membranes. Although H$_2$O$_2$ is not a free radical, it is detectable by DCF-DA. Moreover, its intracellular concentration is decreased by several enzymes (Test & Weiss, 1984). In contrast to hydrogen peroxide, superoxide is not transported across the cell membrane. Superoxide is a poorly reactive oxide radical made from the reaction of molecular oxygen with catecholamines, tetrahydrofolates and some constituents of mitochondrial and other electron-transport chains. Additionally, activated phagocytes generate large amounts of superoxide as part of bacterial phagocytosis (Halliwell, 1994).

During chronic inflammation, this normal and protective mechanism of killing foreign microorganisms could be damaging. Therefore, the inhibition of NADPH oxidase may be necessary.

Many antibiotics have been shown to interfere with various aspects of host defence, including phagocytic ingestion and killing (Gemmel, 1984; Mandell, 1982; Milatovic, 1982) by either up- or down-regulating phagocyte function. Tobramycin sulphate (2 – 12 µg/ml) alone and in a combination with pencillin G sodium and metronidazole hydrochloride was previously reported to significantly and dose-dependently reduce NADPH oxidase activity in human PMNs stimulated with fMLP (Moon et al, 1986). However, tobramycin in the concentration range 1 – 500 µM, approximately 0.5 – 250 µg/ml, was unable to decrease superoxide anion detected from OPZ-stimulated neutrophils. Also, tobramycin at 10 – 500 µM (5 – 250 µg/ml) was ineffective in the inhibition of H$_2$O$_2$ release in the same experimental settings. Likewise, Ottonello and co-workers (1991) showed that penicillin G, ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin did not inhibit superoxide generation by neutrophils.
Moreover, these drugs were capable of inhibiting the neutrophil cytolytic activity by inactivating HOCl generated extracellularly by MPO, crucial to the cell lysis of lymphoblastoid Daudi target cells. No effect of tobramycin on the respiratory burst of neutrophils may be due to the fact that tobramycin was demonstrated not to bind or to be taken up by resting or activated neutrophils (Chapter 3).

Copper-tobramycin, as opposed to tobramycin, reduced superoxide detection from OPZ-stimulated PMNs. This effect was seen at non-cytotoxic concentrations of copper-tobramycin. This could be due to the SOD-like activity of copper-tobramycin complex (Chapter 2), since SOD is a superoxide scavenging enzyme. The same superoxide reducing effect was however also observed for copper sulphate, which could indicate a role for copper. Copper-tobramycin, but not SOD, was also shown to significantly decrease H$_2$O$_2$ detected from both OPZ- and PMA-stimulated neutrophils. SOD as an enzyme catalysing dismutation of superoxide to hydrogen peroxide, may be expected to increase the level of detectable hydrogen peroxide, but this is not always the case (Liochev & Fridovich, 2007). However, large proteins such as SOD do not penetrate cell membranes and are therefore ineffective against intracellular ROS (Szeto, 2006). This experiment raises the possibility that H$_2$O$_2$ is made intracellularly. Since copper-tobramycin does not have catalase activity (Chapter 2), this result might indicate an inhibitory effect on NADPH oxidase. Copper-tobramycin could also act as a pericellular agent (not taken up by neutrophils), while no substantial SOD enzyme activity was observed in the membranous fraction (Rest & Spitznagel, 1977). However, the most probable explanation of the copper-tobramycin effect in reducing detectable hydrogen peroxide and superoxide from activated neutrophils at high concentrations is its ability to elicit neutrophil apoptosis in the same concentration range in assays lacking the protective effect of FBS. Importantly, these results indicate that copper-tobramycin has SOD-like activity in both cell-based and cell-free (Chapter 2) systems.

Copper complexes with amino acids, such as Cu(Lys)$_2$, Cu(His)$_2$ and CuTyr, were shown to inhibit O$_2^-$-dependent NADPH oxidation (Joester et al., 1972; Klug et al., 1972). Additionally, commonly used chelating agents, by interacting with available copper, may artificially increase NADPH oxidation in xanthine oxidase and other superoxide generating systems (Bidwell Goetz & Proctor, 1984). The effect of copper-tobramycin
and, in comparison, copper sulphate on NADPH oxidase could be further investigated after isolation of neutrophil cytoplasts, adapting the method presented by Yazdabakhsh and co-workers (1987) for eosinoplast preparation. Indeed, cytoplasts are granules and internal organelles of neutrophils that contain a fully competent stimulatable NADPH oxidase activity (Henderson et al, 1988). However, as neutrophils do not take up tobramycin or copper-tobramycin (Chapter 3), copper-tobramycin, unlike tobramycin, was demonstrated here to have potent extracellular anti-inflammatory properties.

Neutrophil degranulation is a consequence of neutrophil stimulation with the same activators over time (Bentwood & Henson, 1980). Turner et al (1994) reported that OPZ is a poor stimulus for NE release. Also here, released NE was minimal, whereas the majority (96.76 ± 4.92 %) of NE was bound to the cell surface. Although NE is released to the local environment, human neutrophils express persistently active cell-surface bound human leukocyte elastase (Owen et al, 1995). Moreover, the sulphate groups of HS/CS proteoglycans have been identified as the PMN binding sites for human leukocyte elastase and cathepsin G (CG). HS/CS-containing proteoglycans are low affinity, high volume PMN surface binding sites for NE and CG, which are well suited to bind high concentrations of active serine proteinases released from degranulating PMN and protecting them from inactivation (Campbell & Owen, 2007). Neutrophil elastase, proteinase 3 (PR3) and cathepsin G bound to bronchoalveolar proteoglycans are also active (Shum et al, 2000). Moreover, the active serine proteases in CF sputum were shown to bind to NETs secreted by the activated neutrophils and genomic DNA released from senescent and dead neutrophils. Due to this binding, NE was resistant in part inhibition by exogenous protease inhibitors (Dubois et al, 2012).

In the current study, tobramycin significantly increased the released NE level up to 3-fold. However, copper-tobramycin induced a 30-fold increase in free NE and additionally, CuSO₄ increased NE release by 9 fold. In agreement, copper ions were shown to stimulate neutrophils to degranulate in a concentration-dependent manner (Hunt et al, 1992). Although the effects on NE were seen at concentrations of copper-tobramycin and copper sulphate that induce apoptosis, apoptosis is independent of degranulation (Hebert et al, 1996). Moreover, only copper-tobramycin significantly
decreased cell pellet-associated NE activity, which suggests displacing elastase from its HS binding sites, while the released NE was protected by copper-tobramycin.

α1-AT is the major inhibitor of serine proteases, including NE, which is secreted by the liver and accesses the local inflammatory site by simple diffusion from plasma. Indeed, during an inflammatory response, there is plasma exudation into the airway tissue and lumen (Persson, 1991) and α1-AT is measured in airway BAL or sputum (Hilliard et al, 2002). More importantly, α1-AT is synthesised and secreted by neutrophils (du Bois et al, 1991), like two other low-molecular weight inhibitors, SLPI and elafin, which were also shown to be synthesized locally in the lung (Sallenave et al, 1997). Thus, neutrophils respond to the surface stimulation not only by secreting NE, but also by secreting its inhibitor, α1-AT, suggesting that these cells have an inherent mechanism for suppressing the local effects of NE (Paakko et al, 1996). However, the rate of α1-AT synthesis within neutrophils is low compared with that of hepatocytes and in the current experiment α1-AT was supplemented at the concentrations found in human plasma, 2 mg/ml (Fregonese & Stolk, 2008). α1-AT inhibits free NE, but catalytically active cell surface-bound human NE is remarkably resistant to inhibition by serine proteinase inhibitors, such as α1-AT (Owen et al, 1995). This membrane-bound leukocyte elastase activity may have potent pro-inflammatory effects (Owen et al, 1997). Interestingly, α1-AT in the presence of copper-tobramycin was able to inhibit both free and cell-associated NE. These results suggest that copper-tobramycin not only releases NE from HS/CS binding sites on neutrophils, but also protects α1-AT from degradation by NE and ROS.

NE was demonstrated to bind to the cell surface by a charge-dependent mechanism, since incubation of cells with cationic molecules (protamine and L-lysine) downregulates membrane-bound elastase (Owen et al, 1997). Protamine sulphate, similar to aminoglycoside antibiotics, is a positively-charged molecule at pH 7.4 and here mirrored the effects of tobramycin, copper-tobramycin and copper sulphate in increasing the release of NE activity. Protamine sulphate also induced a degree of neutrophil necrosis, not apoptosis. However, necrotic neutrophils will release IL-8 and NE and here they did not.
Although OPZ stimulation of neutrophils released a small percentage of NE, it had no significant effect on IL-8. IL-8, similarly to NE was mostly associated with the cell pellet. Indeed, the level of cell-bound IL-8 was 40 times higher compared to the IL-8 concentration in the supernatant. Tobramycin at 50 µM increased the level of detected released IL-8, but had no effect on cell-bound IL-8. In contrast, the IL-8 concentration was diminished in both supernatants and cell pellets of neutrophils stimulated with OPZ in the presence of copper-tobramycin, copper sulphate and protamine sulphate.

Neutrophils release the content of the granules in the reverse order to the biosynthesis of their characteristic proteins. Indeed, IL-8 stored in neutrophil secretory vesicles is released long before NE from the primary granules (Borregaard & Cowland, 1997). During that time IL-8 could be degraded by extracellular ROS generated by OPZ-activated neutrophils, but this explanation is not probable. Copper chloride and hydrogen peroxide, a known ROS-generating system, induced a low level of intracellular ROS detected within HLMVEC in a full growth medium. Copper chloride and hydrogen peroxide were also shown to have no effect on IL-8, when incubated in medium deprived of antioxidants, such as FBS or ascorbic acid (results not shown). Also, MMPs were not shown so far to degrade IL-8. For instance, gelatinase B was found to truncate six amino acids from the aminoterminus of IL-8 converting it into 10 - 20 times more potent chemokine that the full length chemokine (Opdenakker et al, 2001). However, NE could degrade a variety of proinflammatory cytokines including IL-8 (Witherden et al, 2004), or the protein could be destroyed by other proteases, such as CG or PR3. Copper-tobramycin and copper sulphate could also interfere slightly with the IL-8 ELISA, as those treatments diminished the detectable IL-8 concentration by approximately 30 %, whereas protamine sulphate diminished IL-8 level by approximately 20 %. Alternatively a recognition site of IL-8 antibody detection had been affected by those treatments.

In vivo, HS functions as an antioxidant in the basement membrane, protecting different molecules from degradation by ROS (Raats et al, 1997). This antioxidant effect has also been shown for heparin and hyaluronic acid (Hiebert & Liu, 1990, Sato et al, 1990). Recently, HS was demonstrated to protect the chemokine from oxidative damage,
promoting dityrosine cross-links and multimer formation under oxidative conditions (MacGregor et al, 2011).

α1-AT was demonstrated to bind IL-8 and thus prevented its interaction with CXCR1, eliciting an anti-inflammatory effect (Bergin et al, 2010). Importantly, the IL-8 level was increased in supernatants from OPZ-stimulated neutrophils in the presence of tobramycin and α1-AT compared to the absence of this inhibitor. However, α1-AT had no effect on neutrophil IL-8 in the presence of copper-tobramycin. α1-AT does not appear to protect IL-8 from degradation, therefore it is not an effect of NE, CG or PR3.

Previous studies have identified abnormalities in the oxidative responses of the neutrophil in CF (Witko-Sarsat et al, 1996, Vaisman et al, 1994). In contrast, McKeon (2010) reported that CF neutrophils have normal intrinsic ROS generation and the expression of NADPH oxidase components is identical in the CF and non-CF neutrophils. Hence, CF neutrophils in BALF respond abnormally to TNF-α and IL-8 and react to opsonised bacteria by releasing increased amounts of NE (Taggard et al, 2000). More importantly, peripheral circulating neutrophils in CF have been characterized by delayed apoptosis. Defective apoptosis, which is suggested to be related to primary innate factors rather than infectious state or CFTR mutation, may explain PMN persistence at the inflammation site (Moriceau et al, 2010). Blood neutrophils have been shown to have reduced expression of Fas and FasL, and thus delayed apoptosis, due to intravenous administration of antibiotics (Downey et al, 2007). Moreover, reduced expression of the tumor necrosis factor receptor 1 (TNF-R1), which is a prerequisite for TNF-α-induced neutrophil apoptosis (Gon et al, 1996), was observed on purified circulating neutrophils (Downey et al, 2007). Also, defective airway clearance of apoptotic cells by macrophages in CF may be due to elastase-mediated cleavage of the phosphatidylserine receptor on phagocytes and may contribute to ongoing airway inflammation (Vandivier et al, 2002). CF neutrophils may therefore not respond to tobramycin and copper-tobramycin treatment in the same way as normal neutrophils. It should therefore be investigated further.

Copper-tobramycin and copper sulphate at 0.5 mM concentration induced nearly 100 % apoptosis in neutrophils in the absence of FBS, ie in HBSS (+ Ca/Mg) containing 20
mM HEPES, pH 7.4 within 30 minutes incubation. However, no neutrophil apoptosis was induced by those drugs at any concentration tested (0.01 – 0.5 mM) in RPMI 1640 supplemented by 2.5 % FBS (Chapter 5). It therefore indicates the deleterious effect of non physiological HBSS buffer with no addition of FBS. FBS could not be used in the assay to measure superoxide and hydrogen peroxide release from neutrophils or NE activity, because bovine serum albumin, the major component of FBS, exerts some important anti-oxidant activities (Wayner et al, 1985). In vivo, phagocytic clearance of intact apoptotic cells is a safe disposal route for the organism (Ren & Savill, 1998). Therefore, apoptosis triggered by copper-tobramycin and copper sulphate would have a favourable effect. However, this effect would not happen in vivo, as neutrophils are not in plasma-free conditions.

Platelets were reported to undergo a respiratory burst by releasing superoxide and hydrogen peroxide (Del Principle et al, 1983; Finazzi-Agro et al, 1982) or augment the respiratory burst of neutrophils (Miedzybrodzki et al, 2008). Also NADPH oxidase appears to be the major source of ROS in ECs (Frey et al, 2009). However, the level of superoxide and hydrogen peroxide released from activated platelets and HLMVEC were not statistically significant upon stimulation in comparison to an untreated control. Also, the co-cultures of HLMVEC with platelets and/or neutrophils demonstrated neutrophils to be the main source of superoxide and hydrogen peroxide.

The current research showed that TNF-α generates intracellular ROS in HLMVEC, confirming the observations of Rahman and coworkers (1998) that TNF-α generates intracellular ROS in human pulmonary artery endothelial cells. Intracellular ROS are here involved in signal transduction after binding of TNF-R1 (Shen & Pervaiz, 2006). In most cells and tissues mitochondria are the major generators of ROS (Boveris, 1984). TNF-α may stimulate ROS production by mitochondria (Chandel et al, 2001), but the major source of ROS is a phagocyte-type NADPH oxidase that is constitutively expressed in EC (Gu et al, 2002; Bayraktutan et al, 2000; Gorlach et al, 2000). In the current study, TNF-α induced intracellular oxidative stress in HLMVEC, which was located mainly in the mitochondria and cytoplasmic organelles, presumably ER (Zhang & Kaufman, 2008). It was however impossible to identify the exact ROS location.
CFTRinh-172 as well as another highly potent CFTR inhibitor, GlyH-101 was able to induce a rapid increase in ROS levels and depolarise mitochondria in epithelial cells and those effects were independent of their CFTR inhibition (Chen et al, 2008; Maiuri et al, 2008). Also here, CFTRinh-172 induced ROS generation to a level similar to TNF-α in HLMVEC. A combination of CFTRinh-172 and TNF-α elicited a stronger response in comparison to CFTRinh-172 or TNF-α alone.

On the contrary, copper in the absence or presence of hydrogen peroxide did not stimulate ROS generation in HLMVEC, although it is known that, in the presence of heavy metals, H_2O_2 undergoes the Fenton reaction to form the highly reactive hydroxyl radical (Biaglow et al, 1997). It is therefore possible that these reactions require longer incubation times and/or higher concentrations of both copper chloride and hydrogen peroxide. Moreover, the CuCl_2/H_2O_2 effect was not receptor-mediated.

Importantly, tobramycin and copper-tobramycin were shown to concentration-dependently reduce intracellular oxidative stress generated in HLMVEC by TNF-α or CFTRinh-172, and copper-tobramycin was more effective than tobramycin. Another antioxidant, NAC, de-acetylated to cysteine on the cell surface or inside of the cell, promotes glutathione formation, while intracellular cysteine limits and/or enhances the antioxidant activity of the cytoplasm (Arakawa & Ito, 2007). Also here, NAC reduced oxidative stress induced by CFTRinh-172, however its effect was not concentration-dependent. Even though NAC is an efficient antioxidant and an anti-inflammatory agent, known to protect lung (Moldeus et al, 1986) and used in the treatment of CF and COPD (Sadowska et al, 2007; Duijvestijn & Brand, 1999), great care has to be taken, because under certain circumstances, anti-oxidants, such as NAC may also serve as pro-oxidants (Childs et al, 2001; Oikawa et al, 1999; Sprong et al, 1998).
5.1. INTRODUCTION

5.1.1. NEUTROPHIL-PLATELET-ENDOTHELIAL CELL INTERACTIONS

CF patients have increased levels of circulating activated platelets. Since the plasma level of ATP is increased in CF, the platelet hyperactivation may be due to ATP. ATP is both transported by CFTR and acts as an agonist of platelets (Birk et al., 2002; Lader et al., 2000). Enhanced lipid peroxidation is an important feature of CF that may also contribute to the persistent platelet activation. The urinary excretion of 11-dehydrothromboxane B2, a marker of in vivo platelet activation, was shown to be significantly higher in CF patients than controls. Moreover, the supplementation of vitamin E, which is also deficient in CF, reduces 11-dehydrothromboxane B2 excretion (Ciabattoni et al., 2000). The platelet reactivity has been determined by greater aggregability with monocytes and neutrophils as well as increased expression of surface P-selectin in response to agonists than in normal platelets. Also increased release of platelet-derived mediators could play an important role in the pathogenesis of CF lung disease (O’Sullivan et al., 2005).

In normal physiologic conditions, circulating platelets do not interact with nonactivated endothelium, but endothelial denudation induces immediate platelet adhesion and aggregation at the site of injury (Clementson, 1999). Platelet-endothelial cell interactions are comprised of an initial platelet tethering, followed by rolling and subsequent firm adhesion of platelets, mediated by selectins, integrins and adhesion molecules.
Two platelet receptors for endothelial P-selectin were identified to be PSGL-1 and a glycoprotein GPIbα to mediate platelet rolling on the endothelial cell layer (Frenette et al, 2000; Romo et al, 1999). Also, vWF, which is stored in Weibel-Palade bodies, similarly to P-selectin, was shown to be an important ligand for platelet GPIbα (Wagner et al, 1982). Platelets may also loosely interact with E-selectin expressed on activated endothelial cells, but the counter-ligand on the platelet surface is unknown (Frenette et al, 1998).

The adhesion of activated platelets to activated endothelium involves platelet integrin αIIbβ3 and endothelial αVβ3 and ICAM-1 (Bombeli et al, 1998; Li et al, 1996). Platelet adhesion to post-ischaemic walls of endothelial cells in vitro revealed a contribution of fibrinogen that bridges endothelial ICAM-1 with αIIbβ3 on platelets (Massberg et al, 1999). Moreover, studies of Lagadec and coworkers (2003) shown that CD47 expressed on human and mouse platelets significantly contributes to platelet adhesion on TNF-α-stimulated vascular endothelial cells by binding of thrombospondin-1 on EC surface. Also, PECAM sites on the endothelium are involved and may be exposed by the injury to promote platelet adhesion and aggregation (Rosenblum et al, 1996).

Platelet firm adhesion induces signalling pathways, which promotes the secretion of platelet-derived mediators from their dense α-granules, such as RANTES and IL-1β (Schober et al, 2002; Lindemann et al, 2001). ADP-activated platelets were shown to induce both endothelial MCP-1 secretion and ICAM-1 surface expression via an NF-κB-dependent mechanism (Gawaz et al, 1998). Additionally, CD40L expression on platelets induces ECs to secrete chemokines and to express adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at the site of injury (Henn et al, 1998).

The above discussed interactions are summarised in Table 5.1 (over).
Rolling Firm adhesion

Leukocyte receptor
- PSGL-1
- LFA-1
- MAC-1
Platelet counter-receptor
- P-selectin
- ICAM-2
- αIIbβ3 via fibrinogen
- ICAM-2 via fibrinogen
- JAM-2

Table 5.2. Adhesion molecules involved in leukocyte adhesion to activated platelets (Bergmeir & Wagner, 2007).

Table 5.1. Adhesion molecules involved in platelet rolling along activated or inflamed endothelium and firm adhesion to inflamed endothelium. The endothelial ligand mediating rolling of activated platelets is unknown. ICAM-1 – intracellular adhesion molecule-1; PECAM-1 – platelet-endothelial cell adhesion molecule 1; PSGL-1 – P-selectin glycoprotein ligand-1; TPS-1 – thrombospondin-1; vWF – von Willebrand factor (Bergmeir & Wagner, 2007).

Activated platelets can bind to leukocytes to form platelet–leukocyte aggregates and therefore potentiate inflammation. The initial adhesion step is mediated by platelet P-selectin (formerly termed GMP-140 or PADGEM) and leukocyte PSGL-1 (Rinder et al., 1992; Hamburger & McEver, 1990). Also rolling of neutrophils on immobilized platelets is selectin-dependent (Buttrum et al., 1993). These interactions increase firm adhesion, mediated by leukocyte integrins, LFA-1 and MAC-1. Moreover, cell surface expression of MAC-1 increased upon cross-linking of PSGL-1 (Blanks et al., 1998). Weber & Springer (1997) demonstrated that a stable arrest and adhesion strengthening of PMN on thrombin-stimulated, surface-adherent platelets in flow required interactions of MAC-1 with fibrinogen, which was bound to platelets via αIIbβ3. Also, JAM-3 and GPIbα serve as a counter-receptor for MAC-1 mediating leukocyte-platelet interactions (Wang et al., 2005; Ehlers et al., 2003; Santoso et al., 2002). ICAM-2, highly expressed on platelets and endothelium was shown to be a counter-receptor for the leukocyte integrin LFA-1 (Table 5.2) (Weber et al., 2004).
Platelet-leukocyte aggregates lead to endothelial activation and the leukocyte migration cascade (Figure 5.3) (Dole et al, 2005). However, *vice versa*, Weissmuller et al (2008) reported that PMNs facilitated translocation of platelets across human and mouse epithelium.

Platelet-neutrophil complexes represent a large subpopulation of neutrophils with higher expression of adhesion molecules, greater capacity of production of reactive oxygen metabolites and phagocytosis (Peters et al, 1999). After leukocytes are recruited to tissue, platelets intensify the inflammatory process by inhibiting apoptosis of PMNs, monocytes and eosinophils (Raiden et al, 2003; Brunetti et al, 2000; Andonegui et al, 1997).

Figure 5.1. Platelets enhance leukocyte rolling on the endothelium. Formation of platelet–leukocyte aggregates (far left) in the circulation enhances recruitment of polymorphonuclear cells (and other leukocytes) to the endothelial surface. There, platelet and endothelial surface P-selectin bind P-selectin glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on the leukocyte surface. This leads to margination and rolling of leukocytes, the first step in leukocyte recruitment. P-selectin/PSGL-1 engagement, via leukocyte signaling pathways, up-regulates integrin adhesion molecule expression on leukocytes (not shown), leading to firm adhesion and diapedesis (O’ Sullivan & Michelson, 2006).
5.1.2. AN IN VITRO MODEL OF LEUKOCYTE TRANSENDOTHELIAL MIGRATION (TEM)

Boyden (1961) originally described an in vitro assay technique, which uses a chamber separated into two compartments by a porous filter membrane to allow the study of the chemotactic activity of soluble substances on motile cells. In this assay, the cells are seeded on one side of the membrane, while a solution to be tested for chemotactic activity is placed on the opposing side. After an incubation period, the membrane is fixed and stained. The number of cells which have migrated through the pores to the underside of the membrane in response to the chemotactic agent is counted microscopically. Antal Rot showed, in fact, cells were migrating in response to membrane-bound chemotactic stimulus in the process called haptotaxis (Rot, 1993).

The Boyden chamber technique subsequently underwent many modifications (Richards & McCullough, 1984; Harvath et al, 1980). For example, a membrane insert was used to establish two compartments in the wells of 24- or 96-well tissue culture plates. Assay options also included coating the membrane with a variety of matrix proteins (Eccles et al, 2005). One of them is collagen IV, which is the most abundant in basement membrane (Timpl et al, 1979).

However, new drug discovery methods measure the ability of motile cells to traverse physiological barriers, such as endothelium and epithelium (Eccles et al, 2005). A growing number of studies on the mechanism of leukocyte transendothelial migration and leukocyte-endothelial interactions use ECs grown on microporous filters as an in vitro model of endothelium (Mackarel et al, 1999). The transendothelial assay was first established by Huber and Weiss (1989). Their data indicated that neutrophil extravasation and reversible basement membrane disruption are coordinated events that occur as a consequence of vessel wall transmigration.

ECs line the blood vessels of the entire circulatory system, ranging from the aorta to capillaries, and act as the barrier between circulating blood and the rest of the vessel wall. While large veins and arteries remain a quick and convenient source of ECs, the great morphological, biochemical and functional heterogeneity that ECs express has necessitated the development of techniques to isolate microvessel ECs from different tissues to create more realistic in vitro models (Hewett & Murray, 1993a).
Although, neutrophil migration in CF takes place mainly in bronchial tissue, there is some evidence of alveolar CF inflammation (Ulrich et al, 2010). In the pulmonary circulation, leukocytes marginate and transmigrate in alveolar capillaries, rather than postcapillary venule, as in the bronchial circulation. Many leukocyte-EC interactions take place in the pulmonary capillary bed, which generally contain many more leukocytes compared to other tissue vascular beds. In fact, the concentration of leukocytes in the pulmonary capillary blood is 35 to 100 times more than that of systemic vessels (Shepro, 2006).

Key studies have demonstrated that neutrophilic inflammation is a hallmark of CF lung disease. However, the exact mechanism of neutrophilic inflammation remains unclear (Tirouvanziam, 2006). Although the genetic basis of CF as well as the molecular structure of CFTR have been well defined, a clear relationship between the genetic defect and the pulmonary pathophysiology, has not been established (Witko-Sarsat et al, 1999). Interestingly, human platelets were found to express a biologically active CFTR and thus platelets from CF patients are affected by the molecular defect of CFTR (Mattoscio et al, 2010). Therefore, platelets are an important, albeit generally underappreciated, component of the inflammatory cascade. Moreover, platelet dysfunction in CF could contribute to pulmonary inflammation and tissue destruction (O’Sullivan & Michelson, 2006). Therefore, to investigate neutrophil-endothelium-platelet interaction, a novel cell culture model of transendothelial migration (TEM) of neutrophils towards thrombin-activated platelets has been developed. Platelets have not previously been used as a source of neutrophil chemoattractant, however they were used before in a model of T cell TEM (Shute et al, 2011, abstract). In the current model, HLMVEC (Figure 5.2, over) were grown on an uncoated polyethylene terephthalate (PET) microporous membrane until confluency over 14 days during, which time they are expected to lay down a basement membrane (Huber & Weiss, 1989). Platelets isolated from healthy donors were activated with a potent platelet stimulus, thrombin and added to the lower compartment of the Transwell. Neutrophil TEM started when neutrophils isolated from healthy donors were added to the upper compartment. After incubation, neutrophils were collected from the lower compartment and counted microscopically. The outline of a transwell chamber is schematically presented in Figure 5.3 (over).
Aminoglycosides are commonly used for the treatment of CF infection, especially tobramycin, which has the highest *in vitro* activity against *Pseudomonas aeruginosa* strains among all aminoglycosides (Laxer *et al.*, 1975). Tobramycin has been suggested to have anti-inflammatory properties beyond its anti-pseudomonal effect (Ramsey *et al.*, 1999). Additionally, it was shown to effectively inhibit T-cell migration (McGregor *et al.*, 2009, abstract). Therefore, the anti-inflammatory effect of tobramycin and copper-tobramycin, a derivative with antioxidant properties (Chapter 4), was tested in this novel neutrophil TEM model.

![Figure 5.2. HLMVEC (400 x).](image1)

![Figure 5.3. The outline of a Transwell.](image2)

5.2. HYPOTHESIS, AIMS AND OBJECTIVES

It was hypothesized that tobramycin and its complex with copper have anti-inflammatory properties.

In order to investigate platelet-neutrophil-endothelial cell interactions, an *in vitro* model of neutrophils migrated across TNF-α-stimulated endothelium towards thrombin-activated platelets was created. To mimic neutrophilic inflammation in CF, CFTRinh-172 was used. A further objective was to test the effect of tobramycin and a copper-tobramycin complex on transendothelial migration of neutrophils in the PMN TEM model and decipher the mechanism of action of these drugs.

The aim was to investigate the anti-inflammatory effect of tobramycin and copper-tobramycin.
5.3. MATERIALS

Multiwell 24-well companion plates and biotin-FITC conjugate were from Becton Dickinson Biosciences (Oxford, Oxfordshire, UK) and 3 µM culture inserts (PET) were supplied from Marathon Labs Supplies (London, UK).

Anti-human IL-8 and NAP-2 antigen affinity purified polyclonal antibodies, TNF-α as well as human IL-8, PAI-1 and NAP-2 development kit were purchased from Peprotech EC (London, UK). DuoSet® ELISA development system for human CCL5/RANTES and human ICAM-1/CD54 was from R&D Systems Ltd. (Abingdon, Oxfordshire, UK). PAI-1 activity assay was from Hyphen BioMed (Neuville-sur-Oise, France).

Nunc MaxiSorb 96-well ELISA plates and ethylenediaminetetraacetic acid (EDTA) were provided by Fisher Scientific (Loughborough, Leicestershire, UK). Superoxide dismutase (SOD), catalase, fetal bovine serum (FBS) heat inactivated and Triton-X100 were from Sigma Aldrich (Dorset, UK). Complete mini® protease inhibitor (double strength) was purchased from Roche Ltd. (Wewlyn Garden City, Hertfordshire, UK).

Calbiochem/Merck (Nottingham, Nottinghamshire, UK) provided MnTBAP (Mn(III)tetrakis(4-benzoic acid)porphyrin chloride), staurosporine from Streptomyces sp., XR5118, PAI-1 inhibitor was a gift from Xenova, UK. WEB 2086 was from Boehringer Ingelheim Inc. (Bracknell, Berkshire, UK). Plasmin substrate was from Chromogenix (Lexington, MA, USA).

Trevigen Inc. (Helgerman City, Gaithersburg, MD, USA) supplied TACS™ annexin V kit and Promega Inc. (Rocky Hill, NJ) supplied Caspase-Glo® 3/7 assay.

5.4. METHODS

5.4.1. SUBCULTURING INTO TRANSWELLS

Polyethylene terephthalate (PET) 3 µM uncoated Transwells cell culture inserts were used to grow HLMVEC for transmigration experiments. The inserts were placed in wells of 24-well companion plates, containing 800 µl of growth medium underneath. 250 µl
of HLMVEC suspension (2 x 10⁵/ml) was added per transwell (5 x 10⁴/well) and topped up to 300 µl with growth medium. The plates were covered and incubated at 37°C and 5 % CO₂.

5.4.2. A CELL CULTURE MODEL OF NEUTROPHIL TRANSENDOTHELIAL MIGRATION (TEM)

Primary HLMVEC were cultured in monolayers on PET, 3 μM uncoated Transwells cell culture inserts for two weeks to confluency (Figure 5.2 & 5.3). EGM-2MV (300 µl in upper well and 800 µl in lower well) was changed every other day.

Where indicated, HLMVEC were stimulated overnight with 10 ng/ml TNF-α and/or pre-treated overnight (16 hours) prior to the migration assay with tobramycin, copper-tobramycin and copper-sulphate at 0.01 – 0.5 mM, catalase (250 U/ml), SOD (500 – 2000 U/ml) and MnTBAP (1 – 50 µM). The compounds were added to both top and bottom compartments at the same concentrations.

On the day of experiment, full growth medium was removed and replaced with RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine supplemented with 2.5 % (v/v) FBS.

Platelets were isolated as previously described in Section 2.4.2, re-suspended in RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine and 2.5 % (v/v) FBS, diluted to 1 x 10⁸ cells/ml and applied to the basal compartment of Transwells with/without 2 U/ml thrombin or with/without 100 ng/ml LPS for 30 minutes preincubation at 37°C and 5 % CO₂. The volume of platelets in the bottom compartment of Transwells was 800 µl (0.8 x 10⁸ cells/well).

Where indicated, TNF-α, copper chelators and antioxidants were refreshed before the experiment. Copper chelators, antioxidants, PAI-1 inhibitor (XR5118) (1, 10, 100 µM) anti-IL-8 and anti-NAP-2 (100 µg/ml) were also added to both top and bottom compartment for 30 minutes or 16 hours pre-incubation. Where indicated, IL-8 (6.25 x 10⁻⁸ M) was also pre-incubated with endothelial cells in the bottom compartment for 30 minutes before neutrophils were added to start the migration assay.
Neutrophils were isolated as previously described in Section 2.4.1, re-suspended in RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine and 2.5 % (v/v) FBS, diluted to 2 x 10^6 cells/ml and applied to the apical compartment of Transwells. The volume PMNs in the top compartment of Transwells was 300 µl (0.66 x10^6 cells/well).

Following 3 hours incubation at 37°C and 5 % CO2, the plate was placed on ice for 10 minutes. 80 µl 100 mM EDTA (Sigma, UK) was added to every bottom well, resulting in a 10 mM final concentration. The inserts were shaken gently to loosen adherent cells. The plate was placed into the incubator for 10 – 15 minutes, followed by another gentle shake of the inserts. The apical supernatant was collected into separate microcentrifuge tubes and cleared by centrifugation at 900 x g for 10 minutes at 4°C. HLMVEC on cell culture inserts were washed two times with 200 µl ice-cold PBS (-Ca/Mg), followed by cell lysis with 300 µl 1 % (v/v) Triton X-100 containing double strength protease inhibitors (Roche). The cell lysates were placed at -80°C and were subjected to 3 freeze-thaw cycles. Basal supernatants containing migrated neutrophils were collected into separate microcentrifuge tubes. The bottom wells were washed with 320 µl RMPI 1640 containing 25 mM HEPES and 2 mM L-glutamine and 2.5 % (v/v) FBS to wash off any cells left behind and added to the recovered cells (the total volume of 1200 µl). Basal supernatants were centrifuged at 450 x g for 10 minutes at 4°C. All supernatant samples were stored at -80°C for further analysis. The remaining cell pellets were re-suspended in 100 µl PBS (-Ca/Mg) and diluted 1/10 in Trypan blue. At least two aliquots were counted using a haemocytometer. The cell number was calculated in two 25-square fields using x 40 microscope magnification and then averaged.

5.4.3. OBTAINING A DOSE RESPONSE CURVE TO TNF-α AND IL-8 IN PMN TEM

To obtain a dose response curve for TNF-α, HLMVEC were stimulated for 16 hours with TNF-α at the final concentration range 1 – 10 ng/ml. For this purpose, TNF-α was added to the upper (3 µl/300 µl) and to the lower (8 µl/800 µl) compartment of Transwells at 0.1 – 1 µg/ml working concentrations. On the day of experiment, TNF-α was refreshed.
In another set of experiments, IL-8 in the concentration range $1.25 \times 10^{-9}$ – $1.25 \times 10^{-7}$ M was prepared in RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine and 2.5 % (v/v) FBS and added to the lower compartment for 30 minutes pre-incubation before starting the experiment.

5.4.4. SETTING UP A CF MODEL OF PMN TEM USING CFTRinh-172

CFTRinh-172 was added to the top (3 µl/300 µl) and bottom (8 µl/800 µl) compartment of Transwells at 20 µM final concentration prepared from 2 mM working concentration 6 hours before the overnight addition of TNF-α. It was refreshed 30 minutes before the experiment. Thrombin-stimulated platelet and neutrophils were added to appropriate compartments as described earlier in Section 5.4.2.

5.4.5. THROMBIN PLATELET ACTIVATION

Platelets were isolated according to the procedure shown in Section 2.4.2, re-suspended in PBS (+ Ca/Mg) and diluted to $2 \times 10^8$/ml. 1250 U/ml thrombin stock (Sigma) was used to obtain 0.1 – 3 U/ml final concentrations of thrombin by 1/100 dilution of its working concentration. Platelets were incubated for 30 minutes at 37°C with or without thrombin stimulation. The material was then centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant was removed and the cell pellet was lysed in an equal volume of 1 % (v/v) Triton X-100 containing double strength protease inhibitor (Roche). The supernatant and cell pellets were stored at -80°C before ELISA analysis.

5.4.6. ELISA

IL-8, PAI-1 and NAP-2 were measured using human IL-8, PAI-1 and NAP-2 ELISA Development Kit (PeproTech), respectively and human CCL5/RANTES and sICAM-1 were measured by Duo Set ELISA development systems (R&D Systems) according to the manufacturer’s instructions.

5.4.7. PLASMIN ACTIVITY ASSAY

This assay is based on data in the Chromogenix catalogue. 3 mM plasmin substrate stock solution (Chromogenix) was made by dissolving 25 mg in 11.33 ml distilled water. Assay buffer was 50 mM TRIS-HCl containing 130 mM NaCl, pH 8.5. Standard enzyme,
0.5 mg plasmin from human plasma (Sigma) was dissolved in 0.5 ml assay buffer to the concentration of 1 mg/ml. Standards were diluted from the 1 mg/ml stock for the assay in the range: 0 – 100 µg/ml using assay buffer. The reaction well of a 96-well plate contained: 75 µl of TRIS buffer, pH 8.5, 25 µl standard enzyme/sample and 25 µl substrate (0.8 mM final concentration). The reaction was started by the addition of the substrate to all wells of the plate, which was incubated at 37°C for 30 minutes before reading on a Dynex plate reader at 405 nm.

5.4.8. PMN VIABILITY ASSESSMENT

A FACS Calibur Immunocytometry System was used to assess neutrophil apoptosis by using TACSTM annexin V-biotin apoptosis detection kit (Trevigen) and biotin-FITC conjugate (Becton Dickinson Biosciences) (Section 4.4.6). Isolated neutrophils were re-suspended in RPMI 1640 supplemented with 2.5 % (v/v) FBS and diluted to 5 x 10⁶ cells/ml. Neutrophils were then challenged with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) and SOD (500 – 2000 U/ml), catalase (250 U/ml), MnTBAP (1 – 50 µM), XRS118 (1 – 100 µM), TNF-α (10 ng/ml) and CFTRinh-172 (20 µM) in the total volume of 250 µl for 3.5 hours at 37°C.

5.4.9. HLMVEC VIABILITY ASSESSMENT

Apoptosis of HLMVEC was assessed using a CytoTox-Glo™ assay and Caspase-Glo® 3/7 assay (Promega) (Section 3.4.16 and 4.4.19, respectively).

5.4.10. STATISTICAL ANALYSIS

Graph Pad Prism 4.0 Software was used to analyse the results. One-way ANOVA with Dunnett’s multiple comparison test as a post hoc test was performed on the data. Where appropriate, two-tailed unpaired t-test was used. The results were expressed as average plus/minus SEM. p<0.05 was the minimum accepted level of significance.
5.5. RESULTS

5.5.1. NEUTROPHIL TEM TOWARDS IL-8 ACROSS UNACTIVATED ENDOTHELIUM

IL-8 in the concentration range 1.25 x 10^{-9} – 1.25 x 10^{-7} M added to the lower compartment of Transwells attracted PMN to migrate across HLMVEC in a dose-dependent manner (Figure 5.4). IL-8 in the range 6.25 x 10^{-9} – 1.25 x 10^{-7} M induced a significant (p<0.01) neutrophil TEM. 6.25 x 10^{-8} M IL-8 concentration induced the highest level of neutrophil migration and 86.65 ± 5.60 % of the neutrophils added to the upper chamber migrated. This concentration was therefore chosen as an optimum concentration to use in further experiments to test the effect of drugs on TEM of PMN. Neutrophil migration was considered in regard to spontaneous migration in the absence of added IL-8, which was 5.15 ± 0.49 %.

![Graph showing dose response curve for IL-8 induced neutrophil TEM.](image)

Figure 5.4. Dose response curve for IL-8 induced neutrophil TEM. Results are presented as neutrophils migrated as % of those added to upper well (mean ± SEM, n=4). Data was analysed using one-way ANOVA and Dunnett’s multiple comparison test, ** p<0.01.

5.5.2. NEUTROPHIL TEM ON TNF-α-ACTIVATED ENDOTHELIUM

There was a low level of spontaneous neutrophil migration, 3.06 ± 1.21 %, across unactivated endothelium (Figure 5.5, over). EC stimulated with TNF-α in a concentration range of 1 – 10 ng/ml were found to induce a significantly higher PMN
TEM compared with the unstimulated control. The neutrophil response was not dose-dependent and there was no significant difference between the response to any concentration of TNF-α tested. The response to 10 ng/ml TNF-α was 27.94 ± 6.18 % and 10 ng/ml TNF-α was chosen as an optimum concentration to induce TEM of PMN in further drug testing experiments, based on literature values for comparison. Additionally, TNF-α (10 ng/ml) was a significantly (p<0.001) weaker stimulus for PMN migration across HLMVEC in comparison to IL-8 (6.25 x 10^{-8} M) (Figure 5.4), 27.94 ± 6.18 % versus 86.65 ± 5.60 %.

![Figure 5.5](image-url) Dose response curve for TNF-α-induced neutrophil TEM. Results are presented as neutrophils migrated as % of cells added to the upper chamber (mean ± SEM, n=3). Data was analysed using one-way ANOVA and Dunnett’s multiple comparison test, * p<0.05, ** p<0.01.

5.5.3. NEUTROPHIL TEM TOWARDS THROMBIN-ACTIVATED PLATELETS THROUGH TNF-α-STIMULATED ENDOTHELUM COMPARED TO SUBENDOTHELIAL IL-8

Platelets (1 x 10^8/ml, 300 µl) added to the top compartment of Transwells did not stimulate, but rather inhibited neutrophil TEM, both upon thrombin and LPS stimulation in the absence and presence of TNF-α activation of EC (data not shown). Neutrophil TEM was observed only when platelets were added to the lower compartment (1 x 10^5/ml, 800 µl) (Figure 5.6, over). A low level of spontaneous neutrophil migration (2.03 ± 0.92 %) was observed over monolayers of unactivated EC control. The addition of unstimulated platelets induced a non-significant increase to
4.90 ± 1.02 %, whereas thrombin (2 U/ml)-activated platelet induced a significant (p<0.01) increase in PMN TEM to 10.98 ± 1.76 %, compared to spontaneous PMN migration. This increase was due to thrombin acting on the platelets, as thrombin alone, acting on EC, did not induce a significant neutrophil TEM.

Stimulation of endothelial cell layers with TNF-α (10 ng/ml) induced a significant (p<0.001) increase in the level of migrating neutrophils to 23.63 ± 3.53 % compared to no stimulus control. A similar result was obtained in a previous set of experiments with TNF-α at 10 ng/ml (Figure 5.5). The addition of platelets, alone, to the lower compartment induced a further significant (p<0.05) increase in neutrophil TEM, 50.25 ± 8.46 %, across TNF-α-stimulated endothelium. Thrombin activation of platelets induced a neutrophil response on activated endothelium (74.96 ± 5.71 %) that was not significantly different to the response to IL-8 on unactivated EC (86.65 ± 5.6 %). IL-8 is a well known and potent neutrophil chemoattractant, therefore the novel contribution of platelets to PMN TEM is the subject of this study.

Figure 5.6. The effect of activated platelets on neutrophil transendothelial migration on unactivated and TNF-α-activated HLMVEC. Transendothelial migration of neutrophils was measured in response to subendothelial platelets to thrombin alone and thrombin-stimulated platelets in the absence or presence of TNF-α (10 ng/ml) compared with the response to IL-8 over 3 hours (n≥3). The data is expressed as mean ± SEM and was analysed using unpaired two-tailed t-test. * represents comparison to control, + represents comparison to TNF-α alone, # represents comparison between bars, * p<0.05, ** p<0.01, *** p<0.001.
Since the effect of thrombin on platelet-induced neutrophil TEM across unactivated (10.98 ± 1.76 %) or TNF-α-activated (74.96 ± 5.71 %) endothelium was significant, it was compared to the effect of LPS activation of platelets on PMN TEM across HLMVEC (Figure 5.7). However, platelets activated with LPS (100 ng/ml) did not induce a significant effect on neutrophil migration across unactivated endothelium (5.33 ± 2.52 %), but did (p<0.05) across TNF-α-activated EC (33.51 ± 7.72 %). Moreover, platelets activated with LPS induced significantly (p<0.01) smaller neutrophil migration across activated HLMVEC than platelets stimulated with thrombin.

![Figure 5.7. TEM of neutrophils induced by platelets ± thrombin (2 U/ml) and LPS (100 ng/ml), n≥3. The data is expressed as a mean ± SEM and was analysed using unpaired two-tailed t-test. * represents comparison to control, + represents comparison to TNF-α, # represents comparison between bars, * p<0.05, ** p<0.01, *** p<0.001.](image)

To investigate further the PMN TEM driving force, IL-8 in both apical and basal supernatants was measured in experiments, where platelets were absent or present in the basal compartment (Figure 5.8, over). IL-8 is the major neutrophil chemoattractant known to be released by activated HLMVEC. Here, IL-8 was mostly detected in the basal compartment of Transwells, confirming endothelial polarisation. IL-8 was negligible in the absence of TNF-α activation. However, even unactivated platelets induced a small, but not significant, increase in IL-8 level in basal supernatant to 191.79 ± 96.82 pg/ml. The presence of thrombin-activated platelets induced a further non-significant increase to 308.11 ± 186.96 pg/ml. LPS was without any effect. TNF-α not-significantly stimulated HLMVEC to release IL-8 to the apical (829.47 ± 316.00
pg/ml) or to the basal (1957.73 ± 916.98 pg/ml) compartment of the Transwells in comparison to the unstimulated control, 1.85 ± 1.85 pg/ml. The addition of platelets in the absence of thrombin on TNF-α-stimulated EC did not increase the level of IL-8 in the apical or basal supernatant, while the presence of thrombin elicited a significant (p<0.05) effect only in the apical supernatant. No further increase with LPS-activated platelets on stimulated or unstimulated HLMVEC was observed. The concentration of IL-8 in Transwells with TNF-α-activated EC was not significantly higher in the basal compartment, because of high data variability between experiments.

Figure 5.8. IL-8 measurement in TEM of neutrophils induced by thrombin- or LPS-activated platelets with TNF-α-stimulated or unstimulated HLMVEC. The data is expressed as a mean ± SEM and was analysed using two-tailed paired t-test. + indicates p<0.05 compared to TNF-α, where, n=3.

Because PAI-1 is pro-inflammatory and supports IL-8-driven PMN TEM, the active form of PAI-1 was measured in both apical and basal supernatants collected after TEM of PMN (Figure 5.9, over). Unlike IL-8, the majority of PAI-1 activity was found in the apical compartment. PAI-1 activity released from unactivated EC was higher in the apical (0.41 ± 0.13 ng/ml) than the basal (0.04 ± 0.02 ng/ml) compartment, although the difference was not significant. The addition of unactivated or thrombin- or LPS-activated platelets did not affect the level of PAI-1 activity. However, TNF-α induced a significant (p<0.01) increase in active PAI-1 release to the apical supernatant, 2.36 ± 0.30 ng/ml. In the presence of platelets, in the absence or presence of thrombin, a non-significant decrease in the apical level of active PAI-1 compared to the TNF-α
control was seen. LPS-activated platelets underneath TNF-α-stimulated EC induced a further non-significant decrease in PAI-1 activity in apical supernatants compared to TNF-α control. The active form of PAI-1 released to the basal supernatant was not significantly different to the unactivated control in any condition tested.

It was of interest to analyse the role of chemoattractants involved in TEM of PMN towards thrombin-activated platelets in the presence of TNF-α. The response was normalised in every experiment to 100 % and the effect of antibodies was compared to this value. IL-8 was released from TNF-α-activated HLMVEC (Figure 5.8) and NAP-2 is one of the main neutrophil chemoattractants found in platelets. Therefore, the effects of neutralizing antibodies to IL-8 and NAP-2 were tested (Figure 5.10, over). Neutrophil migration was significantly (p<0.05) inhibited by anti-IL-8 (17.15 ± 2.06 %, n=4) and by anti-NAP-2 (30.04 ± 5.91 %, n=5). In addition, the anti-IL-8 effect on PMN TEM was not statistically different to the effect of anti-NAP-2. However, IL-8 and NAP-2 antibodies together inhibited only approximately 50 % of TEM of PMN, indicating a contribution of other chemokines, such as ENA-78 or PF-4 or neutrophil attracting lipids such as PAF.
However, a specific PAF antagonist, WEB2086, used at a final concentration of 10 µM was not effective in limiting neutrophil migration across HLMVEC stimulated with TNF-α towards thrombin-activated platelets, as it reduced the migration only by 8.32 ± 5.77%. This result suggests no role of PAF in the tested model of PMN TEM.

![Figure 5.10. The effect of anti-IL-8, anti-NAP-2 and PAF antagonist, WEB2086 on neutrophil TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets. 100 µg/ml of anti-IL-8 and anti-NAP-2 significantly inhibited TEM of neutrophils in the conditions tested. The data is expressed as a % of control, mean ± SEM and was analysed using unpaired two-tailed t-test. * indicates p<0.05. 100 % value represents % neutrophils migrated of those in the upper well, 78.64 ± 6.65 %.

5.5.4. IL-8, NAP-2 AND RANTES RELEASE BY THROMBIN-ACTIVATED PLATELETS

In order to evaluate the role of platelets in PMN TEM, platelets were activated with a range of thrombin concentrations (0.1 – 3 U/ml) for 30 minutes at 37°C. The 30 minutes incubation time reflected the pre-incubation time of platelets with thrombin in the neutrophil transmigration experiment.

Platelets were shown to release very small amounts of IL-8, detected at pg/ml levels (Figure 5.11, over). Unactivated platelets released 1.73 ± 0.34 pg/ml IL-8, while 2.91 ± 0.55 pg/ml IL-8 was still associated with the cell pellet. However, 1, 2 and 2.5 U/ml thrombin induced a significant (p<0.01) IL-8 release of 4.74 ± 0.67, 4.81 ± 0.46 and 5.31 ± 0.28 pg/ml IL-8, respectively compared to the unactivated platelets. Thrombin at 3 U/ml had no significant effect on IL-8 release from platelets. Additionally, reduced
levels of IL-8 were measured in association with the cell pellet following thrombin stimulation. 1 U/ml thrombin significantly (p<0.01) decreased the level of IL-8 bound to the cell pellet to 0.81 ± 0.31 pg/ml in comparison to the untreated control.

Figure 5.11. Platelet dose response curve to thrombin, IL-8. A) IL-8 released from platelets in the absence and presence of thrombin was measured by ELISA in supernatant. B) IL-8 remaining in the cell pellet in the absence and presence of thrombin was measured by ELISA. Results are presented as mean ± SEM, n=7. ** indicates p<0.01 compared to unstimulated control.
Platelets were shown to be a rich source of NAP-2, which was detected in µg/ml levels (Figure 5.12). Unactivated platelets released 6.41 ± 4.23 µg/ml of NAP-2, while 9.05 ± 1.18 µg/ml NAP-2 was still associated with the cell pellet. Thrombin-stimulation increased the level of released NAP-2, however only 2 U/ml thrombin concentration induced a significant (p<0.05) effect in releasing 18.97 ± 3.13 µg/ml NAP-2 compared to the untreated control. A non-significant trend towards a decreasing NAP-2 level in the pellet with increasing concentrations of thrombin was also observed.

![Figure 5.12. Platelet dose response curve to thrombin, NAP-2. A) NAP-2 released from platelets in the absence and presence of thrombin was measured by ELISA in supernatant. B) NAP-2 remaining in the absence and presence of thrombin was measured by ELISA in cell pellet. Results are presented as mean ± SEM, n=3. * indicates p<0.05 compared to unstimulated control.](image-url)
Platelets also contained ng/ml concentrations of RANTES (Figure 5.13). Unactivated platelets released RANTES (9.16 ± 2.04 ng/ml) into supernatants, but most was retained in the cell pellet (19.87 ± 2.92 ng/ml). 1 U/ml thrombin non-significantly increased RANTES release, but significantly (P<0.05) decreased cell-associated RANTES. 2 and 3 U/ml thrombin significantly (p<0.05) increased release of RANTES to 20.85 ± 4.55 and 20.77 ± 3.69 ng/ml, respectively and significantly (p<0.01) decreased cell associated RANTES to 13.44 ± 2.50 and 12.79 ± 2.67 ng/ml, respectively in comparison to the appropriate non-activated platelet control.

Figure 5.13. Platelet dose response curve to thrombin, RANTES. A) RANTES released from platelets in cell pellet in the absence and presence of thrombin was measured by ELISA in supernatant. B) RANTES remaining in the absence and presence of thrombin was measured by ELISA. Results are presented as mean ± SEM, n=4. * indicates p<0.05 and ** indicates p<0.01 compared to unstimulated control.
5.5.5. THE EFFECT OF TOBRAMYCIN AND COPPER-TOBRAMYCIN ON PMN TEM

One of the main objectives of this thesis was to investigate the anti-inflammatory effects of tobramycin. Therefore, tobramycin was tested in models of neutrophil transendothelial migration, as this may be a process affected by intravenous tobramycin. Tobramycin was tested in three in vitro models of neutrophil migration. Firstly, the response of neutrophils to IL-8 added underneath an unactivated endothelium, secondly, the response of neutrophils to TNF-α-activated endothelium, and thirdly, the response of neutrophils to thrombin-activated platelets underneath a TNF-α-activated endothelium. Tobramycin has been shown to bind copper and to have SOD-like properties (Chapter 2), therefore the anti-inflammatory properties of copper-tobramycin were also investigated in the same settings.

Table 5.3 presents an overview of the results of experiments in which the effect of tobramycin and copper-tobramycin in 3 models of PMN TEM was investigated. Only statistically significant data is present in the figures that follow.

The effect of tobramycin and copper-tobramycin were tested in the TEM experiments (3 hours) and following pre-treatment of HLMVEC for 30 minutes or 16 hours.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>HLMVEC activator</th>
<th>Total time of drug treatment</th>
<th>Tobramycin (0.01 – 0.5 mM)</th>
<th>n</th>
<th>Copper-tobramycin (0.01 – 0.5 mM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>-</td>
<td>3.5 h</td>
<td>ns</td>
<td>4</td>
<td>↑* (0.5 mM)</td>
<td>3</td>
</tr>
<tr>
<td>-</td>
<td>TNF-α</td>
<td>3.5 h</td>
<td>ns</td>
<td>3</td>
<td>ns</td>
<td>4</td>
</tr>
<tr>
<td>Platelets + thrombin</td>
<td>TNF-α</td>
<td>3.5 h</td>
<td>ns</td>
<td>3</td>
<td>ns</td>
<td>4</td>
</tr>
<tr>
<td>Platelets + thrombin</td>
<td>TNF-α</td>
<td>19 h</td>
<td>↓** (0.01 and 0.1 mM)</td>
<td>5</td>
<td>↓* (0.5 mM)</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.3. The effect of tobramycin and copper-tobramycin on TEM of PMN. ns – non-significant effect; ↑ - increased cell migration; ↓ - decrease cell migration, * indicates p<0.05; ** indicates p<0.01.

In the short-term, tobramycin in the concentration range of 0.01 – 0.5 mM had no significant effect on neutrophil migration across unstimulated HLMVEC towards IL-8 or across TNF-α-stimulated endothelium, in the absence or presence of thrombin-
activated platelets. However, pre-treatment of TNF-α-stimulated HLMVEC for 16 hours with tobramycin, significantly decreased neutrophil migration towards thrombin-activated platelets (Figure 5.14). Indeed, tobramycin (0.01 mM) significantly (p<0.01) reduced PMN TEM by 23.33 ± 3.63 %. PMN TEM was also significantly (p<0.05) reduced by 12.29 ± 5.23 % by 0.1 mM tobramycin treatment compared to an untreated control. However, 0.5 mM tobramycin concentration did not have a significant effect on TEM of PMN in the conditions tested.

Figure 5.14. The effect of tobramycin pre-treatment for 16 hours of HLMVEC stimulated with 10 ng/ml TNF-α on TEM of neutrophils towards thrombin-activated platelets for 3 hours. Results are expressed as % of control ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test. 100 % = 74.96 ± 5.71 % neutrophils migrated of those added to the upper well.

To understand the mechanisms of tobramycin inhibition of PMN TEM in the conditions tested, the levels of IL-8, PAI-1 and sICAM-1 as markers of the inflammatory response were measured by ELISA in supernatants from both apical and basal compartments collected after each experiment, n=5.

There were significantly higher levels of IL-8 measured in the basal supernatants compared to the apical ones (Figure 5.15, over). The control apical IL-8 concentration was 1.48 ± 0.25 ng/ml, while the control basal supernatant contained 3.88 ± 0.96 ng/ml IL-8. However, tobramycin had no significant effect on IL-8 concentration at any dose tested.
Figure 5.15. IL-8 measurement in supernatants from PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets following tobramycin pre-treatment for 16 hours. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. # indicates p<0.05 and ## indicates p<0.01 as a comparison between apical and basal supernatant IL-8 values.

The total PAI-1 was measured and, unlike active PAI-1 (Figure 5.9), was found to be in greater concentration in basal compartments. The total PAI-1 concentration in the apical Transwell compartment of the control was 11.47 ± 2.68 ng/ml when thrombin-activated platelets were present underneath the TNF-α-stimulated HLMVEC (Figure 5.16, over). In comparison, the active form of PAI-1 in the apical supernatant for the same condition was 1.75 ± 0.34 ng/ml (Figure 5.9), suggesting that the major part of PAI-1 was inactive. PAI-1 in the apical supernatant of the control was significantly (p<0.001) lower than in the basal compartment, which was 45.47 ± 3.50 ng/ml (Figure 5.16). Under the same conditions, the active form of PAI-1 in the basal compartment was only 0.62 ± 0.14 ng/ml (Figure 5.9). Tobramycin pre-treatment for 16 hours did not change the level of total PAI-1 in apical supernatants. However, tobramycin (0.01 mM and 0.5 mM) significantly decreased the total PAI-1 in the basal supernatants compared to the untreated control to 25.52 ± 3.80 ng/ml (p<0.05) and 21.49 ± 7.36 ng/ml (p<0.01), respectively. Although, 0.1 mM tobramycin reduced the total PAI-1 in the basal supernatant, the effect was non significant.
Figure 5.16. Measurement of PAI-1 in PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets in the presence of tobramycin treatment for 16 hours. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. * represents comparison with untreated control, # represents comparison between the apical and basal supernatant. * indicates p<0.05 and ** indicates p<0.01.

ICAM-1 is shed during HLMVEC activation and neutrophil migration and therefore soluble ICAM-1 was measured in supernatants. Figure 5.17 presents the level of sICAM-1 measured in both apical and basal supernatants from the same experiments.

Figure 5.17. sICAM-1 measurement in PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets following tobramycin pre-treatment for 16 hours. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. # indicates p<0.05 and ## indicates p<0.01 as compared between the apical and basal supernatant.
For the control, this was 769.55 ± 137.10 pg/ml in the apical and 1870.73 ± 314.08 pg/ml in the basal compartment. Moreover, sICAM-1 was significantly (p<0.05 or p<0.01) lower in the apical supernatant compared to the basal supernatant, not only for the untreated control, but also for tobramycin-treated samples. sICAM-1 concentration was significantly (p<0.05) decreased in the apical supernatant after addition of 0.5 mM tobramycin compared to the sICAM-1 concentration in untreated control. Tobramycin-treatment did not affect sICAM-1 level in the basal supernatants, however at 0.1 mM it showed a tendency to lower sICAM-1, which may reflect the inhibitory effect of tobramycin on PMN TEM.

Since tobramycin binds copper, which is elevated in the blood and sputum in CF (Gray et al, 2010; Percival et al, 1999), it is conceivable that tobramycin binds copper spontaneously to form a copper-tobramycin complex. Therefore, the anti-inflammatory effect of copper-tobramycin was tested in all 3 in vitro models of neutrophil migration.

Interestingly, copper-tobramycin present with HLMVEC for 3.5 hours demonstrated a tendency to inhibit TEM of PMN. 0.5 mM copper-tobramycin concentration reduced neutrophil migration by 19.05 ± 7.6 %, however this effect was not statistically significant (Figure 5.18, over). This effect became significant when copper-tobramycin was added to HLMVEC 0.5 hour before TNF-α and then each drug was present for 16 hours before TEM. Indeed, 0.5 mM copper-tobramycin concentration significantly (p<0.05) reduced neutrophil migration by 33.78 ± 10.37 % in comparison to the untreated control in the conditions tested.
Figure 5.18. Copper-tobramycin (0.01 – 0.5 mM) effect on neutrophil TEM in the presence of $1 \times 10^8$ platelets/ml stimulated with 2 U/ml thrombin across EC stimulated with 10 ng/ml TNF-α. A) Copper-tobramycin was present for 3.5 hours, n=4. B) Copper-tobramycin was present overnight and was added 0.5 hour before TNF-α, n=5. Results are presented as % of control ± SEM. * indicates p<0.05 compared to unstimulated control. 100 % = 74.96 ± 5.71 % cells migrated of those added to the upper well.

Again, IL-8, PAI-1 and sICAM-1 concentrations were measured in apical and basal supernatants from the above experiments, n=5.

IL-8 was detected in the apical supernatants of control well at 1.04 ± 0.10 ng/ml and 0.5 mM copper-tobramycin induced a small, but significant (p<0.05) increase in IL-8 level to 1.22 ± 0.13 ng/ml, compared to the untreated control (Figure 5.19, over). The
control basal supernatant contained 2.23 ± 0.61 ng/ml IL-8, which was not statistically different with copper-tobramycin treatment.

Figure 5.19. IL-8 concentration in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. * indicates p<0.05.

The PAI-1 concentration in the apical supernatant of control experiment was 9.05 ± 1.31 ng/ml, while significantly higher levels were measured in the basal supernatant was 37.65 ±4.21 ng/ml (Figure 5.20, over). However, the drug treatment did not affect the amount of total PAI-1 level in the basal supernatant.

The level of sICAM-1 measured in the apical and basal compartment of control wells was 534.42 ± 141.68 pg/ml and 1382.13 ± 359.04 pg/ml, respectively and drug treatment did not influence these values, however it tended to decrease in basal supernatant (Figure 5.21, over), possibly reflecting the decrease in neutrophil TEM under these conditions.
Figure 5.20. PAI-1 concentration in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. ## - p<0.01 and ### - p<0.001.

Figure 5.21. sICAM-1 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant.

Tobramycin had no effect on neutrophil TEM towards subepithelial IL-8 (Figure 5.22A, over). Unlike the effect on neutrophil TEM across TNF-α-stimulated endothelium towards thrombin-activated platelets underneath, copper-tobramycin had an
opposite, stimulatory effect on neutrophil TEM with recombinant IL-8 as the chemoattractant, (Figure 5.22B). Indeed, when IL-8 was the chemoattractant, a short-term exposure to copper-tobramycin (0.5 mM) induced a significant (p<0.05) increase in TEM of neutrophils to 116.79 ± 6.02 %. It suggests that all neutrophils have migrated from the upper to the lower compartment of the Transwells.

Figure 5.22. The effect of tobramycin and copper-tobramycin (0.01 – 0.5 mM) on PMN TEM across unstimulated HLMVEC towards IL-8. A) The effect of tobramycin, n=4. B) The effect of copper-tobramycin, n=3. Results are presented as % of control ± SEM. The data was analysed in comparison to the untreated control. 100 % = 86.65 ± 5.60 % of cells added to the apical compartment that migrated to the lower compartment.
To understand the mechanism of the effect of copper-tobramycin on neutrophil migration towards subendothelial IL-8, the levels of IL-8, PAI-1, sICAM-1 and plasmin activity were measured in apical and basal supernatants of the above experiments, n=3.

IL-8 in control apical and basal supernatant was 6.80 ± 0.32 ng/ml and 490.76 ± 21.74 ng/ml, respectively (Figure 5.23), reflecting the addition of IL-8 to the basal compartment. IL-8 levels were unchanged in either apical or basal supernatant after 3.5 hour incubation with a range of copper-tobramycin (0.01 – 0.5 mM).

![Figure 5.23. IL-8 measurement in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=3. The data was analysed using one-way ANOVA and Dunnett`s post hoc test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. # indicates p<0.05 and ## indicates p<0.01.](image)

The level of control PAI-1 was 4.02 ± 1.42 ng/ml in apical supernatant and 6.32 ± 1.30 ng/ml in basal supernatant (Figure 5.24, over) and the concentrations were not significantly different in the presence of copper-tobramycin (0.01 – 0.5 mM).

In parallel with the lack of effect of copper-tobramycin on PAI-1 levels, plasmin activity was also not statistically different upon copper-tobramycin treatment compared to control values of plasmin in apical and basal supernatants (Figure 25, over). The values were 1.09 ± 0.08 µg/ml and 0.99 ± 0.6 µg/ml, respectively.
Figure 5.24. Total PAI-1 level in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=3. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant.

Figure 5.25. Plasmin activity in PMN TEM towards subendothelial IL-8 in the presence of copper-tobramycin. Results are expressed as mean ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant.

The level of sICAM-1 was also measured in apical and basal supernatants (Figure 5.26, over). The apical supernatant from control wells contained 26.64 ± 13.33 pg/ml sICAM-1. This level was unchanged in the presence of copper-tobramycin (0.01 – 0.5 mM). The sICAM-1 in the basal supernatant of the control was 27.93 ± 15.69 pg/ml and both
0.1 and 0.5 mM copper-tobramycin increased sICAM-1 level more than 5 times and 
p=0.08 for 0.5 mM copper-tobramycin (162.57 ± 44.48 pg/ml), possibly reflecting the 
increase in TEM under these conditions with IL-8 as the chemoattractant.

Figure 5.26. sICAM-1 level in PMN TEM towards subendothelial IL-8 in the presence of copper-
tobramycin. Results are expressed as mean ± SEM, n=3. The data was analysed using one-way ANOVA 
and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical 
versus basal supernatant.

5.5.6. THE EFFECT OF CATALASE AND SOD ON NEUTROPHIL TEM

Since tobramycin and copper-tobramycin inhibited TEM without effects on IL-8, PAI-1 
or ICAM-1, an effect on ROS production either by migrating cells or EC was 
hypothesized. Copper-tobramycin was previously shown to have SOD-like activity 
without catalase activity in the range of concentration 0.01 – 0.5 mM (Section 2.2.4). 
To understand the effect of copper-tobramycin on PMN TEM across TNF-α-stimulated 
HLMVEC towards thrombin-activated platelets, SOD alone and in the presence of 
catalase was tested in this model (Figure 5.27, over). SOD in the range 500 – 2000 U/ml 
clearly had no effect on TEM of PMN across TNF-α-stimulated HLMVEC across platelets 
activated by thrombin, and neither did catalase alone at 250 U/ml. Also SOD alone was 
not effective in TEM of PMN when IL-8 was added underneath (n=3) or across TNF-α-
activated HLMVEC (n=5) (results not shown). However, 2000 U/ml SOD in combination 
with 250 U/ml catalase induced a significant (p<0.05) decrease in the level of migrating
neutrophils by 25.73 ± 11.00 % compared to untreated control. This effect was dependent not solely on SOD. It had to be due to a combination of SOD and catalase activities, that can be found in the Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) complex.

Figure 5.27. The effect of SOD in the absence and presence of catalase on PMN TEM across HLMVEC stimulated with 10 ng/ml TNF-α towards thrombin-activated platelets. A) The effect of SOD alone (500 – 2000 U/ml), n=3. B) The effect of SOD in the presence of 250 U/ml catalase, n=4. Results are represented as % of control ± SEM, n=5. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test. 100 % = 74.96 ± 5.71 % cells migrated of those added to the upper well.
5.5.7. THE EFFECT OF MnTBAP ON TEM OF NEUTROPHILS

MnTBAP is a cell permeable SOD mimetic, which also has catalase activity. MnTBAP was used in the following range of concentrations, 1 – 50 µM in the model of PMN TEM, where neutrophils migrated towards thrombin-activated platelets in the presence of TNF-α (Figure 5.28).

Figure 5.28. The effect of MnTBAP (1 – 50 µM) on neutrophil TEM across TNF-α-stimulated endothelium towards thrombin-activated platelets. A) MnTBAP was present on HLMVEC for 3.5 hours, n=3. B) MnTBAP was added to the upper and lower compartment of Transwells 0.5 hour before the addition of TNF-α and was present overnight, n=3. The data is expressed as a % of a control ± SEM and were analysed using one-way ANOVA and Dunnett’s as a post hoc test. * indicates p<0.05. 100 % = 74.96 ± 5.71 % cells migrated of those added to the upper well.
MnTBAP (50 µM) present with TNF-α-stimulated HLMVEC for 3.5 hours significantly (p<0.05) reduced PMN TEM by 43.46 ± 15.32 % towards thrombin-activated platelets (A). Additionally, when MnTBAP was present overnight (B), 50 µM MnTBAP also significantly (p<0.05) reduced PMN TEM by 37.37 ± 5.68 %.

The level of IL-8, PAI-1, sICAM-1 was measured in both apical and basal supernatant collected from PMN TEM in presence of MnTBAP for 16 hours to understand the mechanism of inhibition of neutrophil migration through EC in presence of 50 µM MnTBAP.

The level of IL-8 found in the control apical and basal compartment of Transwells was 1.28 ± 0.92 ng/ml and 1.33 ± 0.41 pg/ml, respectively (Figure 5.29). IL-8 concentration tended to decrease in a dose-dependent manner with increasing concentrations of MnTBAP, although this effect was not statistically significant.

![Figure 5.29](image_url)

Figure 5.29. IL-8 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of MnTBAP. Results are expressed as mean ± SEM, n=3. The data was analysed using one-way ANOVA and Dunnett’s multiple comparison test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant.

PAI-1 concentration in the apical supernatant of the control well was 3.69 ± 0.81 ng/ml. PAI-1 level was significantly (p<0.01 and p<0.05) increased in the apical supernatant by incubation with 1 and 10 µM MnTBAP to 6.41 ± 0.29 ng/ml and 5.49 ± 0.34 ng/ml, respectively, but not significantly increased with 50 µM MnTBAP.
treatment (Figure 5.30). In the basal supernatant of control wells PAI-1 was 18.76 ± 1.97 ng/ml and was unchanged upon incubation with MnTBAP. Moreover, the amount of PAI-1 measured in apical supernatant was significantly (p<0.05 or p<0.01) lower compared to PAI-1 concentration in basal supernatant.

![Figure 5.30. PAI-1 level in PMN TEM across TNF-α-stimulated HLMVEC towards subendothelial thrombin-activated platelets in the presence of MnTBAP. Results are expressed as mean ± SEM, n=3. The data was analysed using one-way ANOVA and Dunnett’s post hoc test and paired two-tailed t-test to compare IL-8 level in apical versus basal supernatant. # - p<0.05 and ## - p<0.01.](image)

In control wells the level of sICAM-1 was 485.74 ± 156.19 and 1511.09 ± 302.15 pg/ml in apical and basal Transwell compartment, respectively (Figure 5.31, over).

MnTBAP at 50 µM significantly (p<0.05) increased sICAM-1 concentration in apical supernatant, while MnTBAP at 10 µM significantly (p<0.05) decreased sICAM-1 concentration in the basal supernatant compared to the untreated control. Moreover, significantly (p<0.05) more sICAM-1 was detected in basal than in apical supernatant in the untreated control and following 50 µM MnTBAP treatment.
5.5.8. THE EFFECT OF COPPER SULPHATE ON TEM OF NEUTROPHILS

To test whether the effect of copper-tobramycin on PMN TEM was due to copper, CuSO$_4$ was tested in the same way as copper-tobramycin. Copper-tobramycin increased the level of neutrophils migrated towards subendothelial IL-8 (Figure 5.22), but there was no effect of copper sulphate in the same concentration range (0.01 – 0.5 mM) (Figure 5.32, over). Also, no effect of copper sulphate was observed on PMN migration across the layer of TNF-α-stimulated ECs towards platelets activated with thrombin (Figure 5.33, over), in comparison to copper-tobramycin (0.01 – 0.5 mM) (Figure 5.18).
Figure 5.32. The effect of copper sulphate on neutrophil TEM towards subendothelial IL-8. The results are expressed as % of control ± SEM. The data was analysed in comparison to the untreated control, n=4. 100 % = 86.65 ± 5.60 % cells migrated of those added to the upper well.

Figure 5.33. The effect of copper sulphate on neutrophil TEM on TNF-α-stimulated HLMVEC towards thrombin-activated platelets. The data is expressed as a % of a control and was analysed in comparison to the untreated control, n=5. 100 % = 74.96 ± 5.71 % cells migrated of those added to the upper well.

5.5.9. THE EFFECT OF PAI-1 INHIBITOR (XR5118) ON TEM OF NEUTROPHILS

PAI-1 inhibitor, XR5118, was used to investigate the role of PAI-1 in TEM of PMN in the presence of TNF-α and activated platelets. XR5118 (100 µM) induced a significant
(p<0.01) inhibition of cell migration by 72.00 ± 10.90 %, while lower XR5118 concentrations (1 and 10 µM) had no effect (Figure 5.34).

![Graph showing XR5118 effect on neutrophil TEM](image)

Figure 5.34. XR5118 effect on neutrophil TEM in response to platelet activated with thrombin across stimulated EC. Results are presented as mean ± SEM. ** indicates p<0.01 compared to unstimulated control, n=4. 100 % = 74.96 ± 5.71 % neutrophils migrated of those added to the upper well. ** - p<0.01.

5.5.10. THE EFFECT OF CFTRinh-172 ON NEUTROPHIL TRANSENDOTHELIAL MIGRATION

As a new in vitro model of neutrophil transendothelial migration across TNF-α-stimulated endothelial cell layer towards thrombin-activated platelets was established, it was of interest to examine the role of CFTR, using the CFTR inhibitor, CFTRinh-172 to mimic vascular inflammation in CF.

In order to set up an in vitro CF model of PMN TEM, the effect of pre-treatment with CFTRinh-172 (20 µM) for 20 hours was tested on neutrophil migration in the absence of any stimuli or with platelets (1 x 10^8/ml) activated with thrombin, with or without endothelial stimulation of HLMVEC with TNF-α (10 ng/ml) or subendothelial IL-8 (6.25 x 10^-8 M) (Figure 5.35, over).

In experiments where the CFTR inhibitor was tested (Figure 5.35, over), the neutrophil response in control wells in the absence of inhibitor (A) was similar to that reported in previous experiments (Figure 5.6). The spontaneous response of PMN across unactivated EC was the same in the absence (A) or presence (B) of the inhibitor.
CFTRinh-172 did not have a significant effect on PMN TEM. However, CFTRinh-172 significantly (p<0.05) decreased PMN TEM across unactivated HLMVEC in the presence of platelets activated with thrombin from 11.15 ± 2.71 % to 2.92 ± 0.92 %. Moreover, the significantly increased neutrophil response to thrombin-activated platelets compared to TNF-α-activated endothelium alone (A) was not seen in the presence of CFTRinh-172 (B).

Figure 5.35. The effect of CFTRinh-172 on neutrophil transendothelial migration. A) PMN TEM in the absence of CFTRinh-172. B) PMN TEM in the presence of 20 µM CFTRinh-172. The results are presented as an average % of migrating neutrophils ± SEM and analysed by comparison to the appropriate experiment control and by using paired, two-tailed t-test, n=4. * is comparing to the control and # is comparing ± CFTRinh-172 under the same conditions. * - p<0.05 and ** p<0.01.
To understand why CFTRinh-172 did not cause the expected pro-inflammatory profile of CF endothelium with increased PMN TEM, the cytokine level in both apical and basal supernatant was measured.

Figure 5.36 (over) presents the apical IL-8 concentration from the above experiment in the absence (A) and presence (B) of CFTRinh-172. A low level of IL-8 measured in the apical supernatant of the untreated control was significantly increased to 2.86 ± 0.73 ng/ml in the presence of subendothelial thrombin-activated platelets plus TNF-α (p<0.05) or IL-8, 3.30 ± 1.40 ng/ml (p<0.05). The addition of CFTRinh-172 significantly (p<0.05) increased apical IL-8 levels only in the presence of thrombin-activated platelets plus TNF-α. There was no statistically significant difference in IL-8 concentration measured in the apical supernatant in the absence versus the presence of CFTRinh-172.

The basal IL-8 concentration in the absence (A) and presence (B) of CFTRinh-172 is shown in Figure 5.37 (page 229). IL-8 levels detected in the basal compartment was much higher than IL-8 in the apical compartment, but not significantly different. IL-8 detected in the basal compartment of Transwells containing already approximately 7500 ng/ml IL-8 added as a neutrophil chemoattractant, was significantly (p<0.01) higher compared to the untreated control both in the absence and presence of the CFTRinh-172, 5704.14 ± 1367.95 ng/ml and 8792.78 ± 3369.87 ng/ml, respectively and not significantly different between each other. When thrombin-activated platelets were present on unactivated endothelium, IL-8 was significantly lower in the presence of the inhibitor.
Figure 5.36. The level of IL-8 in neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in apical supernatants. A) IL-8 level in PMN TEM upon different conditions. B) IL-8 level in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by comparing to appropriate untreated experiment control and by using paired, two-tailed t-test, n=4. * indicated p<0.05 and ** indicates p<0.01.
Figure 5.37. The level of IL-8 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in basal supernatants. A) IL-8 level in PMN TEM upon different conditions. B) IL-8 level in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by comparison to appropriate untreated experiment control and by using paired, two-tailed t-test, n=4.

Also, total PAI-1 levels were measured in the apical and basal compartments of Transwells in the absence and presence of the CFTRinh-172. Additionally, PAI-1 was measured in endothelial cell pellets collected after each of 4 experiments.

PAI-1 detected in the apical supernatant from PMN TEM did not differ significantly from apical PAI-1 level in the presence of CFTRinh-172 in PMN TEM with one exception (Figure 5.38, over). Under TNF-α-activation of HLMVEC and in the presence of
platelets, the PAI-1 level was significantly (p<0.05) lower in the presence of CFTRinh-172, 31.20 ± 6.93 ng/ml versus 36.59 ± 7.62 ng/ml, while in the absence of platelets the p value was 0.067.

![Figure 5.38](image-url)

Figure 5.38. The level of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in apical supernatants. A) PAI-1 in PMN TEM upon different conditions. B) PAI-1 in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by comparison to appropriate untreated experiment control and by using paired, two-tailed t-test, n=4. * represents comparison to control and # is comparing ± CFTRinh-172 under the same conditions. * indicates p<0.05.

The PAI-1 level measured in the basal Transwell compartment was approximately 5 times higher in comparison to the PAI-1 level in the apical one, however there was no
significant difference between PAI-1 detected in the basal supernatant in the absence and presence of CFTRinh-172 (Figure 5.39).

![Figure 5.39. The level of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in basal supernatants. A) PAI-1 in PMN TEM upon different conditions. B) PAI-1 in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by comparison to appropriate untreated experiment control and by using paired, two-tailed t-test, n=4.](image)

Moreover, the absence and presence of CFTRinh-172 did not show any statistically significant difference in PAI-1 measured in endothelial cell pellets (Figure 5.40, over).
Figure 5.40. The effect of PAI-1 on neutrophil transendothelial migration in the absence and presence of CFTRinh-172 measured in endothelial cell pellets. A) PAI-1 in PMN TEM upon different conditions. B) PAI-1 in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by comparison to appropriate untreated experiment control and by using paired, two-tailed t-test, n=4.

Plasmin activity was measured in the apical supernatant of PMN TEM in the absence and presence of CFTRinh-172 (Figure 5.41, over). The untreated control had 1.31 ± 0.08 µg/ml and 1.50 ± 0.13 µg/ml plasmin, depending on the absence or presence of the inhibitor, respectively. The plasmin activity was higher in the apical supernatant upon CFTRinh-72 treatment and reached significance (p<0.05) in the case of thrombin-activated platelets, platelets in the presence of TNF-α and subendothelial IL-8 as neutrophil chemoattractant.
The plasmin activity in neutrophil TEM in the absence and presence of CFTRinh-172 measured in apical supernatants. A) Plasmin activity in PMN TEM upon different conditions. B) Plasmin activity in PMN TEM upon different conditions in the presence of 20 µM CFTRinh-172. The results are expressed as a mean ± SEM and analysed by using unpaired two-tailed t-test, n=4.

There was approximately 5 times more plasmin activity detected in basal Transwell compartment than apical compartment (Figure 5.42, over). Plasmin activity in the basal control supernatant was 5.44 ± 1.45 µg/ml and this activity was significantly (p<0.01) increased in PMN TEM towards thrombin-activated platelets across TNF-α activated (9.16 ± 1.40 µg/ml) and unactivated (7.72 ± 0.97 µg/ml) HLMVEC and in platelets plus TNF-α (9.00 ± 1.56 µg/ml) conditions, respectively. The presence of CFTRinh-172 enhanced plasmin activity in the basal control supernatant to 7.39 ± 1.79 µg/ml.
µg/ml, which was significantly (p<0.05 and 0<0.01) increased with thrombin-activated platelets on unstimulated or stimulated endothelium 9.90 ± 2.05 µg/ml or 11.17 ± 1.03 µg/ml, respectively, in comparison to the untreated control.

Interestingly, plasmin activity detected in the basal supernatant upon CFTRinh-172-treatment was statistically (p<0.05) higher in the untreated control, platelets plus TNF-α, thrombin-activated platelets in the presence of TNF-α and subendothelial IL-8, than in the absence of this inhibitor.
In addition, correlations between the measured parameters were assessed in the previous experiments (Figure 5.35 – 5.42). Only significant correlations are presented in the graphs below. There was a significant (p=0.0002 and p=0.0391) correlation found between the level of both IL-8 and PAI-1 detected in basal supernatants of PMN TEM and the percentage of neutrophils migrated through HLMVEC (R²=0.8293 and R²=0.4314, respectively) (Figure 5.43 and 5.44).

**Figure 5.43.** The correlation between a percentage of migrated neutrophils and IL-8 level detected in the basal supernatant of PMN TEM.

**Figure 5.44.** The correlation between a percentage of migrated neutrophils and PAI-1 level detected in the basal supernatant of PMN TEM.
A significant positive correlation was also found between plasmin activity in the apical supernatants versus the percentage of PMN migrated transendothelially, IL-8 and PAI-1 levels measured in the basal compartments (results not shown).

Additionally, Figure 5.45 presents a significant (p=0.0420) correlation between the level of basal IL-8 and PAI-1, where $R^2$ equal 0.4223.

![Graph showing correlation between IL-8 and PAI-1 levels](image)

Figure 5.45. The correlation between IL-8 and PAI-1 level measured in the basal supernatant of PMN TEM.

5.5.11. VIABILITY OF NEUTROPHILS

The cytotoxic effect of drugs on neutrophil viability was tested in separate experiments, in which freshly isolated neutrophils were exposed to the drugs for 3.5 hours to mimic the effect of the drugs in TEM experiments. The viability of neutrophils was examined by annexin V-biotin assay and measured by flow cytometry. This technique allows analysis of neutrophil populations into 3 groups, such as necrotic, apoptotic and live cells. Every experiment had its own control of untreated neutrophils for comparison with drug-treated cells. The assay was done once in triplicate or three times in single measurements, as indicated on the graphs.

The following drugs were incubated with freshly isolated neutrophils for 3.5 hours in RPMI 1640 supplemented with 2.5 % (v/v) FBS medium for TEM experiments: tobramycin, copper-tobramycin and copper-sulphate (0.01 – 0.5 mM), SOD (500 –
2000 U/ml), catalase (250 U/ml), MnTBAP (1 – 50 µM), PAI-1 inhibitor, XR5118 (1 – 100 µM) and CFTRinh-172 (20 µM).

Tobramycin (Figure 5.46), copper-tobramycin (Figure 5.47) and copper sulphate (Figure 5.48, over) were shown to be non-toxic for neutrophils in the conditions tested.

Figure 5.46. Viability of neutrophils in 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS with tobramycin treatment (0.01 - 0.5 mM), n=1, in triplicate.

Figure 5.47. Viability of neutrophils in 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS in the presence of copper-tobramycin (0.01 – 0.5 mM), n=3, independent experiments.
Figure 5.48. Viability of neutrophils during 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS in the presence of copper sulphate (0.01 – 0.5 mM), n=3, independent experiments.

None of the antioxidants used in neutrophil transmigration experiments were harmful to neutrophils. Among them were SOD (Figure 5.49), catalase (Figure 5.50, over) and MnTBAP (Figure 5.51, over).

Figure 5.49. The effect of SOD (500 – 2000 U/ml) on viability of neutrophils during 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS, n=3, independent experiments.
Figure 5.50. The effect of catalase (250 U/ml) on neutrophil viability during 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in triplicate.

Figure 5.51. Viability of neutrophils during 3.5 hours incubation of PMN with MnTBAP (1- 50 µM) in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in triplicate.

Only XR5118 at the highest concentration of 100 µM diminished the population of live neutrophils from 95.86 ± 0.96 % to 55.76 ± 3.87 %, increasing the amount of both apoptotic (from 2.41 ± 0.25 % to 34.33 ± 2.93 %) and necrotic (from 1.73 ± 0.72 % to 9.91 ± 1.60 %) cells (Figure 5.52, over).
CFTRinh-172 had no effect on neutrophil viability in the conditions tested (Figure 5.53).
5.5.12. VIABILITY OF HLMVEC

The viability of HLMVEC after 3.5 hours incubation in RPMI 1640 supplemented with 2.5 % (v/v) FBS with drugs used in PMN TEM was assessed luminometrically. The results were compared to the following controls, a negative control of apoptosis, which was untreated HLMVEC and a positive control of apoptosis, HLMVEC treated with 1 µM staurosporine concentration for 24 hours. The negative and positive apoptosis controls were 25,046 and 102,303 CPS (count per second) luminescence units, respectively.

Tobramycin, copper-tobramycin and copper sulphate in the range of 0.01 – 0.5 mM were not found to be toxic (Figure 5.54). Also, extracellular SOD in the concentration range 500 – 2000 U/ml (Figure 5.55, over) and XR5118 at 1 – 100 U/ml (Figure 5.56, over) were not toxic to HLMVEC for 3.5 hours incubation in RPMI 1640 supplemented with 2.5 % (v/v) FBS.

![Figure 5.54](image.png)

Figure 5.54. Viability of HLMVEC at 3.5 hours incubation with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporine-induced apoptosis, n=1, in duplicate. CPS = count per second.
Moreover, the effect of the above mentioned drugs was assessed on HLMVEC in the presence of 10 ng/ml TNF-α for 3.5 hours in RPMI 1640 + 2.5 % (v/v) FBS in the regard to the appropriate negative and positive apoptosis controls, which were measured to be 35,787 and 104,740 CSP luminescence units, respectively (Figure 5.57 – 5.59). None of the tested drugs were toxic to HLMVEC activated with TNF-α, with the exception of 100 U/ml of XR5118, which induced apoptosis in 22.15 % of HLMVEC.
Figure 5.57. Viability of TNF-α-activated (10 ng/ml) HLMVEC at 3.5 hours incubation with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporine-induced apoptosis, n=1, in duplicate. CPS = count per second.

Figure 5.58. Viability of TNF-α-activated (10 ng/ml) HLMVEC at 3.5 hours incubation with SOD (500 – 2000 U/ml) in RPMI 1640 + 2.5 % (v/v) FBS and a staurosporine-induced apoptosis, n=1, in duplicate. CPS = count per second.
Some of the drugs tested in PMN TEM were incubated on HLMVEC for 24 or 16 hours. Therefore, the viability of HLMVEC was also measured using luminescence following 24 hours incubation of these drugs in RPMI 1640 supplemented with 2.5 % (v/v) FBS. The results below are presented as a percentage of cell viability.

Figure 5.60 (over) presents the effect of 10 ng/ml TNF-α and 20 µM CFTRinh-172 on HLMVEC viability after 24 hours treatment in RPMI 1640 supplemented with 2.5 % (v/v) FBS. Tobramycin in the range of concentrations 0.01 – 0.5 mM was not toxic to HLMVEC following 24 hour incubation in RPMI 1640 plus 2.5 % (v/v) FBS. Similarly, 0.01 and 0.1 mM copper-tobramycin and copper sulphate did not decrease endothelial cell viability. However 0.5 mM concentration of copper-tobramycin caused 16.64 % cytotoxicity, whereas 0.5 mM copper sulphate caused a 41.25 % decrease (Figure 5.61, over).
Figure 5.60. Viability (%) of HLMVEC upon 24 hours incubation with TNF-\(\alpha\) (10 ng/ml) and CFTRinh-172 (20 \(\mu\)M) in RPMI 1640 + 2.5 % (v/v) FBS, n=1, in duplicate.

Figure 5.61. Viability (%) of HLMVEC treated with tobramycin, copper-tobramycin and copper sulphate (0.01 – 0.5 mM) in RPMI 1640 + 2.5 % (v/v) FBS for 24 hours, n=1, in duplicate.

Catalase (250 U/ml) and SOD in a range of 500 – 2000 U/ml (Figure 5.62, over) as well as MnTBAP at 10 – 50 \(\mu\)M were not toxic to HLMVEC after 24 hours incubation in RPMI 1640 supplemented with 2.5 % (v/v) FBS (Figure 5.63, over). 86.39 % of HLMVEC were alive after treatment with 50 \(\mu\)M MnTBAP.
Figure 5.62. Viability (%) of HLMVEC treated with catalase (250 U/ml) and SOD (500 – 2000 U/ml) in RPMI 1640 + 2.5 % (v/v) FBS for 24 hours, n=1, in duplicate.

Figure 5.63. Viability (%) of HLMVEC for 24 hours in RPMI 1640 + 2.5 % (v/v) FBS after treatment with MnTBAP (1 – 50 µM), n=1, in duplicate.

5.6. SUMMARY OF RESULTS

A new *in vitro* model of neutrophil migration across TNF-α-activated HLMVEC towards thrombin activated platelets was established. The measured response was equivalent to the response elicited by IL-8 added alone under inactivated EC. Neutrophil migration was partly IL-8- and NAP-2-dependent, however other cytokines are likely to be
involved. Tobramycin was more potent and copper-tobramycin was more effective in inhibiting PMN TEM in this model. The effect is likely to be due to the SOD-like activity of copper-tobramycin, since SOD plus catalase and MnTBAP also inhibited PMN TEM. The inhibition of PMN TEM by any drug was apparently not due to a cytotoxic effect, however copper-tobramycin caused a decrease in EC viability in 24 hours. Additionally, copper-tobramycin (0.5 mM) increased PMN TEM when IL-8 was the chemoattractant.

An in vitro model of neutrophil migration in the presence of CFTRinh-172 was set up to mimic CF conditions, however no significant difference in neutrophil migration was observed compared to PMN TEM in the absence of this inhibitor.

5.7. DISCUSSION

This study introduced three independent in vitro models of neutrophil transendothelial migration based on the use of HLMVEC grown in culture for 14 days. This time period allowed EC to form their own basement membrane. Similarly, Huber & Weiss (1989) cultured HUVEC for 21 days and the EC monolayer displayed in vivo-like intracellular borders and junctions and deposited a single-layered, continuous basement membrane. This basement membrane was impenetrable to colloidal particles and supported neutrophil extravasation in a physiologic manner.

IL-8 is the most potent known neutrophil chemoattractant and also here induced the maximum level of PMN TEM, 86.65 ± 5.6 % of cells added to the upper chamber migrated to the lower. Smart & Casale (1993) previously showed IL-8-induced neutrophil migration through naked filters and through both endothelial (HUVEC) and epithelial (A549) cells cultured on these filters. Moreover, the IL-8 concentration they used (1 x 10^{-8} M) is similar to the one reported in this study, 6.25 x 10^{-8} M. Rot et al (1996) suggested that subendothelial IL-8 is internalized by EC, transported transcellularly and released onto the luminal surface to be localized on membrane protrusions. However, IL-8 was also shown to bind to endothelial HS and this binding creates a chemotactic gradient and enhances neutrophil responses to IL-8 (Webb et al, 1993).
TNF-α-induced TEM of PMN was reported to be dependent on TNF-α-stimulated production of IL-8 (Smart & Casale, 1994) and supports the suggestion that, in this model, TNF-α stimulates $2.38 \times 10^{10}$ M IL-8 release from EC. PAI-1 has been shown to support IL-8 gradients (Marshall et al, 2003) and TNF-α is known to induce release of both IL-8 and PAI-1 from endothelial cells (Mantovani et al, 1997; Van der Poll, 1991). In the model of PMN TEM, discussed in this chapter, pro-inflammatory mediators, such as IL-8 and PAI-1 were detected in cell culture supernatants and their levels were increased by TNF-α. Moreover, IL-8 and PAI-1 release were polarized to the basal aspect of the HLMVEC layer and levels of both correlated with the extent of neutrophil migration.

IL-8 was mostly released from activated HLMVEC, since activated platelets released only small amounts of IL-8. TNF-α-activation of non-adherent neutrophils is controversial. It was shown that TNF-α induces a classical respiratory burst in neutrophils (Dusi et al, 1996). However, neutrophil respiratory burst induced by TNF-α in this model is unlikely. TNF-α is a prominent priming agent (Onnhein et al, 2008) and in the discussed model, there is no agonist in the apical compartment of Transwells capable of activating neutrophils, except for soluble and apical membrane bound IL-8. Neutrophil activation is therefore due to apical membrane bound IL-8.

*In vitro*, TNF-α-stimulated EC secrete large amounts of PAI-1 over a period of hours (Handt et al, 1996) and endothelium considerably contributes to release of PAI-1 during an inflammatory process (Binder et al, 2002). In the blood, the main storage of PAI-1 is platelet α-granules, where it is released to the plasma (Nordenhem & Wiman, 1997), therefore platelets could contribute to the PAI-1 released into the basal compartment. However, PAI-1 released to the basal compartment of Transwells was mostly inactive, compared to PAI-1 in the apical compartment, which was active. PAI-1 is known to exist in at least three different conformations, including an active, latent and proteolytically or oxidatively inactivated form (Edelberg et al, 1994). Low levels of the active form of PAI-1 in the basal supernatant suggests that ROS (He, 2010) and proteases (Harlan, 1985) released from migrating neutrophils may subsequently inactivate PAI-1.
The possibility that PAI-1 plays a direct role in inflammatory diseases (Arndt et al, 2005) makes it an attractive target for small molecule drug development, such as PAI-039, known as tiplaxtinin (Gorlatova et al, 2007). XR5118 is a low-molecular-weight inhibitor of PAI-1 that binds to PAI-1 at the area between amino acids 110 and 145 of the PAI-1 molecule or at least affecting the accessibility to this region, which contains the t-PA-PAI-1 interaction site (Friederich et al, 1997). XR5118 (100 µM) was shown to significantly reduce PMN TEM across TNF-α-stimulated HLMVEC towards thrombin-activated platelets, suggesting a role of PAI-1 in the given PMN TEM model. However, XR5118 at 100 µM was found to induce a degree of apoptosis, not necrosis, of both neutrophils and HLMVEC.

To understand the mechanisms of transendothelial migration of neutrophils across TNF-α-stimulated HLMVEC, a comparison to other, similar models was made. Casale et al (1998 & 1999) created an in vitro model of neutrophils migration through endothelial (HUVEC) monolayers grown on the top of permeable filter and epithelial (A549) monolayers on the underface of the filters. A sequential migration of human neutrophils through endothelium and epithelium was observed towards basally added TNF-α. Morzycki et al (1990) reported that the apical or basal exposure of HUVEC to TNF-α induced comparable PMN adherence. However, basal exposure alone induced significantly higher PMN migration than apical exposure alone. Here, TNF-α was added to both apical and basal compartment of Transwells, to allow TNF-α molecules to interact with both sides of the HLMVEC membrane as well as with neutrophils.

An upregulation of adhesion molecules, such as ELAM-1 and ICAM-1 in the response to TNF-α was also reported (Morzycki et al, 1990). Indeed, TNF-α and other cytokines were reported to increase the expression of adhesion molecules on endothelial cells (Ohira et al, 2003; de Vries et al, 1998). Also, treatment of human EC with TNF-α results in marked changes in cell shape and cytoskeletal organization (Camussi et al, 1991) and synthesis of cytokines, such as IL-8 (Elner et al, 1990) and mediators, such as PAF (Bussolino et al, 1988). Also, both endothelium and PMNs contribute to the generation of low levels of ROS (Lum & Roebuck, 2001).
Endothelial IL-8 and platelet NAP-2 were demonstrated to contribute approximately 50% to the neutrophil migration in this PMN TEM model, however other cytokines of endothelial (ENA-78, GRO-α, GCP-2) and/or platelet (NAP-2, PF-4, ENA-78) origin could be involved. Specific neutrophil chemokines express different chemotactic potency and the following pattern was observed for adult and neonatal neutrophils at 100 ng/ml: IL-8/CXCL8 > GRO-α/CXCL1 > GCP-2/CXCL6 > NAP-2/CXCL7 > ENA-78/CXCL5 > GRO-γ/CXCL2 > GRO-β/CXCL3 (Fox et al., 1995). In addition, PAF, generated by various cells including platelets and thrombin-activated EC (Lynch et al., 1986; Ludwig et al., 1984; Prescott et al., 1984), was reported to induce neutrophil migration (Carolan & Casale, 1990), however neutrophil migration in the model showed here was not due to PAF.

This study introduced also an in vitro model of neutrophil transendothelial migration across TNF-α-stimulated HLMVEC towards thrombin activated platelets. Here, platelets were recognised as a source of chemokines. Indeed, platelets, in addition to a role in haemostasis, contribute to inflammation by secreting chemokines that attract neutrophils to the site of inflammation. PF-4 and β-TG, which is proteolytically cleaved to NAP-2, are the most abundantly expressed platelet chemokines. However platelets also contain MIP-1α, RANTES, MCP-3, GRO-α, ENA-78 and IL-8 (Gear & Camerini, 2003).

Thrombin is a potent activator of platelets (Sambrano et al., 2001) and activates G protein-coupled protease activated receptors, PAR-1 and PAR-4 on human platelets. Thrombin initiates signaling cascades leading to increases in intracellular Ca²⁺, secretion of autocrine activators, trafficking of adhesion molecules to the plasma membrane and shape change, which promote platelet aggregation (Brass, 2003). Interestingly, there is increased coagulation in CF, which generates thrombin (Komp & Selden, 1970). Human platelets were previously demonstrated to release NAP-2 (Piccardoni et al., 1996) and RANTES (Kameyoshi et al., 1992) upon thrombin activation. Platelets from rabbit have been reported to secrete IL-8 after thrombin stimulation (Su et al., 1996). Here, thrombin-activated platelets were found to release high (µg/ml) NAP-2, moderate (ng/ml) RANTES and low (pg/ml) IL-8 levels. Among them, NAP-2 and IL-8 are chemotactic for neutrophils.
An inflammatory state, which is often a result of endothelial dysfunction, as seen in CF (Romano et al, 2001), predisposes platelets to interact directly with leukocytes for their efficient recruitment to inflamed tissue. Platelet-leukocyte aggregates have been observed in CF and asthmatic patients and a murine model of allergic inflammation and it is suggested that platelets might prime leukocytes for subsequent adhesion and transmigration into tissues (O`Sullivan et al, 2005; Pitchford et al, 2003). Moreover, Weissmuller et al (2008) reported that PMNs facilitated translocation of platelets across human and mouse epithelium. On the contrary, the current research indicated the possibility that platelets in the apical compartment of Transwells arrested the movement of neutrophils when platelets and neutrophils were added to the upper compartment of Transwells together.

Moreover, platelets not only contribute to the migration of other cells, but actively participate in this process themselves. Platelets have been recognised to be activated in CF and asthma (O`Sullivan et al, 2005; Page et al, 1985) and accumulated within the guinea pig lung after PAF infusion (Morley et al, 1985). They were also found in BALF from asthmatic patients and allergic rabbits after allergen challenge (Metzger et al, 1987). Platelets were also shown to participate directly in allergic tissue inflammation as they migrate extravascularly in response to a sensitized allergen (Pitchford et al, 2008).

Random migration of human platelets has been recognised as a parameter of platelet function (Valone et al, 1974) and platelets were reported to migrate directly to added collagen, which was not a result of diffusion or Brownian movement (Lowenhaupt et al, 1973). Metabolic inhibitors such as iodoacetic acid, sodium fluoride, and 2,4-dinitrophenol inhibited platelet movement further proving platelet migration to be an energy-dependent process (Lowenhaupt et al, 1977). Czapiga and co-workers (2005) demonstrated gradient-driven chemotaxis of platelets toward formyl peptides, derived from bacteria.

In the novel in vitro model of neutrophil migration across TNF-α-activated EC towards thrombin activated platelets, thrombin could also activate HLMVEC, as HLMEC and HUVEC express several PAR, including PAR-1, PAR-3 and thrombin independent PAR-2
PAR-1 and PAR-2 signalling on HUVEC activated by thrombin was potentiated by LPS and TNF-α to release an inflammatory cytokine, IL-6 (Chi et al., 2001). Although TNF-α is known to induce release of IL-8 from endothelial cells (Mantovani et al., 1997), thrombin in the presence of TNF-α could be responsible for exaggerating this response. Indeed, thrombin-activated platelets underneath a layer of HLMVEC activated by TNF-α induced significantly higher apical and non-significantly basal release of IL-8 compared to unactivated endothelium. However, thrombin alone did not induce a significant increase of PMN TEM compared to spontaneous neutrophil migration.

LPS is a major component of the outer surface of Gram-negative bacteria that forms a complex with LPS-binding protein (Schimann et al., 1990). This complex further binds to CD14, a high-affinity LPS receptor present on the surface of the immune and inflammatory system (Wright et al., 1990). The putative LPS signaling receptor, Toll-like receptor 4 (TLR4), is expressed in many cells, including platelets (Cognasse et al., 2005). LPS was shown to promote platelet activation by inducing secretion of the contents of both α and dense granules and thus amplifying secretion-dependent platelet aggregation (Zhang et al., 2009). It is still unknown whether EC TLR4 is expressed within the HUVEC and human coronary artery endothelial cells (HCAEC) or on the cell surface (Andreakos et al., 2004, Dunzendorfer et al., 2004). LPS was also reported to activate neutrophils via TLR2 and TLR4 (Sabroe et al., 2002). Indeed, treating peripheral PMNs with LPS induces the production of TNF-α mRNA (Palma et al., 1992). However, endothelium rather than neutrophil TLR4 was important in neutrophil sequestration into the lungs (Andonegui et al., 2003). Additionally, LPS increases neutrophil adherence mostly via an effect on HUVEC (Thomas et al., 1988). It is also known that LPS stimulates the interaction between platelets and neutrophils, leading to robust neutrophil activation via TLR4-dependent mechanisms (Clark et al., 2007; Semple et al., 2007). In this study, LPS could act on both platelets and EC. However, thrombin was demonstrated to be a more potent stimulus than LPS. Indeed, thrombin-activated platelets significantly increased PMN TEM in the absence or presence of TNF-α, whereas LPS induced a small increase in PMN TEM versus TNF-α alone.
There are indications of a role of ICAM-1 in the discussed model of TEM of PMN. ICAM-1 is expressed on endothelial cells, but is greatly increased at inflammatory sites by stimulation with LPS and pro-inflammatory cytokines such as IL-1 and TNF-α (Lawrence & Springer et al, 1991). ICAM-1 engagement is involved in ROS generation (Wang & Doerschuk, 2000). TNF-α is known to increase ROS production and stimulates lung vascular barrier function by engaging TNF-α receptor-1, leading to the increased endothelial permeability in cultured EC (Partridge et al, 1993; Hocking et al, 1990). Moreover, under activation, the soluble form of ICAM-1 (sICAM-1) can be released from different cells including HUVEC, human saphenous vein EC, human aortic SMCs, melanoma cells and haematopoetic cell lines (Lawson & Wolf, 2009). In the current research, significantly more sICAM-1 was detected in the basal than apical supernatant and reflected levels of neutrophil migration.

In the model of transendothelial migration of neutrophils with platelets activated with thrombin underneath TNF-α-stimulated HLMVEC, 78.64 ± 6.65 % neutrophils migrated. This response was not significantly different to the response elicited by IL-8 and represents a more physiological model of the inflammatory response.

Copper-tobramycin (0.5 mM) significantly potentiated PMN TEM when IL-8 was added subendothelially. Indeed all neutrophils added to the top well migrated through the EC layer. Copper-tobramycin was shown to have SOD-like activity (Chapter 2) and to bind to HSPGs (Chapter 3). There are number of ways in which the SOD-like activity of copper-tobramycin may enhance neutrophil TEM. The binding of copper-tobramycin to HSPGs could be responsible for protecting HSPGs from degradation by ROS (Klebanoff et al, 1993) or elastase released from migrating neutrophils (Heeringa et al, 1996). Moreover, PAI-1 supports IL-8-mediated PMN TEM by inhibition of plasmin shedding of IL-8/HS complexes (Marshall et al, 2003). Copper-tobramycin might therefore also protect PAI-1 activity, especially without changing the amount of PAI-1, as there is no change in the amount of PAI-1 measured in apical or basal levels in the conditions tested.

However, the neutrophil migration towards IL-8 in the presence of copper-tobramycin could also involve adhesion molecules, such as ICAM-1. The results indicate that
copper-tobramycin increased neutrophil migration, which is followed by shedding of ICAM-1 from the endothelial cell surface. After copper-tobramycin treatment, ICAM-1 was increased in the basal supernatant, although not significantly, due to the variability in results. Alternatively, copper-tobramycin may enhance actin cytoskeleton rearrangements to aid in neutrophil migration (Yoshida et al., 2006). Indeed, ICAM-1 is localized at the apical surface on EC and is organized in microdomains along with VCAM-1 around adherent leukocytes. The cytoplasmic C-terminus of ICAM-1 associates to α-actin and the actin binding ezrin-moesin-radixin family of proteins (Carpen et al., 1992).

Additionally, there is a possibility that early stages of HLMVEC apoptosis contributed to the enhancement of neutrophil migration in the presence of copper-tobramycin for 24 hours.

Although, copper-tobramycin is a potent anti-oxidant agent (Chapter 3 & 4), it had no effect on neutrophil migration when HLMVEC were stimulated with TNF-α alone. In the current study, stimulation of HLMVEC layers with TNF-α induced 23.63 ± 3.53 % of neutrophil migration. Presumably the level of neutrophil migration was too low to observe an effect elicited by copper-tobramycin.

Incubation of tobramycin or copper-tobramycin on TNF-α-activated EC for 3.5 hours indicated a trend towards an inhibition of PMN TEM towards activated platelets. However, low concentrations of tobramycin (0.01 and 0.1 mM) as well as a high concentration of copper-tobramycin (0.5 mM) was shown to significantly inhibit PMN TEM in the model across TNF-α-stimulated HLMVEC towards platelets activated with thrombin when both drugs were present on EC for 19 hours. Moreover, these drugs were added 0.5 hour earlier than TNF-α. Indeed, tobramycin and copper-tobramycin were shown to be potent intracellular antioxidants (Chapter 4), which were believed to protect endothelial cells from ROS, generated by TNF-α.

It is conceivable, in the view of the results in Chapter 2, that tobramycin binds copper present in the culture medium in a 1:1 metal:ligand ratio. The copper level in RPMI 1640 supplemented with 2.5 % (v/v) FBS was measured by GF-AAS to be 23.11 ± 1.98 ng/ml (Chapter 2), which corresponds to approximately 0.37 µM. This small amount of
copper may bind to tobramycin, which is in excess, by 30 and 300 fold, at 0.01 and 0.1 mM concentration to form a copper-tobramycin complex, which significantly inhibited PMN TEM. Tobramycin at the concentration 500 µM was not effective in inhibiting PMN TEM. This may be because free tobramycin in excess competes with copper-tobramycin for HS binding sites for cellular uptake (Chapter 2).

Copper-tobramycin significantly inhibited PMN TEM only at 0.5 mM, while lower concentrations of this complex were not effective. Moreover, the copper-tobramycin complex was demonstrated to have SOD-like activity without catalase activity at the concentration 0.01 – 0.5 mM (Section 2.5.4). SOD or catalase alone did not affect PMN TEM, however the combination of SOD (2000 U/ml) and catalase (250 U/ml) significantly inhibited neutrophil migration in the conditions tested. MnTBAP (50 µM), which is known to have both intracellular SOD and catalase activities (Faulkner et al, 1994) produced a similar effect. These results may suggest that SOD plus catalase as well as MnTBAP are stronger antioxidants than copper-tobramycin, as they potently inhibited PMN TEM without overnight preincubation. Also, MnTBAP demonstrated the same degree of PMN TEM inhibition when present on HLMVEC for 3.5 and 19 hours.

Oxidative stress was demonstrated to promote leukocyte transendothelial migration. Neutrophil activation by releasing their granular contents, containing digestive enzymes and producing ROS, allow their interaction with endothelium. In turn, neutrophil adhesion to the endothelium triggers multiple endothelial signalling events, including those, mediated by ROS. It then leads to widened intracellular space and facilitating plasma leakage and neutrophil migration (Yuan et al, 2012). Removal of superoxide inhibits the infiltration of neutrophils at sites of inflammation, as demonstrates by the use of the liposomal SOD (Niwa et al, 1985) and synthetic SOD mimetics M40403 and SC-55858 (Salvemini et al, 2001; Salvemini et al, 1999). However, SOD3 was shown to inhibit monocyte and T cell infiltration in the model of acute ischaemia, but have little effect on neutrophil migration (Laurila et al, 2009). Indeed, T-cells utilize the VCAM-1 pathway of cell adhesion to the endothelium, whereas neutrophils, instead of VCAM-1, use the ICAM-1-dependent pathway with no role for NADPH oxidase (reviewed by Cook-Mills et al, 2011). Moreover, NADPH
oxidase-dependent ROS have been identified as key regulators of neutrophil chemotactic migration (Hattori et al., 2010).

ROS could also alter vascular permeability involving regulation of junctional protein phosphorylation. ROS inhibits phosphatase activity by reversible oxidation of the catalytic cysteine residue and the resulting increase in tyrosine phosphorylated junctional proteins might increase TEM as an effect of junctional disruption (Sallee et al., 2006). ROS may also regulate junctional permeability by affecting the organisation of the actin cytoskeleton (Moldovan et al., 2000). Copper-tobramycin as a potent intracellular antioxidant (taken up by HLMVEC, Chapter 3 and 4) may inhibit ROS generation and therefore inhibit TEM of PMN.

Also, the extracellular antioxidant activity of copper-tobramycin (not taken up by neutrophils, Chapter 3 and 4) may contribute to the inhibition of ROS generation via a ROS scavenging mechanism or inhibition of NADPH oxidase and thus prevent neutrophil-endothelial interactions leading to TEM. Moreover, by inhibition of NADPH oxidase or superoxide scavenging, copper-tobramycin prevents superoxide undergoing further conversion into hydroxyl radical or peroxynitrate.

NE elastase has also been demonstrated to contribute to the human PMN migration across basement membrane by activating pro-gelatinase B (Delclaux et al., 1996). It was also reported that migrating PMN localise their membrane-bound elastase to the migrating front, where it facilitates TEM (Cepinskas et al., 1999). Interestingly, carbon monoxide (CO)-releasing molecule (CORM) was reported to reduce the level of cell-bound elastase, which contributes to the suppressed PMN TEM in a mice model of sepsis (Mizuguchi et al., 2009). Also, copper-tobramycin could displace NE from its cell surface-binding sites, where it is persistently active and cannot be inhibited by α1-AT (Chapter 4). This is therefore another possible mechanism of the inhibition of PMN TEM in the presence of copper-tobramycin.

Copper-tobramycin was not toxic to neutrophils or HLMVEC in medium with FBS. Moreover, PMN were unactivated in the viability experiments, as it took place in TEM of PMN. Indeed, TNF-α and other inflammatory stimuli, such as IL-6 and LPS, prolong the neutrophil lifespan. Additionally, transmigration across the endothelium signals in
further delay of neutrophil apoptosis (reviewed by Mayadas & Cullere, 2005). Therefore, PMN viability in TEM of PMN could be greater than demonstrated in the actual viability experiments. These results cannot be also explained by a cytotoxic effect of copper-tobramycin on HLMVEC, as there was an inhibitory effect of this drug. Also copper sulphate elicited some cytotoxicity on HLMVEC, however copper sulphate had no effect on PMN TEM at any concentration tested.

Interestingly, copper ions are known to inactivate a variety of enzymes and lactate dehydrogenase (LDH) is exceptionally sensitive to this metal. Moreover, NADH, found in neutrophil cell membranes, strongly enhances the Cu(II)-mediated loss of LDH activity (Pamp et al, 2005). For this reason, the LDH assay could not be used to assess the cytotoxicity of copper containing compounds. The cytotoxicity of test compounds on neutrophils and HLMVEC was assessed based on annexin V and caspase-3 and -7 activities, which allow detection of the early stages of apoptosis.

The quantitative measurement of inflammatory mediators, such as IL-8, PAI-1 and an inflammatory marker, sICAM-1 was undertaken to investigate the mechanism of action of tobramycin and copper-tobramycin in the inhibition of PMN TEM. More total IL-8, PAI-1 and sICAM-1 were detected in basal than apical supernatants collected from appropriate experiments with no indication for inhibition of PMN TEM. However, both IL-8 and PAI-1 correlate with neutrophil migration, demonstrating their involvement in TEM of PMN and confirming a good model of PMN TEM.

CFTR inhibitors are so far the only known tools to mimic CF endothelium, as there is no CF endothelial cell line available. CFTRinh-172 specifically inhibited CFTR chloride channel function (He et al, 2004, Thiagarajah et al, 2004). However, it expresses other effects, which are independent of CFTR inhibition. Indeed, CFTRinh-172 and GlyH-101 were able to depolarize mitochondria and induce a rapid increase in ROS levels. CFTRinh-172 was previously shown (Chapter 3) to upregulate endothelial HS expression on the HLMVEC surface and generate intracellular ROS (Chapter 5). CFTRinh-172 significantly attenuated lung endothelial cell apoptosis induced by staurosporine or H$_2$O$_2$ (Noe et al, 2009). Furthermore, CFTRinh-172 alters the organization of cytoskeletal proteins, F-actin and α-tubulin, in the following epithelial
cell lines, 16HBE14o⁻ and CFBE41o⁻ (Nilsson et al, 2010). CFTRinh-172, but not GlyH-101, induced NF-κB activation (Kelly et al, 2010; Perez et al, 2007). Additionally, CFTRinh-172 enhanced secretion of IL-1, IL-6 and IL-8 release from lung epithelial cells (Bartling & Drumm, 2009). It was therefore expected that CFTR inhibition would induce an exaggerated inflammatory response, as observed in CF epithelial cells (Chen et al, 2008; Perez et al, 2007) and subsequently increase PMN TEM. The percentage of PMNs migrated across HLMVEC in the presence of CFTRinh-172 tended to decrease with a significant effect with thrombin-activated platelets underneath unstimulated HLMVEC. This response was, however, too low to be further investigated. The level of IL-8, PAI-1, sICAM-1 and plasmin activity showed no change when compared to the absence of the inhibitor.

It is also highly controversial as to whether neutrophils and platelets in CF are different to normal cells (Mattoscio et al, 2010, O´Sullivan et al, 2005; Witko-Sarsat et al, 1999). However, it is possible to improve this in vitro model of CF PMN TEM by the use of neutrophils and platelets from CF patients.

Normal and CF neutrophils express CFTR at the mRNA and protein level and extracellular HOCl production by CF neutrophils is normal, however the chlorination of ingested P. aeruginosa is impaired (Painter et al, 2006). It may therefore indicate a lack of functional CFTR in neutrophils in CF. However, no role of CFTR in controlling neutrophil oxidative activity was suggested by McKeon et al (2010). In contrast, Witko-Sarsat et al (1996) reported that MPO-dependent oxygenation activity and chloramine release were significantly increased in both CF homozygotes and heterozygotes as compared with controls. Also, Vaisman et al (1994) observed that neutrophils from CF patients exhibit increased chemiluminescence activity, not related to increased ROS production. Moreover, CF neutrophils release increased amounts of NE elastase compared to normal neutrophils upon stimulation with TNF-α, IL-8 and opsonised bacteria (Taggart et al, 2000). Pharmacologic inhibition and genetic mutation of CFTR in neutrophils facilitates production of MIP-2 and TNF-α and activates NF-κB upon LPS stimulation (Su et al, 2011). CF neutrophils were reported to express a reduced responsiveness to IL-8 (Dai et al, 1994) and to LTB4 (Lawrence & Sorrelli, 1992). Reduced L-selectin responsiveness could be responsible for defective adhesion of CF
neutrophils (Russell et al., 1998). However, CF neutrophils co-cultured with bronchial epithelial cells deficient with CFTR expressed increased adherence and the level of both IL-6 and IL-8 measured in the cell supernatants. These effects may contribute to the sustained and exaggerated inflammatory response in CF airways (Tabary et al., 2006).

The hyperactivity of CF platelets has been suggested to be a consequence of CFTR dysfunction, although neither the mRNA nor the CFTR protein has been found in platelets from CF or normal subjects (O`Sullivan et al., 2005). On the contrary, a more recent study of Mattoscio et al. (2010) found that platelets express biologically active CFTR. Moreover, platelets from CF patients generated 40% less LXA4 than platelets from healthy subjects. LTXA4 were shown to be involved in resolution of inflammation and therefore are anti-inflammatory compounds (McMahon et al., 2001). Interestingly, CFTR inhibition increased platelet-dependent PMN viability and suppressed nitric oxide generation.
6.1. INTRODUCTION

6.1.1. NORMAL AND CF PULMONARY EPITHELIUM

The entire pulmonary tree is lined by a continuous layer of epithelial cells. The relative distributions and abundance of epithelial cell types vary significantly between airway regions. The epithelial cells lining the luminal surface of the proximal airway can be further classified as ciliated cells, non-ciliated secretory cells and basal cells. A characteristic pseudostratified two-layered epithelium persists through the major bronchi, while a multi-layered structure is seen in the more distal, narrow bronchi, which have fewer cartilage rings and more submucosal glands. Ciliated cells and secretory cells attach to the basal lamina via desmosine adhesions and to one another via tight junctions at the luminal surface. The underlying basal cells lie in contact with most of basal membrane (Jeffery, 1983; Breeze & Wheeldon, 1977).

The pulmonary epithelium is important for maintaining the normal functions of the respiratory system, which include acting as a barrier for various insults (Widdicombe, 1987b), facilitating mucociliary clearance (Sleigh et al, 1988), secreting substances, such as surfactant proteins, mucus and antimicrobial peptides for airway surface production (Widdicombe, 1987a), repairing and regenerating epithelial cells to restore normal airway function (Evans et al, 1976) and modulating the response of other airway components, such as airway smooth muscle cells and inflammatory cells (Flavahan et al, 1985; Holtzman et al, 1983).

Mutations in CFTR lead to unique alterations in the microenvironment of the lung, including the respiratory epithelium, that facilitate bacterial infection (Smith et al, 1996). Reported abnormalities in the CF lung manifest by altered fluid and ionic fluxes
across the respiratory epithelium, diminished glutathione level, excessive and dehydrated luminal mucus, diminished mucociliary clearance, altered patterns of epithelial surface molecule glycosylation and decreased activity of bacterial factors, such as lysozyme, lactoferrin, defensins and cathelicidins in the airway surface liquid (Pilewski et al, 1999; Travis et al, 1999, Matsui et al, 1998; Cheng et al, 1989).

6.1.2. EPITHELIAL CELL LINES FOR CF RESEARCH

The development of immortalized cell lines has been a significant benefit to the study of human disease, including CF (Gruenert et al, 1995). The immortalization of cells from CF patients as well as cells from non-CF individuals from tissues relevant to CF has been critical to enhancing our understanding of the physiological, biochemical and genetic mechanisms underlying CF and for the development of therapeutic strategies designed to manage CF pathology. Gruenert et al (2004) presented and discussed a comprehensive list of immortalized cells from various tissues and species, with an emphasis on epithelial cells that have played a significant role in advancing our knowledge of CF. Among them, two cell lines were chosen in the current study. The normal human bronchial epithelial cell line 16HBE 14o- was recognized as a model system of the airways for studying drug transport (Forbes et al, 2003). The CFBE 41o- cell line was generated by transformation of CF tracheo-bronchial cells with SV40 and has been reported to be homozygous for the ΔF508 mutation over multiple passages in culture. It has been recognised as useful tool for studies of CF gene transfer or alternative treatment with small drug molecules and for the gathering of further information about the disease at the cellular level, without the need for primary culture (Ehrhardt et al, 2006).

6.2. AIMS AND OBJECTIVES

The predominant aim in this pilot study was to evaluate the effects of the antibiotic tobramycin and the copper-tobramycin complex on CFTR expression in normal and CF epithelial cells using immunocytochemistry.
6.3. MATERIALS

16HBE 14o− and CFBE 41o− cell lines were kindly gifted by Dieter Gruenert, University of Davis, CA. Basal MEM (minimal essential medium) was supplemented with 10 % (v/v) FBS, 1 % (v/v) L-glutamine and 1 % (v/v) antibiotic/antimycotic from Invitrogen Ltd. (Paisley, UK). Human plasma fibronectin and albumin bovine fraction V 35 % (v/v) were purchased from Sigma-Aldrich Inc. (Poole, Dorset, UK). BD Biosciences (Oxford, Oxfordshire, UK) provided collagen type I.

6.4. METHODS

6.4.1. EPITHELIAL CELL CULTURE

16HBE 14o− and CFBE 41o− cell lines were seeded into collagen I/ fibronectin coated 75 cm² flasks at a seeding density of 5 x 10³ cells/ cm². Cells were grown until confluent in MEM containing 10 % (v/v) FBS, 1 % (v/v) L-glutamine and 1 % (v/v) antibiotic/antimitotic. The feeding and subculturing into 8-well chamber slides were the same as stated for HLMVEC line (Section 2.4.4.1, 3.4.2, respectively).

6.4.2. COLLAGEN I/FIBRONECTIN COATING

Fibronectin from human plasma (Sigma) was reconstituted in 1 ml sterile water to obtain a 1 mg/ml working concentration and stored in 100 µl aliquots at -20°C. The collagen I/fibronectin solution was prepared in basal MEM containing a final concentration of 0.1 mg/ml albumin bovine fraction V 35 % (v/v) (Sigma), 0.03 mg/ml collagen type I (BD biosciences) and 0.01 mg/ml fibronectin. 2ml of solution per T-75 flask and 1 ml per well of a 6 well plate was added and incubated for 2 – 3 hours at 37°C. Coating solution was aspirated and flasks/plates were stored maintaining sterility at room temperature.

6.4.3. OTHER METHODS

Epithelial cells were treated with tobramycin and copper-tobramycin (0.01 – 0.5 mM) in the same way as endothelial cells (Section 3.4.3). A method for immunocytochemical detection of CFTR was previously described in Chapter 3.
6.5. RESULTS

6.5.1. CFTR LOCALISATION IN EPITHELIAL CELLS

Untreated 16HBE 14o⁻ showed intracellular CFTR detection (panel 2B, Figure 6.1). Tobramycin at 0.01 mM had no effect of the distribution or the amount of CFTR (panel 3B), unlike at other tobramycin concentrations. Tobramycin at 0.1 mM highly upregulated the level of CFTR protein, located in the epithelial cell cytoplasm, excluding nucleus and big vesicles (panel 4B). CFTR was also present in the cytoplasm of 16HBE 14o⁻ treated with 0.5 mM tobramycin, but to a much smaller extent compared to the presence of 0.1 mM tobramycin (panel 5B). Copper-tobramycin at 0.01 mM increased the level of CFTR protein located in the cell cytoplasm and in the perinuclear area (panel 6B). Copper-tobramycin at 0.1 and 0.5 mM had similar effects on the level and distribution of CFTR in the epithelial cell line (panel 7B and 8B, respectively). Both concentrations highly increased the level of intracellular CFTR. The cells showed no autofluorescence in the absence of CFTR antibody (panel 1B).

Figure 6.1. The effect of tobramycin and copper-tobramycin on CFTR in 16 HBE 14o⁻. A) nuclear stain (Hoechst blue); B) CFTR stain (Mr. Pink); C) merged image. 1) no primary antibody; 2) no treatment; 3) 0.01 mM tobramycin; 4) 0.1 mM tobramycin; 5) 0.5 mM tobramycin; 6) 0.01 mM copper-tobramycin; 7) 0.1 mM copper-tobramycin; 8) 0.5 mM copper-tobramycin. Scale bar 25 μm.
A Z-stack of the untreated 16HBE 14o− revealed some surface staining of CFTR on the apical membrane and CFTR was detected intracellularly, especially within cell vesicles, presumably ER (Figure 6.2). Figures 6.3 and 6.4 (over) demonstrate the maximal upregulation of the CFTR level within 16HBE 14o- under treatment with tobramycin (0.1 mM) and copper-tobramycin (0.5 mM), respectively. Additionally both treatments moved CFTR presumably from the ER to the cytoplasm. There is no nuclear stain in the case of tobramycin at 0.1 mM, whereas copper-tobramycin (0.5 mM) intensified CFTR staining within all regions of the cell, including the apical cell membrane.

Figure 6.2. Z-stack of the untreated control of 16HBE 14o− with 1 µm intervals. Scale bar 25 µm. Optical slices were obtained through the cell from the apical to the basal, adherent cell surface.
Figure 6.3. Z-stack of 16HBE 14o– treated with 0.1 mM tobramycin with 1 µm intervals. Scale bar 25 µm.

Figure 6.4. Z-stack of 16HBE 14o– treated with 0.5 mM copper-tobramycin with 1 µm intervals. Scale bar 25 µm.
The untreated control CFBE 41o` expressed a low level of CFTR protein detected in the perinuclear area (panel 2B, Figure 6.5) compared to the negative control (1B). The incubation of CFBE 41o` with 0.01 mM tobramycin had no effect on CFTR (panel 3B). However, 0.1 mM tobramycin highly increased the level of CFTR located perinuclearly and in the cell cytoplasm (panel 4B). In the presence of 0.5 mM tobramycin the amount of CFTR within CFBE 41o` was decreased (panel 5B) compared to the treatment of 0.1 mM, but slightly enhanced compared to the untreated control. Copper-tobramycin at 0.01 and 0.1 mM was ineffective (panel 6B and 7B, respectively). In contrast, 0.5 mM copper-tobramycin upregulated CFTR levels mainly around the cell nucleus (panel 8B) and, additionally, a condensation of DNA was observed (panel 8A), suggesting an effect of top copper-tobramycin concentration on CFBE 41o` apoptosis.

Figure 6.5. The effect of tobramycin and copper-tobramycin on CFTR in CFBE 41o`. A) nuclear stain (Hoechst blue); B) CFTR stain (Mr. Pink); C) merged image. 1) no primary antibody; 2) no treatment; 3) 0.01 mM tobramycin; 4) 0.1 mM tobramycin; 5) 0.5 mM tobramycin; 6) 0.01 mM copper-tobramycin; 7) 0.1 mM copper-tobramycin; 8) 0.5 mM copper-tobramycin. Scale bar 25 µm.

Figure 6.6 (over) shows some degree of surface staining within CFBE 41o` treated with 0.1 mM tobramycin, however CFTR was mostly located in the cytoplasm and around the nucleus and was highly upregulated.
Figure 6.6. Z-stack of CFBE 41o- treated with 0.1 mM tobramycin with 1 µm intervals. Scale bar 25 µm.

6.6. DISCUSSION

Like other glycoproteins, CFTR is synthesized on ER-associated ribosomes and core-oligosaccharide chains are attached to the protein co-translationally at the asparagines (N) residues 894 and 900 located in the 4th extracellular loop (Chang et al, 1994). A large portion of CFTR is degraded from the ER. Retained misfolded proteins undergo ER-associated degradation (ERAD) through the ubiquitin-proteasome pathway (Farinha & Amaral, 2005). The remaining protein fragments are transported to the Golgi. Along the secretory pathway, the polypeptidic chain undergoes different post-translational modifications at its glycidic residues to produce the fully glycosylated form. Monitoring the glycosylation state of CFTR allows the wild type protein to be distinguished from the several processing mutants. According to the glycosylation status, 3 forms of the CFTR can be identified: the non-glycosylated polypeptidic chain known as band A (130 kDa), the ER core-glycosylated form of the protein known as band B (150 kDa) and the fully glycosylated mature form known as band C (170 – 180 kDa) (Cheng et al, 1990).
In normal human airway surface epithelium, CFTR is restricted to the apical plasma membrane of ciliated cells (Puchelle et al, 1992) and it is directly linked to cellular polarization (Morris et al, 1994). The deletion of the amino acid Phe 508 (ΔF508) is a CFTR mutation characterized by an abnormal intracellular maturation and trafficking (Cheng et al, 1990). Mutated CFTR has been localized diffusely in the cytoplasm of epithelial respiratory cells, as a consequence of its retention in the ER. It is therefore unable to accumulate at normal levels at the apical plasma membranes (Dupuid et al, 1995; Puchelle et al, 1992).

In the current study, both 16HBE 14o− and CFBE 41o− lines were observed to express low levels of surface CFTR stain, similarly to HLMVEC (Chapter 3). Higher levels of cytoplasmic CFTR was detected within 16HBE 14o− than CFBE 41o−. Interestingly, CFTR was located in cell vesicles neighbouring the nucleus, indicating ER. However, to find out what is the exact cellular location of CFTR in the given cell lines, specific markers of intracellular organelles, such as ER (calnexin), trans Golgi network (58K-9), endosomes (Rab4) (Drevillon et al, 2011) or lysosomes (LAMP-1) (Luciani et al, 2010) should be used. However, mature CFTR can travel continually between the apical plasma membrane and the recycling endosome (Rab11 and EHD1) (Drevillon et al, 2011). Therefore, the protein distribution depends on conditions, such as cell type, differentiation and phosphorylation (Bertrand & Frizzell, 2003). Moreover, the epithelial cells in the current study were not polarized, which can be achieved by growing them on permeable filters (Denning et al, 1992a; Denning et al, 1992b). Indeed, epithelial and endothelial cells (Chapter 3) were grown on collagen IV-coated chamber slides in submerged culture.

Stop mutations, such as G542X or W1282X, are the class I of CFTR mutations that consist of only about 5 % of total mutations, resulting in most severe CF phenotype (Chapter 1). It was previously demonstrated that, after topical gentamicin treatment, a significant increase in peripheral and surface staining for CFTR was observed in the nasal epithelial cells of patients carrying stop mutations in vivo (Wilschansky et al, 2003). Indeed, gentamycin was proposed to reduce translation fidelity, caused mRNA misreading and insertion of alternative amino acids at the site of mutated codons. In contrast, the studies of Clancy et al (2007) indicated no difference observed in CFTR
expression of nasal epithelial cells obtained from both healthy and CF subjects carrying heterozygous CFTR stop mutations before and during aminoglycoside (tobramycin and gentamycin) treatment in vivo. The study of Wilschansky et al (2003) was carried out on homozygous epithelial cells and the local concentrations of drugs used were higher by using single drops (0.9 mg daily) compared to the research done using an inefficient spray system (1.8 mg daily) by Clancy et al (2007). These disparities could be responsible for the different effect of aminoglycosides on epithelial CFTR level. However, the nasal epithelium demonstrates different pattern of gene expression compared to bronchial epithelium, therefore it is not recommended for assessing the effect of a new treatment on inflammation in the lung (Ogilvie et al, 2011).

In the current study, the role of tobramycin and its complex with copper was examined on the ΔF508 mutation in vitro. Importantly, the ΔF508 mutation is the most common CFTR mutation, belonging to the class II of CFTR mutations. Here, tobramycin at 0.1 mM upregulated the level of CFTR in both normal and CF epithelial cells in one of the mechanisms discussed below. Also, copper-tobramycin at 0.5 mM had a similar enhancing effect within 16HBE 14o, but not within CFBE 41o. Copper-tobramycin at the top concentration may therefore induce CF epithelial cells apoptosis, which could be indicated by nuclear granularity and possible DNA condensation. However, an assessment of epithelial cell viability (16HBE 14o and CFBE 41o) in the presence of used drugs has to be carried out.

Moreover, tobramycin and copper-tobramycin should be examined in terms of correcting CFTR function by measuring the transepithelial electric potential difference across ΔF508-CFTR-transfected epithelial cells and/or epithelial cells isolated from patients carrying the ΔF508 mutation. Also, specific CFTR inhibitors (CFTRinh-172 or GlyH-101) could be used to indicate that Cl movement across the membrane is via CFTR.

CFTR expression is susceptible to oxidative stress and subsequent degradation (Cantin et al, 2006). Epithelial cells and/or culture medium are assumed to contain copper,
which is exchangeable and binds tobramycin to give a copper-tobramycin complex. Copper-tobramycin is therefore believed to be an antioxidant that should increase CFTR expression. Tobramycin and copper-tobramycin could interfere in at least two steps of the CFTR cycle of maturation, trafficking and plasma membrane insertion and degradation process (Figure 6.7).

![Figure 6.7. Intracellular locations of CFTR during maturation, plasma membrane insertion and degradation. Properly folded, nascent CFTR leaves the rough endoplasmic reticulum and travels through the Golgi, where it undergoes glycosylation to mature protein. Mature CFTR leaves the Golgi in vesicles that can travel directly to the apical plasma membrane or to the recycling endosome. CFTR-containing vesicles continually traffic between recycling endosomes and plasma membrane. CFTR eventually follows the late endosome-to-lysosome pathway for degradation. Misfolded CFTR mutants and significant wildtype nascent CFTRs are degraded by the proteosome (Bertrand & Frizzell, 2003).](image)

Tobramycin and copper-tobramycin could inhibit the proteosomal degradation of CFTR. Indeed, several copper-binding compounds have been found to spontaneously complex with copper and form active proteasome inhibitors and apoptosis inducers
(Daniel et al, 2007). For example, a copper chelator, disulfiram, induces apoptotic cell death in breast cancer cultures and xenografts via inhibition of the proteasome activity (Chen et al, 2006). Misfolded, damaged or mutant proteins are eliminated by ERAD via the ubiquitin proteasome system. Ubiquitination is essential for proteasome degradation and endosomal recycling of native CFTR from the plasma membrane (Belcher & Vij, 2010; Ward et al, 1995). Interestingly, ubiquitination is also inhibited by copper chelators and may increase intracellular and membrane-associated CFTR. Clioquinol (5-chloro-7-iodoquinolin-8-ol), an anti-cancer drug, inhibits ubiquitination in a Cu(II)-dependent manner (Choi et al, 2006) and it is capable of directly inhibiting the proteasome at higher concentrations (Schimmer et al, 2011). The copper metabolism gene MURR1 domain 1 (COMMD1) is involved in copper metabolism and was suggested to inhibit proteasomal degradation, thereby affecting protein trafficking (Drevillon et al, 2011). Tobramycin as a copper-binding compound (Chapter 2) could have the same role as disulfiram, clioquinol or COMMD1 and therefore increase intracellular CFTR protein in the epithelial cells and restore CFTR on the HLMVEC membrane (Chapter 3).

To find out whether tobramycin and copper-tobramycin inhibits the endothelial proteasome, immunoprecipitation of ubiquitin immunoreactive polypeptides with CFTR antibody could be done in a similar way to the study of Ward et al (1995), followed by separating and immunoblotting CFTR immunoprecipitates with a polyclonal ubiquitin antibody. Ward and co-workers used two potent inhibitors of the 20S proteasome, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal and lactacystin, which could be now compared with tobramycin and copper-tobramycin.

Alternatively, tobramycin and copper-tobramycin could promote CFTR escape from ER, thus working as correctors. Correctors may be a useful tool in the treatment of CF caused by the ΔF508 mutation (Pedemonte et al, 2005). Curcumin, has also been described as CFTR corrector, which works by eliciting increased CFTR traffic towards the plasma membrane (Egan et al, 2004). Interestingly, curcumin was reported to have anti-inflammatory properties (Bachmeier et al, 2008; Takahashi et al, 2007). Moreover, curcumin binds copper and the Cu(II)-curcumin complex possesses SOD activity, free radical neutralizing ability and antioxidant potential (Barik et al, 2005). Therefore in these properties, curcumin resembles copper-tobramycin.
Tobramycin is a natural aminoglycoside antibiotic, isolated from *Streptomyces tenebrarius* and discovered in 1967. It has higher *in vitro* activity than other aminoglycosides against *Pseudomonas aeruginosa* strains (Laxer *et al*., 1975; Koch *et al*., 1973). The study of Ramsey *et al*. (1999) shows that long-term, intermittent administration of inhaled tobramycin in addition to standard therapy for CF improves pulmonary function, decreases *P. aeruginosa* colonisation and reduces the requirement for intravenous antipseudomonal antibiotics as well as hospital admissions. Tobramycin inhalation therapy is also clinically effective for severe non-cystic fibrosis bronchiectasis patients who present with increased density of *P. aeruginosa* in expectorated sputum (Schienberg & Shore, 2005). Inhaled tobramycin was shown to not only reduce *P. aeruginosa* counts in sputum over a 28 day cycle, but also to reduce levels of inflammatory cytokines IL-8, IL-6, TNF-α, as a consequence. This study did not however rule out a direct effect of tobramycin on epithelial cells (Husson *et al*., 2005).

In addition to its known antibacterial activity, inhaled tobramycin was suggested to have an anti-inflammatory effect, although the mechanism was not known (Ramsey *et al*., 1999). Also, CF patients may improve clinically, as measured by lung function, FEV1, even if *P. aeruginosa* is present in their sputum and not fully sensitive to the antibiotics they have received (Smith *et al*., 2003). Subsequently, Nakamura and co-workers (2011) reported that high-dose tobramycin inhibits LPS-induced MUC5AC production in human lung epithelial cells, presumably because positively charged tobramycin binds negatively charged LPS and neutralises its effect. Furthermore, the results of Cantin and Woods (1993) indicated that tobramycin and gentamicin protected lung epithelial cells against MPO-dependent oxidant injury by binding to anionic cell surfaces and converting HOCI to hydrophilic noncytotoxic chloramines. Additionally, tobramycin completely inhibited epithelial cell cytotoxicity induced by CF sputum and H₂O₂ (Cantin
Vancomycin and tobramycin at high concentrations also had relaxing effects on vascular smooth muscle in vitro suggesting a protective role on NO (Richter et al., 2010). These results clearly indicate the anti-oxidant effects of tobramycin.

The study of Geller et al. (2002) evaluated the high sputum and low serum concentrations of aerosolised tobramycin that were achieved when administered as 300 mg dose in a regime of 28 days off/28 days on. Mean sputum tobramycin concentrations measured 10 minutes after administration of the first dose of tobramycin for inhalation were approximately 1237 µg/g (~2 mM), while the mean serum tobramycin concentration at 1 hour after the administration of aerosolised tobramycin for inhalation was approximately 1 µg/ml (~2 µM) (Geller et al., 2002). In comparison, the serum concentration of tobramycin 30 minutes after intravenous administration of 2.71 ± 0.38 mg/kg was 10.2 ± 1.2 µg/ml (~20 µM) (Roberts et al., 1993). However, parenteral aminoglycosides are highly polar and penetrate poorly into the endobronchial space, resulting in 1.07 – 2.2 µg/ml (~2 – 4 µM) tobramycin in the airways (Hoogkamp-Korstanje & van der Laag, 1983).

The clinical use of intravenous aminoglycoside antibiotics results in side effects, including ototoxicity and nephrotoxicity, but the participation of free radicals in the adverse renal and cochlear side effects of aminoglycoside antibiotics is controversial (Priuska & Schacht, 1995). Gentamicin was shown to enhance the production of hydrogen peroxide in renal mitochondria (Walker & Shah, 1987). Free radicals are also rapidly produced by avian hair cells in vitro after exposure to gentamicin. A number of studies have demonstrated a protective effect of antioxidants (Hirose et al., 1997). However, since chelators can enhance iron-catalyzed oxidations, it was suggested that gentamicin-dependent radical formation was based upon iron chelation (Priuska & Schacht, 1995). The ability of aminoglycosides to bind Cu(II) is also thought to be responsible for aminoglycoside toxicity (Gaggelli et al., 2010).

Aminoglycosides themselves are redox-inactive. They need a specific medium to participate in the oxidative pathway, where metal ions are present (Sczepanik et al., 2004b). Some unique activities of aminoglycosides depend on metal ions (Kozlowski et
al, 2005). The binding of iron to gentamicin has been postulated to induce free radical formation, responsible for the peroxidation of lipids (Priuska et al, 1998). An erythromycin-iron complex was observed to exhibit superoxide scavenging activity, which was not seen for the antibiotic without the metal (Muranaka et al, 1997). The Cu$^{2+}$-aminoglycoside complexes are observed to exhibit oxidative activity, which can catalyse oxidation of nucleotides in the presence of H$_2$O$_2$ in vitro (Szczepeanik et al, 2003, Jezowska-Bojczuk et al, 2001a, Jezowska-Bojczuk & Lesniak, 2001). Hygromycin B in the presence of Cu(II) was found to damage extracellular DNA in vitro (Gaggelli et al, 2010).

Interestingly, tobramycin can inhibit the activity of copper-dependent enzymes. Indeed, tobramycin and vancomycin significantly inhibited, while their combination almost completely abolished copper-dependent acid sphingomyelinase activity in the renal cortices of rats (Beauchamp et al, 1990). Acid sphingomyelinase generates ceramide from sphingomyelin. It has been reported that ceramide is both increased (Becker et al, 2010) and decreased (Guilbault et al, 2009) in CF. Increased ceramide has been associated with lung inflammation in CF (Becker et al, 2010), while decreased ceramide has been associated with defective host defences against Pseudomonas (Guilbault et al, 2009). Decreased levels of ceramide in CF may also contribute to the known defect in neutrophil apoptosis in CF (Seumois et al, 2007). The contribution of tobramycin therapy to these changes in ceramide levels is unknown, but tobramycin may be associated with the inhibition of acid sphingomyelinase, reduced ceramide levels and defective apoptosis. Conversely, copper-tobramycin would not be expected to have these effects.

The use of aminoglycosides is variably associated with the side effects of ototoxicity and nephrotoxicity (Talaska & Schacht, 2007). Oxidative stress has been suggested to have a key role in these processes, but the mechanism for aminoglycoside-induced ROS formation is not known. Several mechanisms have been suggested, including the binding of metal ions to aminoglycosides and, in particular, the binding of Fe$^{2+}$ ions to gentamicin. However, several physio-chemical factors mitigate against this being a
likely mechanism under physiological conditions (Talaska & Schacht, 2007). Another mechanism, that has been proposed for ROS formation is the activation by aminoglycosides of Rac-1, a member of the family of Rho-GTPases with subsequent activation of the NADPH oxidase complex. In fact, the evidence presented in this thesis indicates that a copper complex of tobramycin has antioxidant properties and would protect against ROS formation via any mechanism and the subsequent toxic effects on cells of the inner ear and renal epithelial and endothelial cells (Lopez-Novoa et al, 2011).

Two opposite structural changes have been described in the CF lung. Firstly, bronchiectasis, which is a common result of an early protease/anti-protease imbalance in CF and, secondly, fibrotic changes, which may be associated with end-stage lung disease (Regamey et al, 2011). The bronchectasis changes have been related to inflammation and anti-inflammatory therapeutic approaches to reduce the protease burden in the lung are suggested (Rottner et al, 2011). This study has shown that copper-tobramycin can displace elastase from sites of encryption on the neutrophil membrane making the enzyme susceptible to inhibition by endogenous anti-proteases, such as α1-AT. Indirectly, therefore, copper-tobramycin may redress the protease/antiprotease imbalance.

Preliminary data has shown that tobramycin can inhibit the copper-dependent enzyme lysyl-oxidase, which is essential for cross-linking newly synthesized elastin and collagen fibres and therefore the tissue matrix repair process. Thus tobramycin may limit bronchial tissue repair and contribute to the development of bronchiectasis.

Similarly, decreased Cu/Zn-SOD and SOD3 activity in mononuclear and polymorphonuclear cells detected in CF patients (Percival et al, 1999) could be an effect of tobramycin use. However, due to diminished SOD3 activity in CF lung (Rottner et al, 2011), copper-tobramycin could be considered as a replacement therapy.
Moreover, tobramycin and other aminoglycoside antibiotics prescribed in CF patients were shown to trigger hypomagnesaemia (Adams et al, 1998), which inhibits magnesium-dependent DNase I activity (Sanders et al, 2006).

This study tested the hypothesis that tobramycin binds copper and becomes a copper-tobramycin complex with anti-inflammatory properties. In the view of the current study, it appears that tobramycin chelates copper present in the cell culture medium and/or cells and forms a safe storage depot for copper. It is also proposed that tobramycin is a pro-drug for the copper-tobramycin complex, which has anti-inflammatory and antioxidant effects not seen in tobramycin.

Azithromycin, a macrolide antibiotic with anti-inflammatory activity was shown to chelate copper in vitro (Sher et al, 1994). The results of Szczepanik et al, (2004a) have shown that the aminoglycoside kanamycin A has higher affinity for Cu(II) ions than other metal ions, which may be available biologically or accessible following the contamination. Moreover, it was suggested that copper(II) complexes of aminoglycosides can easily be formed in vivo, in blood plasma by withdrawing of the metal bound to human serum albumin (Szczepanik et al, 2004a).

Although, the blood plasma copper levels in healthy humans are 808.8 ± 80.0 µg/l (12.84 ± 1.27 µM), copper levels in the circulation of adult men with CF are significantly elevated at 1205.9 ± 255.4 µg/l (19.14 ± 4.05 µM) (Percival et al, 1999). The majority of copper in the human blood is bound to ceruloplasmin (65 %), while, albumin and transcuprein bind 18 % and 12 % of plasma copper, respectively. Cells take up copper from all these pools, but copper bound to albumin and transcuprein appears to be the primary component of the exchangeable plasma copper pool. The remaining 5 % of copper is associated with small peptides and amino acids (Linder & Hazegh-Azam, 1996).

Intravenously administered tobramycin is predicted to bind with the maximum of 30 % of exchangeable endogenous copper. However, the concentration and therefore availability of copper in the blood could be increased by wearing copper bracelets and
in this way increase the efficacy of tobramycin as an anti-inflammatory agent. Indeed, clinical trials using copper bracelets and Cu(II) salicylate complexes together showed efficacy in arthritis (Walker et al, 1981). Moreover, the kanamycin B, amikacin and tobramycin complexes with Cu(II) are more stable in lower pH (Gokhale et al, 2007), which is usually found in CF airway surface fluid and organelles of CF respiratory epithelial cells (Poschet et al, 2002).

Previous in vitro studies demonstrated that the complex of salicylic acid with Cu(II) could not exist in plasma (Arena et al, 1978), presumably because of its stability constant of 10.60 (Furia, 1972) similar to the one reported for Cu(II)-HSA, 11.18 (Masuoka et al, 1993). For comparison, a pK\textsubscript{D} for copper-tobramycin equal to 7.44 was proposed by Jezowska-Bojczuk et al (1998) and 4.30 in the current research (Chapter 2). Because tobramycin forms a stronger complex with copper than salicylic acid, it is more likely that tobramycin would pull copper from albumin, not salicylate. However, copper-tobramycin administered in a pre-formed complex might exert a better anti-inflammatory effect than tobramycin alone. Conversely, a Cu(II)-salicylic acid complex did not have a different therapeutic effect when administered parenterally pre-formed or as a mixture of its individual components at equivalent concentrations, but the preformed complex was postulated to exhibit a greatly enhanced bioavailability (Arena et al, 1978).

An intravenous route for copper-tobramycin administration in CF lung disease to achieve of final blood level of 20 µM, would be beneficial because TEM of PMNs takes place through the blood vessel wall, where the anti-inflammatory and anti-oxidant potential of copper-tobramycin is observed. Indeed, the IC\textsubscript{50} for copper-tobramycin in neutrophil oxidative stress was calculated as 2.5 µM (Figure 4.4B) and in endothelial oxidative stress below 10 µM (Figure 4.37). Moreover, intravenous copper-tobramycin could exert its SOD-like activity in the sputum, if it achieves the same final concentration as tobramycin (~2 µM). Indeed, copper-tobramycin exerts its SOD-like activity at levels as low as 0.2 µM, as demonstrated in a cell-free system.
Copper-tobramycin could be also administered via inhalation and also reach approximately 2 µM in serum, depending on a dose. Copper levels were shown to be increased in CF sputum (0.3 µM) compared to healthy control (0.135 µM) (Gray et al, 2010). The source of copper is not known, however, sputum microorganisms, such as *P. aeruginosa* (Marks et al, 2004) and *Aspergillus* are likely to contain copper in a small copper protein, azurin and in the membrane, respectively (Tseko and Todorova, 2002). The inhalation approach could achieve copper-tobramycin sputum concentrations of about 2 mM, if administered in the same dose as tobramycin. Importantly, copper-tobramycin was shown to be equally effective as tobramycin in inhibiting bacterial growth within 72 hours (unpublished results).

Copper complexes were shown to be much more effective than their parent drugs (Sorenson et al, 1989). High-dose non-steroidal anti-inflammatory drugs, mainly ibuprofen, can slow the progression of lung damage in CF, especially in children (Lands & Stanojevic, 2007). Interestingly, Cu(II) complexes with NSAIDs were shown to be more effective and less toxic than their parent drugs (Wedekind et al, 2002). Indeed, copper-tobramycin might be devoid of the side effects characteristic for tobramycin, because it already contains copper within the complex and it would not inhibit copper-dependent (SOD, lysyl oxidase, sphingomyelinase, tyrosinase) and magnesium-dependent (DNase I) enzymes. Moreover, copper-tobramycin is an antioxidant and therefore protective.

Roberts and Robinson (1984) proved that copper-conjugates of NSAIDs have SOD-like activity, similar to copper-tobramycin. Of them, Alcusal® is still available commercially (Wedekind et al, 2002).

Preclinical studies revealed that endogenous EC-SOD had a protective effect in animal models of lung inflammation (Bowler et al, 2004), asthma (Bowler & Crapo, 2002) or COPD (Barnes, 2000). Also, the use of the native enzymes in clinical trials supported the concept that removal of superoxide had a beneficial outcome. For example, Orgotenin® (bovine Cu/Zn-SOD) showed promising results in human rheumatoid arthritis (Goebel et al, 1981). However, there were drawbacks associated with its use.
The main problem was the nonhuman origin of the enzyme, causing a variety of immunological problems (Muscoli et al., 2003). Therefore, a recombinant human Cu/Zn-SOD was indicated to decrease early pulmonary injury due to bronchopulmonary dysplasia, resulting in improved pulmonary outcome in premature infants (Davis et al., 2003). However, natural human Cu/Zn-SOD is a large molecule (135 kDa) (Marklund, 1982). Its diffusibility through most living tissues is low and is almost negligible through cellular membranes. In the view of potential therapeutic uses, in which a greater ability to diffuse through tissues and capacity of intracellular uptake are required, low molecular weight SOD mimics might be beneficial. Such non-enzymatic systems should reach deeper and faster their targets (Lapluye, 1990). SOD mimetics, such as M40403 and SC-55858 were shown to have anti-inflammatory properties in a rat model of inflammation (Salvemini et al., 2001; Salvemini et al., 1999).

In addition, MnTBAP, which is a synthetic metalloporphyrin with anti-oxidant action prevented the generation of intracellular ROS in LPS-stimulated RAW 264.7 macrophage cells (Tumurkhuu et al., 2007).

Although superoxide is implicated in neutrophil migration and bacterial phagocytosis, it plays a central role in generating other ROS in the human body, contributing to cell and matrix damage and therefore to the pathogenesis of many inflammatory lung diseases (Rahman et al., 2006). For instance, superoxide can immediately react with NO, an important signal molecule and endothelial-derived vasodilator, which also decreases platelet aggregation, to form peroxynitrite. This reaction depletes NO bioactivity and peroxynitrite has detrimental effects on vascular function, by the oxidation of cellular proteins and lipids (Guzik et al., 2002). Therefore, copper-tobramycin might be a better drug than tobramycin to protect the bioactivity of NO (Richter et al., 2010).

In the presence of copper-tobramycin, superoxide ion cannot be converted to its toxic intermediates, including peroxynitrite and, especially because copper-tobramycin was demonstrated not to accumulate within neutrophils, it could act as extracellular ROS scavenger or an inhibitor of NADPH oxidase. Additionally, copper-tobramycin is not a
true SOD or SOD-mimetic, as it had no effect on extracellular H$_2$O$_2$ concentrations, which may be expected following dismutation of superoxide ions. However, inhaled or intravenous copper-tobramycin could be used as SOD3 replacement therapy in CF, additionally offering other multiple effects.

Preliminary data demonstrated that copper-tobramycin degraded extracellular DNA, indicating another possible therapeutic application of copper-tobramycin. Similarly to inhaled human recombinant DNase (Fuchs et al, 1994), it could be considered as a useful mucolytic agent in CF.

Importantly, this study suggested that tobramycin and copper-tobramycin entered the human endothelium, and presumably epithelium, via HS binding, which precedes its accumulation within the cell cytoplasm and in the perinuclear area. Also, both endothelial and epithelial cells were previously demonstrated to express CFTR (Tousson et al, 1998; Puchelle et al, 1992). Tobramycin and copper-tobramycin were shown in the current study to increase the level of epithelial and endothelial CFTR expression as well as endothelial surface CFTR level, which is predicted to have an anti-inflammatory effect. A functional CFTR was demonstrated to suppress NF-κB activation in inflammatory signalling in epithelial cells and IL-8 expression (Verhaeghe et al, 2007). Tobramycin and copper-tobramycin are suggested to inhibit proteasomal degradation of CFTR or correct defective ΔF508-CFTR folding or cellular processing, thus working as a CFTR corrector. Curcumin is a well known CFTR corrector (Egan et al, 2004). Curcumin has been recognised to express anti-inflammatory properties (Bachmeier et al, 2008; Takahashi et al, 2007), being a free radical scavenger and hydrogen donor with both anti- and pro-oxidant activity, respectively. It also binds metals, particularly iron and copper (Hatcher et al, 2008). Moreover, Cu(II)-curcumin complexes possesses SOD activity, free radical neutralizing ability and antioxidant potential (Barik et al, 2005). Copper-tobramycin actions are therefore similar to those of curcumin, but copper-tobramycin is believed not to be a hydrogen donor, because it had no effect on extracellular H$_2$O$_2$. Moreover, the pro-inflammatory effects of copper-tobramycin were not observed in this project. Importantly, rescued ΔF508-CFTR in
epithelial cells is less stable than wild-type CFTR (Hoelen et al, 2010). Therefore to maintain the effect of copper-tobramycin on CFTR, regulation of the channel gating by agents, small molecule CFTR potentiators, such as genistein (Hwang et al, 1997) or capsaicin (Ai et al, 2004) would be required together with the copper-tobramycin complex administered parenterally.

Copper-tobramycin was shown to displace NE from its sites of encryption, making it more susceptible for inhibition by α1-AT, which could be derived from activated neutrophils or the serum in vitro and in vivo. Additionally, α1-AT secretion is believed not to be impaired in CF and functions normally (Cantin et al, 1992), but another report revealed that α1-AT was partly oxidized and could be proteolytically inactivated (Cantin & Bilodeau, 1989). Copper-tobramycin potentially protects α1-AT from proteolysis and ROS-mediated degradation. These actions of copper-tobramycin may improve the protease/antiprotease imbalance observed in CF patients (Birrer et al, 1994). Several studies indicate a promising role of α1-AT inhalation therapy, reducing airway inflammation in patients with CF (Griese et al, 2007, McElvaney et al, 1991). Therefore, inhaled α1-AT could be used in a combination with inhaled copper-tobramycin therapy as an important improvement of CF therapy.

To summarise, since there is no satisfactory anti-inflammatory therapy for lung disease in CF, tobramycin beyond being an effective antibiotic, might fill the niche of anti-inflammatory treatment in CF. It exerts further multiple roles, including anti-oxidant, anti-inflammatory and possibly anti-mucolytic due to binding with copper. Importantly, tobramycin may increase CFTR protein in vivo as observed in cell culture experiments. Intravenous tobramycin could therefore serve as a pro-drug for copper-tobramycin, together with copper bracelets to increase the pool of available endogenous copper. Alternatively, pre-formed copper-tobramycin might be administered intravenously or via inhalation; however it should be closely monitored for possible toxic effects.
APPENDIX

Partial dissociation of the complex “MLH” to M, LH, L’ and H⁺

M – metal, LH – weakly acidic ligand

\[
\text{MLH} \leftrightarrow \text{M} + \text{LH} \quad \text{K}_{D1} = \frac{[\text{M}][\text{LH}]}{[\text{MLH}]} \tag{1}
\]

\[
\text{MLH} \leftrightarrow \text{ML}^- + \text{H}^+ \quad \text{K}_{a1} = \frac{[\text{ML}^-][\text{H}^+]}{[\text{MLH}]} \tag{2}
\]

\[
\text{ML}^- \leftrightarrow \text{M} + \text{L}^- \quad \text{K}_{D2} = \frac{[\text{M}][\text{L}^-]}{[\text{ML}^-]} \tag{3}
\]

\[
\text{LH} \leftrightarrow \text{L}^- + \text{H}^+ \quad \text{K}_{a2} = \frac{[\text{L}^-][\text{H}^+]}{[\text{LH}]} \tag{4}
\]

First calculation: Reactions 2 and 3 are ignored. The aim is to estimate the concentration of undisassociated complex in a solution prepared from a pure sample, allowing for partial dissociation to free metal and partly ionised ligand:

\[
\text{MLH} \leftrightarrow \text{M} + \text{LH} \quad \text{K}_{D1} = \frac{[\text{M}][\text{LH}]}{[\text{MLH}]} \tag{1}
\]

\[
\text{LH} \leftrightarrow \text{L}^- + \text{H}^+ \quad \text{K}_{a2} = \frac{[\text{L}^-][\text{H}^+]}{[\text{LH}]} \tag{4}
\]

Second calculation: All four steps are included, but simplified:

- Both MLH and ML⁻ have the same molar absorbance, \( \varepsilon_{\text{complex}} \)
- \( K_{a1} = K_{a2} \), denoted \( K_a \) and \( K_{D1} = K_{D2} \), denoted \( K_D \)

The same calculation would apply if the ligand was protonated from a weak base, i.e:

\[
\text{MLH}^+ \leftrightarrow \text{M} + \text{LH}^+ \quad (1a)
\]

\[
\text{LH}^+ \leftrightarrow \text{L} + \text{H}^+ \quad (4a)
\]

Let the prepared concentration (molar) of MLH be “c”

Let the equilibrium concentration of M be “y”

\( \therefore \) the equilibrium concentration of MLH is “c-y”

Let the equilibrium concentration of H⁺ be “x”

\( \therefore \) the equilibrium concentration of L⁻ is “x” and the equilibrium concentration of LH is “y-x”
\[ K_D = \frac{y(y-x)}{c-y} \quad (5) \quad K_a = \frac{x^2}{y-x} \quad (6) \]

Rearrange to: \( y = \frac{x^2}{K_a} + x \) and substitute \( y \) in (5)

\[ K_D = \frac{y(y-x)}{c-y} = \frac{x^2}{K_a} + x = \frac{x^2}{K_a} + \frac{x^2}{K_a} \]

Multiply out: \( K_a K_D c - K_D x^2 - K_a K_D x = \frac{x^4}{K_a} + x^3 \)

Rearrange to: \( \frac{1}{K_a} x^4 + x^3 + K_D x^2 + K_a K_D x = K_a K_D c \)

Factorise: \( x^2 \left( \frac{x^2}{K_a} + K_D \right) + x(x^2 + K_a K_D) = K_a K_D c \)

\[ \therefore x^2 \left( \frac{x^2}{K_a} + K_D \right) + xK_a \left( \frac{x^2}{K_D} + K_D \right) = K_a K_D c \]

\( \left( \frac{x^2}{K_a} + K_D \right)(x^2 + xK_a) = K_a K_D c \quad (8) \)

If we have values of \( K_a \) and \( K_D \), then the equation can be solved (in principle) for any chosen value \( c \) to give \( x \), and then \( y \) is obtained by substituting back. Polynominial equations were here solved by making reasonable approximations:

For example:

\( K_a = 1 \times 10^{-6} \) M \((pK_a = 6.0)\)

\( K_D = 1 \times 10^{-4} \) M

\( c = 6.25 \) mM = 0.00625 M

\( \left( \frac{x^2}{K_A} + K_D \right)(x^2 + xK_a) = K_a K_D c \quad (8) \)

\( \left( \frac{x^2}{10^{-6}} + 10^{-4} \right)(x^2 + 10^{-6} x) = 10^{-4} \times 10^{-6} \times 0.00625 \)

\( (10^6 x^2 + 10^{-4})(x^2 + 10^{-6} x) = 6.25 \times 10^{-13} \)

\( 10^6 x^2 \gg 10^{-4} \) and \( x^2 \gg 10^{-6} \quad \therefore \quad x \gg 10^{-6} \)

\( 10^6 x^4 = 6.25 \times 10^{-13} \)

\( x^4 = 6.25 \times 10^{-19} \)

\( x^2 = 7.89 \times 10^{-10} \)

\( x = 2.81 \times 10^{-5} \) M
$y$ is calculated from the ionization of LH:

$$K_a = \frac{x^2}{y-x} \quad (6)$$

Rearranged to:

$$y = \frac{x^2}{K_a} + x$$

$$y = \left(\frac{2.81 \times 10^{-5}}{10^{-6}}\right)^2 + 2.81 \times 10^{-5} = 8.1771 \times 10^{-4} \text{ M}$$

The percentage reduction in the concentration of MLH, and hence in the absorbance of the solution:

$$\frac{y}{c} \times 100 \% = \frac{8.1771 \times 10^{-4}}{6.25 \times 10^{-3}} \times 100 \% = 13.1 \%$$

$$[ML] = 6.25 \times 10^{-3} \times \frac{86.9}{100} = 5.43 \times 10^{-3} \text{ M}$$

Correction for $\varepsilon$ is:

$$\frac{6.25 \times 10^{-3}}{5.43 \times 10^{-3}} = 1.1574 \text{ M}^{-1}\text{cm}^{-1}$$

Corrected $\varepsilon$ is $1062 \times 1.157 = 1229 \text{ M}^{-1}\text{cm}^{-1}$
Calculating dissociation constant for the copper-tobramycin complex

\[ [M_0] = 0.78125 \times 10^{-3} \text{ M} \]
\[ [T]_T = 0.78125 \times 10^{-3} \text{ M} \]
\[ A_0 = 0.024151 \text{ (0.78125 mM CuSO}_4 + 0 \text{ mM Tob)} \]
\[ A_T = 0.38129 \text{ (0.78125 mM CuSO}_4 + 0.78125 \text{ mM Tob)} \]
\[ \varepsilon_{250} = 1229 \text{ M}^{-1} \text{ cm}^{-1} \]

\[ A_{\text{CuSO}_4 + \text{Tob}} = A_{\text{CuSO}_4} + A_{\text{CuT}} \]
\[ A_T = A_M + A_{ML} \]
\[ A_{ML} = A_T - A_0 = 0.38129 - 0.024151 = 0.357139 \]

\[ [ML]^\prime = \frac{A_T - A_0}{\varepsilon_{ML}} = \frac{0.357139}{1229 \times 0.5} = 0.58119 \times 10^{-3} \text{ M} \]

\[ [M]^\prime = [M]_0 - [ML]^\prime = 0.78125 \times 10^{-3} - 0.58119 \times 10^{-3} = 0.20006 \times 10^{-3} \text{ M} \]

\[ A_{AL} = A_0 \times \frac{[M]}{[M_0]} = 0.024151 \times \frac{0.20006}{0.78125} = 0.0061845 \text{ (adjusted baseline)} \]

\[ [ML] = \frac{A_T - A_0}{\varepsilon_{ML}} = \frac{0.38129 - 0.0061845}{1229 \times 0.5} = 0.610424 \times 10^{-3} \text{ M} \]

\[ [M] = [M_0] - [ML]^\prime = 0.78125 \times 10^{-3} - 0.610424 \times 10^{-3} = 0.170826 \times 10^{-3} \text{ M} \]
\[ [L] = [T]_T - [ML] = 0.78125 \times 10^{-3} - 0.610424 \times 10^{-3} = 0.170826 \times 10^{-3} \text{ M} \]

\[ K_D = \frac{[M][L]}{[ML]} = \frac{0.170826 \times 10^{-3} \times 0.170826 \times 10^{-3}}{0.610424 \times 10^{-3}} = 0.048 \text{ mM} \]

\[ pK_D = - \log K_D \]

\[ pK_D = 4.301 \]


Bergmeier W, Wagner DD; Michelson AD, 2007. Inflammation - Chapter 39; Platelets. 713-726.


Cai TQ, Wright SD, 1996. Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. *J Exp Med* 184, 4, 1213-1223.


Cystic Fibrosis Mutation Database: www.genet.sickkids.on.ca/cftr/


Daniel KG, Chen D, Yan B, Dou QP, 2007. Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer. *Front Biosci* 12, 135-144.


copper(II) complex with the unusual aminoglycoside antibiotic hygromycin B. *Dalton Trans* 39, 41, 9830-9837.


Lin SJ, Culotta VC, 1995. The ATX1 gene of Saccharomyces cerevisiae encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc Natl Acad Sci USA* 92, 9, 3784-3788.


McPhail LC, Snyderman R, 1983. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. Evidence that the same oxidase is activated by different transductional mechanisms. *J Clin Invest* 72, 1, 192-200.


Onnheim K, Bylund J, Boulay F, Dahlgren C, Forsman H, 2008. Tumour necrosis factor (TNF)-a primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular granule mobilization. Immunol 125, 591-600.


Szczepeanik W, Kaczmarek P, Jezowska-Bojczuk M, 2004b. Oxidative activity of copper(II)complex with the aminoglycoside antibiotics as implication to the toxicity of these drugs. *Biorg Chem Applicat* 2, 1-2, 55-68.

Szczepeanik W, Swiatek M, Skała J, Jezowska-Bojczuk M, 2004c. ATP, histidine or magnesium ions can protect DNA against sisomicin-induced damage, following stray Cu(II) binding. *Arch Biochem Biophys* 431, 1, 88-94.


