Probing the Role of Organic Cation Transporters in Respiratory Epithelial Cell Proliferation *in vitro*

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Abstract

Introduction: The expression profile of plasma membrane organic cation transporters OCT/Ns has been widely investigated in a variety of cell types including hepatocytes, enterocytes, and renal cells. Their functional characteristics in terms of translocation of a wide range of positively charged compounds have been widely examined as well. Recently, the focus has been on their role in the transportation of inhaled drugs through respiratory epithelial cells. The airway epithelium is considered as a protective barrier against inhaled particulates and infectious agents that may cause damage to epithelial cells resulting in the permeation of harmful agents to the underlying tissue. Therefore, restoration of the lost epithelial cells is essential for maintaining epithelial functions. It has been suggested that OCTs, and particularly OCT1, mediate the release of non-neuronal acetylcholine from the cytosol of airway epithelial cells to the extracellular space where it acts as an auto/paracrine molecule. Furthermore, the OCTN2 gene has been associated with respiratory airway diseases such as asthma. In the current study, we aimed to investigate whether the OCT1 protein plays a role in respiratory wound healing via the transportation of non-neuronal acetylcholine. This has indeed been suggested to play a role in the stimulation of cell proliferation through activation of either muscarinic or nicotinic receptors.

Methods: The alveolar epithelial cell line A549 was used as a model of the airway epithelium as it had been reported to express cholinergic components such as acetylcholine, muscarinic and nicotinic receptors. In a first instance, OCT/Ns expression in the cell line was probed at pre and post transcriptional levels using normal polymerase chain reaction (PCR), quantitative PCR (qPCR) and In Cell Western™ ICW, respectively. The intestinal cell line Caco-2 and the broncho-epithelial cell line Calu-3 were used as positive controls. A scratch wound model was then establised to assess the role of OCT/Ns in epithelial repair following injury. Initially, the healing of wounded A549 monolayers was assessed in presence of a panel of OCT/Ns inhibitors using the PrestoBlue™ cell proliferation assay. In the second stage of the study, the OCT1 gene was transiently silenced using short interfering RNAs (siRNAs) and the proliferation of knocked down cells was monitored using the PrestoBlue and Cyquant® cell proliferation assays.

Results: Traditional PCR showed that OCT1, OCT3, OCTN1 and OCTN2 were expressed in A549 cells, whereas OCT2 was absent. The levels of the OCT1 and OCTN2 proteins were then relatively quantified in these cells using ICW. Some of the OCT/Ns inhibitors employed exhibited proliferation inhibitory effects on unscratched monolayers, while the bronchodilator ipratropium decreased cell proliferation at high concentration without causing toxic effects.

However, it was difficult to determine whether OCT/Ns were likely to be involved in wound repair because the drug exhibits other properties than only an inhibitory effect on these transporters. Following optimisation of the siRNA transfection protocol, the OCT1 gene expression was ultimately decreased to about 60% compared to control cells. Nevertheless, OCT1 knockdown had no effect on cell proliferation post injury as monitored using the PrestoBlue and Cyquant® assays up to 48 hrs post transfection.

Conclusions: Decreased levels of the OCT1 protein to about 60% of control did not affect cell proliferation in the A549 cell line. This might be because the protein still exhibited normal functions in terms of translocation of non-neuronal acetylcholine despite a lower expression. A549 cell proliferation may also be governed by molecules present in the cell culture medium such as growth factors rather than by non-neuronal acetylcholine.

Publications and presentations

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Table of content

Abstract ..... *i*

Publications and presentations ..... *iv*

Acknowledgements ..... *v*

Table of contentList of Abbreviations ..... *vii*

List of Abbreviations ..... *xiv*

List of figures ..... *xviii*

List of tables ..... *xxiii*

Chapter 1 General introduction *1*

1.1 Anatomy of the human respiratory system **2**

1.2 The airway epithelium **4**

1.2.1 Tracheo-bronchial and bronchiolar epithelia 4

1.2.1.1 Ciliated cells 4

1.2.1.2 Goblet cells (mucus producing cells) 5

1.2.1.3 Basal cells 5

1.2.1.4 Serous cells 6

1.2.1.5 Intermediate cells 6

1.2.1.6 Pulmonary neuroendocrine cells (PNEC) 6

1.2.1.7 Club cells 7

1.2.2 Alveolar epithelium 9

1.2.2.1 Pneumocytes I or alveolar epithelium type 1 9

1.2.2.2 Pneumocytes II or alveolar epithelium 2 10

1.3 Functions of the airway epithelium 10

1.4 Role of the bronchial epithelium in asthma and COPD 13

1.5 Mechanisms involved in airway remodelling and repair of respiratory epithelium wounds 16

1.6 Acetylcholine and cholinoceptors in the lung 20

1.6.1 Neuronal origin (parasympathetic system) 21

1.6.2 Non-neuronal origin (respiratory epithelium) 24

1.6.3 Expression of muscarinic and nicotinic receptors in the respiratory tissue 26

1.7 Organic cation transporters OCTs 31

1.7.1 Structure and morphology of the OCTs 32

1.7.2 Regulation of the organic cation transporters 33

1.7.3 Expression of OCTs in the lung 35

1.7.4 OCTs roles in the respiratory epithelium 40

1.8 Aims of the thesis 43

Chapter 2 Expression of SLC22A1-5 genes in the alveolar adenocarcinoma cell line A549 46

2.1 Introduction 46

2.1.1 Respiratory epithelial cell lines 46

2.1.2 Expression of *SLC22A1-5* in respiratory cell lines 49

2.1.3 Muscarinic and nicotinic receptor expression in respiratory epithelial cell lines 51

2.2 Materials and methods.....54

2.2.1 Cell culture maintenance 54

2.2.2 Testing of different types of serum 56

2.2.3 Cell culture conditions..... 56

2.2.4 Trans-epithelial electrical resistance measurement for Calu-3 and Caco-2 cell lines 58

2.2.5 Gene expression 59

2.2.5.1 RNA extraction 59

2.2.5.2 Reverse Transcription polymerase chain reaction RT-PCR…….. 61

2.2.5.3 Purification of the PCR products..... 65

2.2.5.4 Sequencing of DNA 66

2.2.5.5 Validation of the primers for quantitative real time polymerase chain reaction qRT-PCR 67

2.2.6 Protein quantification by In-Cell Western™ Assay..... 73

2.2.6.1 In Cell Western™ assays 73

2.2.6.2 Primary and secondary antibodies employed..... 75

2.2.6.3 Visualization options 76

2.2.6.4 Analysis of data..... 76

2.2.6.5 Validation of primary antibody dilutions..... 77

2.2.6.6 *SLC22A1* and *A5* protein expression in A549 cell line ……. 81

2.2.7 Statistics 82

2.3 Results 82

2.3.1 Effect of the serum on Calu-3 and Caco-2 cell growth .. 82

2.3.2 Semi-quantitative PCR 84

2.3.3 Sequencing 86

2.3.4 Quantitative polymerase chain reaction 88

2.3.5 OCT1 and OCTN2 protein expression in A549 cells 89

2.4 Discussion 90

2.4.1 Effect of the serum on Calu-3 and Caco-2 growth 90

2.4.2 Expression of mRNA 92

2.4.3 Protein expression 94

2.5 Conclusion 95

Chapter 3 Impact of Organic Cation Transporter Inhibitors on cell proliferation 96

3.1 Introduction 96

3.2 Materials and methods 103

3.2.1 Validation of cell proliferation and viability assay PrestoBlue 103

3.2.2 Wounding assay on monolayers 105

3.2.3 Time lapse microscopy 106

3.2.4 Organic cation transporters inhibitors 106

3.2.4.1 Tetraethylammonium TEA 106

3.2.4.2 Quinine 108

3.2.4.3 Corticosterone 109

3.2.4.4 Salbutamol 110

3.2.4.5 Ipratropium 112

3.3 Results 114

3.3.1 Tetraethylammonium 114

3.3.2 Quinine 118

3.3.3 Corticosterone 119

3.3.4 Salbutamol 120

3.3.5 Ipratropium 122

3.4 Discussion 123

3.4.1 The effect of TEA 124

3.4.2 Quinine impact 127

3.4.3 The influence of Corticosterone 128

3.4.4 The impact of salbutamol 130

3.4.5 Ipratropium effect 132

3.4.6 Conclusion 134

Chapter 4 Knock down of SLC22A1 and A5 genes via small interfering RNA 135

4.1 Introduction 135

4.2 Materials and methods 139

4.2.1 Materials 139

4.2.2 Transient knockdown protocols for N-TER peptide/ Fugene HD 141

4.2.3 Transient knockdown protocol for INTERFERin® 142

4.2.4 Measurement of *SLC22A1* and *A5* mRNA levels by qPCR 143

4.2.5 Measurements of OCT1 protein levels by ICW 144

4.3 Validation of the CyQUANT® NF cell proliferation assay 145

4.3.1 Cell proliferation assays on transfected A549 cells 146

4.4 Results 147

4.4.1 *SLC22A1* and 5 mRNA level in A549 cells transfected with N-TER reagent 147

4.4.2 *SLC22A1*mRNA level in A549 cells transfected with N-TER and Fugene transfection reagents 148

4.4.3 *SLC22A1*mRNA level in A549 cells transfected with INTERFERin transfection reagent 148

4.4.4 Protein expression in transfected cells 151

4.4.5 Cell proliferation assay CyQUANT® NF 154

4.4.6 PrestoBlue cell proliferation assay 154

4.5 Discussion 155

4.6 Conclusion 160

Chapter 5 General discussion 161

5.1 Introduction and proposed hypothesis 161

5.2 Synopsis of findings 162

5.3 Selection of the cell culture model 163

5.4 Efficiency of using PrestoBlue reagent to determine cell viability and proliferation/wound healing.....165

5.5 Influence of transient knock down of OCT1 gene on cell proliferation.....168

5.6 Future work 170

5.7 Conclusion 172

References.....173

List of Abbreviations

Ach	Acetylcholine
AchE	Acetylcholine esterase
AE1	Alveolar epithelium type 1
AE2	Alveolar epithelium 2
AFU	Arbitrary fluorescent units
ALI	Air-liquid interface
ASP^+	4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BMSC	Bone marrow-derived stem cell
BSA	Bovine serum Albumin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CC10	Club cell 10-kD protein
ChAT	Choline acetyltransferase
CHL-1	Choline high like transporter-1
CHO	Chines hamster ovary
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CT	Computed tomography
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptors
EMT	Extraneuronal monoamine transporter
EMTU	Epithelial-mesenchymal trophic unit
ERK	Extracellular regulated kinase
EVOM	Epithelial voltohmmeter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOI	Gene of interest
GPCRs	G-protein couple receptors
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney293
hEMT	Human extraneuronal monoamine transporter
HGF	Hepatocyte growth factor
HKG	Housekeeping gene
HNF-4α	Hepatocyte nuclear factor-4 alpha
HRPE	Human retinal pigment epithelial
ICC	Immunocytochemistry
ICW	In Cell Western™
IGFs	Insulin-like growth factors
KGF	Keratinocyte growth factor
LTB4	leukotriene B4
M1-M5	Muscarinic receptors
MAPK	Mitogen-activated protein kinase

MDCK	Madin-Darby Canine Kidney
MMPs	Metalloproteinases
nAChR	Nicotinic acetylcholine receptors
NF-KB	Nuclear factor kB
NHBE	Normal human bronchial epithelial
OCTs	Organic cation transporters
PB	PrestoBlue
PCR	Polymerase chain reaction
PDGFs	Platelet derived growth factors
PFD	Paraformaldehyde
PGE2	Prostaglandins
PI3K	Phosphatidylinositol 3- kinase
PK	Protein kinases
PNEC	Pulmonary neuroendocrine cells
PPAR α	Peroxisome proliferator-activated receptors α
qPCR	Quantitative polymerase chain reaction
RISC	RAN-induced silencing complexes
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinase
SCLC	Small cell lung cancer
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLC22A	Solute link carrier family
TEA	Tetraethylammonium

TEER	Transepithelial electrical resistance
TGF-α	Transforming growth factor alpha
TGF-β1	Transforming growth factor
TJs	Tight junctions
TMD	Transmembrane domains
VAChT	Vesicular acetylcholine transporters
VEGFs	Vascular endothelial growth factors
ZO-1	Zonula occludens 1 protein
ΔCt	Delta cycle threshold

List of figures

Figure 1.1: Division of the human tracheobronchial tree into the conducting and respiratory zone. 3

Figure 1.2: Schematic of the tracheo-bronchiolar and bronchial epithelia. 9

Figure 1.3: Schematic illustrating the adhesion molecules, tight junction, desmosome and adhesive belt in epithelia 13

Figure 1.4: Schematic showing all steps of Ach synthesis and release from the neuron terminal. 23

Figure 1.5: Schematic illustrating all steps involved in the synthesis, release and degradation of non-neuronal Ach. 26

Figure 1.6: The imaginary structure of the organic cation transporters. 33

Figure 1.7: Schematic illustrating the role of organic cation transporters in the respiratory epithelium. 42

Figure 1.8: Schematic presenting the plan of the thesis manuscript 45

Figure 2.1: Air liquid interface conditions for Calu-3 cells. 58

Figure 2.2: Gel electrophoresis of the total RNA. 61

Figure 2.3: Thermal profile, amplification plots, standard curves, and melting curves for 3 genes. 70

Figure 2.4: Amplification plots, standard curves, and melting curves for 2 genes 72

Figure 2.5: ICW assay principles. 75

Figure 2.6: Validation of the primary antibody concentration for OCT1 79

Figure 2.7: Fluorescence intensity as a function of OCT1 primary Abs dilutions. 80

Figure 2.8: Validation of primary antibody concentration for OCTN2 transporter. 80

Figure 2.9: Fluorescence intensity as a function of OCTN2 primary Ab dilutions. 81

Figure 2.10: TEER values for Calu-3 layers cultured on Transwell® supports in presence of two types of serum. 83

Figure 2.11: TEER values for Caco-2 layers cultured on Transwell® supports in presence of two types of serum. 84

Figure 2.12: PCR gel electrophoresis for amplified products of six genes for Caco-2 cell line cultured with two different types of sera and Calu-3 cells cultured with the Sigma serum. 85

Figure 2.13: PCR electrophoresis gel for amplified products of six genes for A549 cells cultured with three different types of serum. 86

Figure 2.14: Identity between sequence of amplicons and the original gene sequence. 87

Figure 2.15: Expression level of SLC22A1 (A) and SLC22A5 (B) mRNA in A549 and Calu-3 cells as measured by qPCR. 88

Figure 2.16: ICW scan images of A549 cultured in Sigma serum batches 1 and 2. 89

Figure 2.17: percentage of OCT1 and N2 protein expression above the background level in A549 cell line cultured with the two batches of Sigma serum. 90

Figure 3.1: Linear regression between A549 cell number and arbitrary fluorescent units. 104

Figure 3.2: Chemical structure of tetraethylammonium ($C_8H_{20}N+$) 108

Figure 3.3: Chemical structure of quinine ($C_{20}H_{24}N_2O_2$) 109

Figure 3.4: Chemical structure of corticosterone ($C_{21}H_{30}O_4$) 110

Figure 3.5: Chemical structure of salbutamol ($C_{13}H_{21}NO_3$)..... 112

Figure 3.6: Chemical structure of ipratropium bromide ($C_{20}H_{30}BrNO_3$) 114

Figure 3.7: Effect of tetraethylammonium (TEA) on cell proliferation in A549 wounded monolayers. 115

Figure 3.8: Effect of tetraethylammonium in cell viability of A549 confluent unwounded monolayers. 116

Figure 3.9: Wound repair in control and TEA treated scratched A549 monolayers. 117

Figure 3.10: Effect of quinine on cell proliferation in A549 wounded monolayers..... 118

Figure 3.11: Effect of quinine on cell viability in A549 unwounded monolayers. 119

Figure 3.12: Effect of corticosterone on cell proliferation in A549 wounded monolayers..... 120

Figure 3.13: Effect of corticosterone on cell viability in A549 unwounded monolayers. 120

Figure 3.14: Effect of salbutamol on cell proliferation in A549 wounded monolayers..... 121

Figure 3.15: Effect of salbutamol on cell viability in A549 unwounded monolayers. 121

Figure 3.16: Effect of ipratropium bromide on cell proliferation in A549 wounded monolayers. 122

Figure 3.17: Effect of ipratropium bormide on cell vaibility in A549 unwounded monolayers. 123

Figure 4.1: Schematic illustrating the processing of synthetic double strand RNA. 139

Figure 4.2: Standard curve of Cyquant cell proliferation assay in A549 cells. 146

Figure 4.3: SLC22A1 and A5 mRNA levels in A549 cells transfected with N-TER reagent at 20 nM siRNA. 147

Figure 4.4: SLC22A1 mRNA level in A549 cells transfected with (A) N-TER and (B) FuGENE ® HD reagent at 20 nM siRNA. 148

Figure 4.5: SLC22A1 mRNA level in A549 cells transfected with INTERFERin® at 1 nM siRNA. 150

Figure 4.6: SLC22A1 level in A549 cells transfected with INTERFERin® at 5 nM siRNA. 150

Figure 4.7: SLC22A1 mRNA level in A549 cells transfected with INTERFERin® at 20 nM siRNA..... 151

Figure 4.8: Percentage of OCT1 protein expression in A549 cells transfected with 1nM siRNA. 152

Figure 4.9: Percentage of OCT1 protein expression in A549 cells transfected with 5 nM siRNA. 153

Figure 4.10: Percentage of OCT1 protein expression in A549 cells transfected with 2 0nM siRNA. 153

Figure 4.11: Cell proliferation assay on A549 cells transfected with SLC22A1 siRNA at 20 nM. 154

Figure 4.12: Cell proliferation assay on A549 cells transfected with SLC22A1 siRNA-A and B at 20 nM. 155

List of tables

Table 1.1: Distribution of cholinoceptors, muscarinic and nicotinic receptors in non-neuronal respiratory cells 31

Table 2.1: OCT/Ns transporter expression in A549 cells using different techniques in published studies and the current study. . 51

Table 2.2: PCR primers details 65

Table 2.3: qPCR primers for SYBR assay. 69

Table 4.1: SLC22A1 and 5 siRNA sequences used with N-TER™ and Fugene® HD reagents 140

Table 4.2: SLC22A1 siRNA sequences used with INTERFERin® reagent 141

Chapter 1 General introduction

Organic cation transporters (OCTs) belong to the solute link carrier family which are classified into subtypes, OCT1-3, and OCTN1 and 2 [1-3]. These transporters are membrane proteins and are ubiquitously distributed in different cellular types such as enterocytes, hepatocytes, renal cells, neurons, skeletal muscle cells, and epithelial cells, including the airway epithelium [4-8]. These transporters translocate a variety of drugs and xenobiotics substances across the cell membrane and eventually contribute to their elimination via secretory and excretory organs, providing a protective and balanced condition [9].

Many investigations have emphasized on probing OCT/Ns functions in various organs; however, less attention has been paid on their functions in the airway epithelium. The respiratory epithelium represents the first physiological barrier which encounters the contents of inhaled air that may be harmful. In airway inflammatory diseases such as asthma, the epithelium is fragile and can be removed partially or completely depending on the severity of the disease, impairing its protective role. Additionally, the denuded area is not effectively compensated as a result of retardation in growth and differentiation of airway epithelial cells. These alterations in the epithelium contribute to the presence of airway wounds [10].

The epithelium is equipped with several mechanisms that participate in repairing the damaged or denuded regions. One of which may involve the synthesis and release of acetylcholine from respiratory epithelial cells via OCTs. It has been suggested that acetylcholine stimulates the migration and proliferation of epithelial cells following binding to the muscarinic and nicotinic cholinergic receptors [11].

The aim of the current study is to screen the role of OCTs in the repair of epithelial injuries that might result from respiratory diseases or from external insults, and to investigate whether the underlying mechanisms involve the non-neuronal acetylcholine produced by epithelial cells.

1.1 Anatomy of the human respiratory system

The respiratory system is divided into two regions: the upper and lower parts (Figure 1.1). The upper part, also known as the conducting region, consists of the nose, part of the pharynx, trachea and the main bronchus. All these parts are responsible for conducting air from the external environment into, the lower part of the pulmonary tract. This part is formed of the bronchioles, and alveoli which are the terminal region of the airways, where gas exchanges occur [12]. The anatomical alterations affect the components of the epithelium as well. The epithelium is thicker in the conducting parts, whilst it converts to a flat or cuboidal layer of

cells in the respiratory zone. Conducting airways are surrounded by a muscular layer which decreases in thickness around alveolar ducts, and completely disappears in the alveolar wall. The bronchi are also surrounded by a connective tissue of cartilage, which lessens towards the deeper parts of the respiratory tract, and cannot be observed any more where the airway diameter falls to approximately 1 millimetre. A lack of cartilage in the deep lung means the bronchioles can be easily constricted, particularly in asthmatic individuals [13].

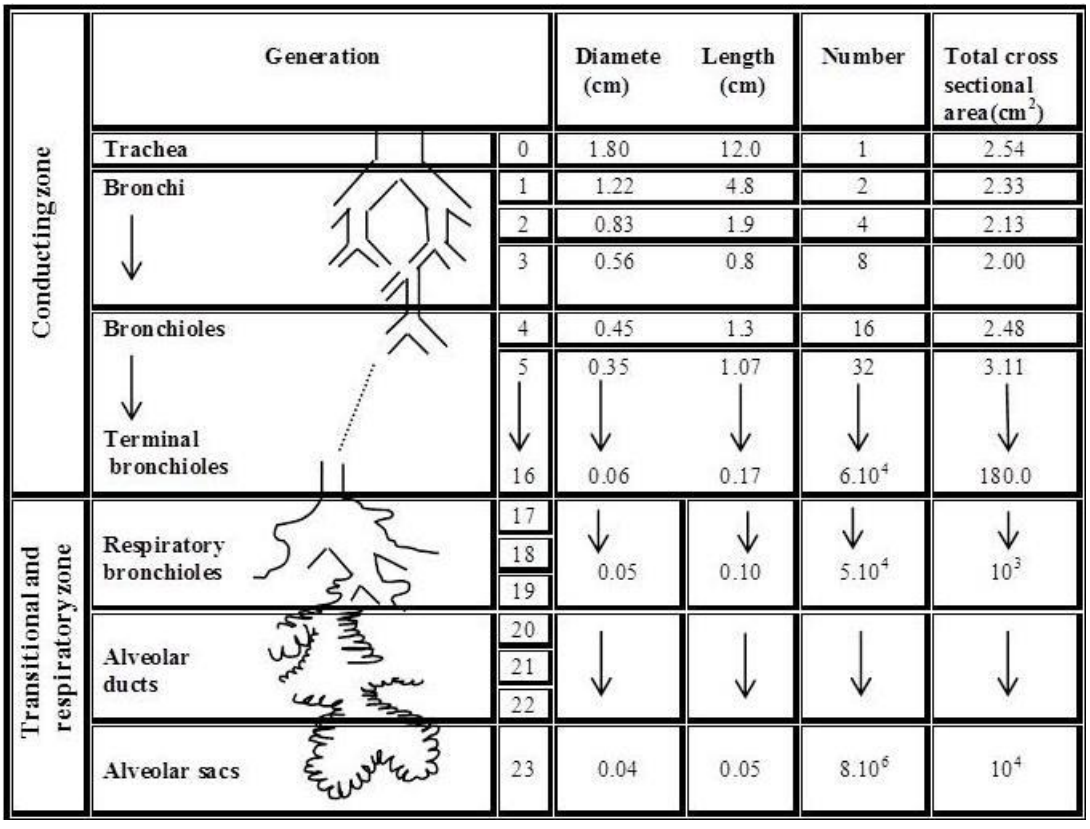

Figure 1.1: Division of the human tracheobronchial tree into the conducting and respiratory zone.

The schematic illustrates the number, length, diameter and total cross sectional area for all parts constituting the tree. Adapted from [12]

1.2 The airway epithelium

The airway epithelium is composed of a diversity of cellular components. These cellular components exhibit different morphological and functional characteristics in the bronchial and alveolar regions.

1.2.1 Tracheo-bronchial and bronchiolar epithelia

The bronchial epithelium is formed of the following cells as shown in Figure 1.2.

1.2.1.1 Ciliated cells

Ciliated cells are highly abundant in the upper conducting passages of the respiratory system. They exhibit a columnar and elongated morphology and present cilia on the luminal surface of the epithelium. These cilia are responsible for removing the mucus that covers the surface of the epithelium. The shape of ciliated cells is converted into a shorter and cuboidal structure in the bronchioles. It is suggested that these cells differentiate from other cell types, such as the goblet and/or basal cells that are lined alongside the ciliated cells [14, 15]. These cells are involved in the mucocilliary clearance which is thought to be controlled by various factors such as histamine and cytokines [16, 17].

1.2.1.2 Goblet cells (mucus producing cells)

The non-ciliated, goblet cells are highly present in the epithelium of the airway ducts in the conducting passages but decrease in number until they are completely replaced by Club cells in the respiratory region [15, 18]. These cells synthesize mucus which is then secreted from their luminal surface, providing a protective mechanism via preventing the entrance of inhaled particulates into the terminal part of the airway ducts [15, 19]. These cells were shown to act as progenitor cells in the rat trachea [20, 21].

1.2.1.3 Basal cells

Basal cells are characterized by a small pyramidal morphology. These cells are located between columnar cells and are lined on the basement membrane which represents the base of the respiratory epithelium. Basal cells are connected to the adjacent cells and the basement membrane by particular molecular structures termed desmosomes and hemi-desmosomes, respectively [22, 23]. It is believed that these structures provide a firm anchorage between the columnar cells, basement membrane and underlying tissue, and that basal cells perform a critical role in the attachment of columnar cells onto the basal membrane [24]. These cells were found to express 15-lipoxygenase enzymes [25], suggesting that they are likely to be involved in inflammation events [26, 27]. It has also been demonstrated that these cells function as progenitor

or stem cells and can differentiate into other epithelial cell types such as goblet (mucus producing) cells, and ciliated cells [15, 28-31]

1.2.1.4 Serous cells

This type of cells has been well identified in the mouse airway epithelium, however, it has also been recognized that serous cells are present in the human respiratory epithelium especially in the bronchioles. Serous cells are responsible for the synthesis of various substances, one of which being mucin [32].

1.2.1.5 Intermediate cells

These cells function as progenitors and can be differentiated into ciliated and goblet cells [15].

1.2.1.6 Pulmonary neuroendocrine cells (PNEC)

Pulmonary neuroendocrine cells, also called Kulchitsky's cells have a small rounded morphology, are based on the basement membrane and rarely protrude to the luminal surface [33]. They are distributed in the different part of the tracheobronchial tree but they are more present in the smaller branches of the bronchi [15]. These cells are characterized by their ability to produce biologically active substances, for example, hormones and peptides which include calcitonin, serotonin, and gastrin [34]. However, the most highly produced substance is gastrin, calcitonin and serotonin

being produced to a lesser extent. PNEC are highly present in the lung during certain stages of embryonic development, notably the period of growing and maturation of the foetus and it has been suggested that these cells play a crucial role in the development of the lung [15]. It has also been demonstrated that these cells are responsible for sensitization to hypoxia as they express oxygen chemoceptors on their membrane [35]. Additional functions of this cell type have been described in a number of studies, and include their ability to renew the respiratory epithelium [36, 37].

1.2.1.7 Club cells

Club cells (formerly called Clara cells) or non-ciliated bronchiolar secretory cells are distributed in the distal or peripheral airway branches in the human respiratory system. These cells contain obvious organelles such as mitochondria and both smooth and rough endoplasmic reticulum [15]. Various substances are produced by these cells, for example, a proteinase inhibitor substance termed antileukprotease ALP [38-40]. These cells have also the ability to detoxify inhaled hydroxylated aromatic substances as they express metabolic enzymes such as P-450 cytochromes [41, 42]. These cells have been shown to synthesize and secret a variety of proteins, one of which was identified as a small molecular weight protein known as Club cell 10-kD protein (CC10) or Club cell secretory protein or secretoglobine family 1A

member 1 (SCGB1A1) [43, 44]. CC10 is considered to be a constitutive protein in the airway surface fluid and it has been identified as a multifunctional protein, playing for instance, a crucial role in the modulation of inflammation and immune responses [44]. In this respect, Hung et al., 2004 proved that CC10 was able to inhibit TH2 cytokine expression and its transcriptional factors, GATA3 in splenocytes were isolated from mice [45]. Additionally, reconstitution of the Club cell 10 gene in CC10-defficient mice was shown to decrease the TH2 cytokine level. In a more recent work, Longe et al., 2012 observed that this protein is involved in the suppression of airway inflammation via inhibition of nuclear factor kB (NF-KB) [46]. A further study reported that CC10 exposed normal human bronchial epithelial (NHBE) cells showed decreased mucin MUC5AC and neutrophils chemotaxis IL-8 production as well as inhibition of phosphorylation of NF-KB and extracellular regulated kinase (ERK)1/2 [47]. It is probable that this protein plays an essential role in the growth and development of the lung in the early period of life as well as airway repair/remodelling [48, 49]. In addition to the above functions, some investigations have confirmed that these cells act as progenitors to other epithelial cell types, particularly ciliated and goblet cells [43, 50-53].

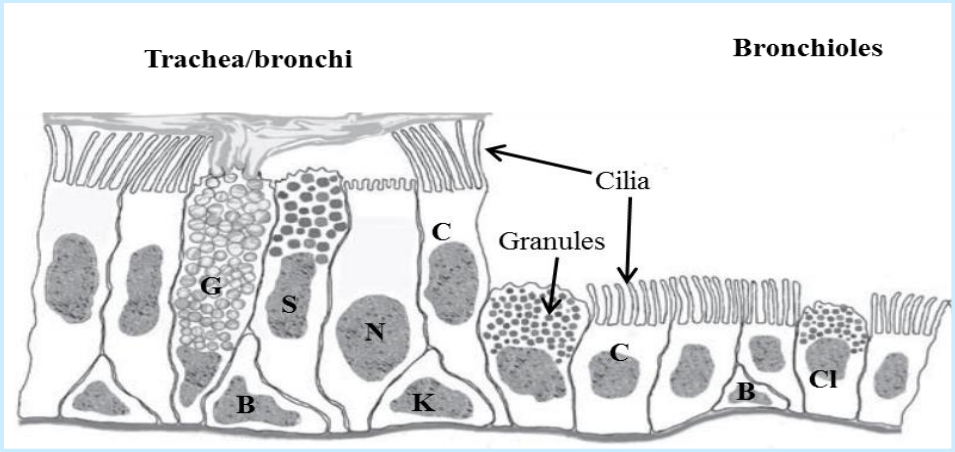

Figure 1.2: Schematic of the tracheo-bronchiolar and bronchial epithelia.

Cells in the tracheo-bronchial epithelium are ciliated (C), goblet (G), secretory (S), neuroendocrine (K), and basal (B). Cells of the bronchiolar epithelium are ciliated (C), basal (B) and Club/Clara (Cl). The image is adapted from [33]

1.2.2 Alveolar epithelium

The alveolar epithelium consists of the following two cell types:

1.2.2.1 Pneumocytes I or alveolar epithelium type 1

Alveolar epithelium type 1 (AE1) cells make up approximately ninety five percent of the alveolar surface and are mainly responsible for gas exchange. These cells contain cytoplasmic plates that are specific for their fundamental function, gas exchange and no organelles, for example, mitochondria have been observed in their cytoplasm [54-57]. Basically, AE type1 originate from AE2 cells. It is thought they may be involved in alveolar wound repair [58].

1.2.2.2 Pneumocytes II or alveolar epithelium 2

The alveolar epithelium 2 (AE2) cells are characterized by a spherical morphology and represent more than fifty percent of the total number of alveolar cells, ten to fifteen of the total lung population, but merely five percent of the alveolar lining surface [56]. They perform multiple vital functions such as production, storage and release of the pulmonary surfactant. This is an essential substance that decreases the surface tension of the alveoli, preventing respiratory collapse during inspiration and expiration. These cells act as progenitors and have the ability to differentiate into AE1 [55, 56, 59]. An additional function of these cells is regulating the movement of water across the alveolar epithelium. It has been shown as well that these cells are involved in remodelling of the alveolar epithelium [56]. They also participate in immunoresponses following airway disturbances by interacting with cytokines and other factors such as growth factors, produced by other types of airway epithelial cells [60].

1.3 Functions of the airway epithelium

The epithelium represents the first barrier that separates the external and the internal environment. The epithelial cells are anchored on the basement membrane, and adhesive molecular structures provide a firm binding between the epithelial cells or between the cells and the basal membrane [61]. Tight junctions

(TJs) connect the apical surface of the columnar cells in a tight manner; desmosomes bind the ciliated/columnar cells with the basal cells, and finally hemi-desmosomes link the basal cells with the underlying tissue (Figure 1.3). Structurally, TJs consist of two different main types of transmembrane proteins known as occludin and claudin both of which composed of four domains [62-64]. TJs are shown to be linked to the cytoskeleton via their basic component occluding with a distinct protein zonula occludin 1, (ZO-1) [62]. In normal individuals, all these adhesive molecular structures provide a firm epithelial layer impermeable to inhaled particulates and regulate the transport of substances across the epithelium or between epithelial cells [61]. Furthermore, the airway epithelium, particularly the bronchial epithelium fulfils a number of functions rather than being just a physical barrier. It is an important absorptive site for inhaled medicines used in the treatment of respiratory diseases such as asthma and chronic obstructive pulmonary diseases [10]. It was previously mentioned that the airway epithelium has the capacity to produce and secrete various substances via different cell types. These substances include cyclooxygenase, lipoxygenase (LO) and monooxygenase which are mainly responsible for eicosanoid synthesis in human airway epithelial cells [65]. Stimulation of cyclooxygenase leads to formation of prostaglandins such as PGE2 and PGI1 that inhibit airway smooth muscle contraction, mucus secretion and nerve

activity [66, 67]. Nitric oxide (NO) is another mediator synthesized by the epithelium and has a dilatation effect on the airways, particularly in asthmatic patients [68, 69].

The airway epithelial cells have the capacity to produce and release a variety of growth factors and pro-inflammatory mediators such as epidermal growth factor EGF, transforming growth factor TGF-β1, platelet-derived growth factors (PDGFs), insulin-like growth factors (IGFs), neurotrophins (e.g.NGF) and vascular endothelial growth factors (VEGFs), cytokines (IL-1, IL-6, and IL-8) and tumour necrosis factor [10, 70-73]. All growth factors were shown to activate fibroblast proliferation and their differentiation into myofibroblasts. The epithelium has also been shown to generate granulocyte macrophage colony-stimulating factor (GM-CSF), particularly in the damaged epithelial cells of asthmatic patients [74, 75].

All these growth factors and mediators generated by the epithelial cell, fibroblasts and immune cells associate in structural alterations that occur during remodelling of the airways.

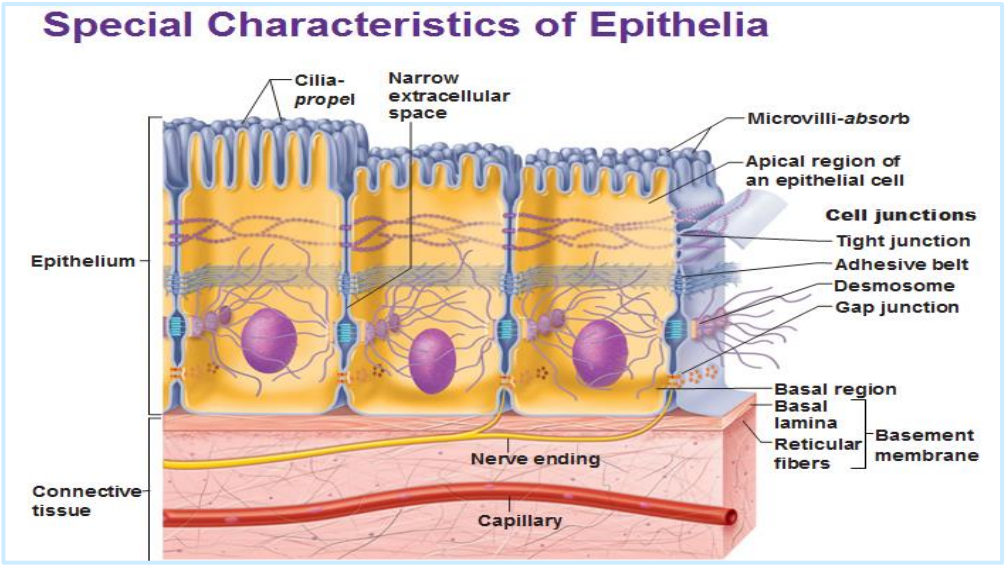

Figure 1.3: Schematic illustrating the adhesion molecules, tight junction, desmosome and adhesive belt in epithelia
Taken from [76]

1.4 Role of the bronchial epithelium in asthma and COPD

Like with other organs, the lungs can be affected by various disturbances or diseases. The most frequent illnesses that impact on the airway epithelium and lung tissue are infectious diseases caused by various agents such as bacteria and viruses, asthma, and chronic obstructive pulmonary disease.

Asthma is defined as a chronic inflammatory disease which mainly targets small airway walls. It causes structural changes in the airway components such as mucus producing cells (Goblet cells), airway smooth muscles as well as underneath the basal membrane. These changes can be described as mucus cells hyperplasia, airway smooth muscles hypertrophy/hyperplasia, and increase in the thickness of the lamina reticularis as a result of

deposition of extracellular matrix (ECM) [77-82]. The main clinical features in asthmatic individuals are bronchoconstriction, and mucus hypersecretion which associated with limitation of the airway passages result in breathing difficulty, wheezing, coughing, and chest pain [83].

It has been shown that the function of the airway epithelium as a protective barrier is impaired in asthmatic patients, which makes it easily penetrated and attacked by various inhaled insults [84]. The fragile epithelium is attributed to a defect in the tight junctions that play an essential role in providing a tight impermeable layer of epithelium in healthy individuals [85, 86]. Accordingly, it has been found that epithelial cells from the bronchial airways were present in sputum, bronchoalveolar lavage, and biopsy of asthmatic patients [19, 87, 88]. In addition to the described characteristic manifestations, interactions between the damaged epithelium and the inhaled insults trigger the production of a range of mediators from the epithelium, fibroblasts, and airway smooth muscles. These mediators are responsible for the recruitment or attract inflammatory cells into the affected airways. The inflammatory cells mainly present in the asthmatic airways are Th2-type or T-lymphocytes (CD4+), eosinophils, mast cells and basophils [81, 84-86]. Infiltrated eosinophils are considered to be a marker of asthmatic patients as well [81]. It was found that these cells

infiltrate from the circulation into the airways of allergen-induced asthmatic patients via eotaxin cytokine [89], and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokine prolongs their presence in the airways of asthmatic patients [71, 84, 90]. GM-CSF is a well-known cytokine principally synthesized and released by bronchial epithelial cells, and to a lesser extent by macrophages, T cells, mast cells and myofibroblasts [75, 91, 92]. In addition to eotaxin, a variety of cytokines play an important role in the attraction of eosinophils to the airway of asthmatic patients [93].These complex interactions between the damaged epithelium, inflammatory cells and the underlying tissue are implicated in abnormal remodelling of the airway epithelium [71, 84, 94].

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the lungs which is characterized by a severe stenosis in the airways of affected individuals. In the early stage of the disease, the epithelium is impaired and no longer tightly connected to the basal membrane [95]. However, in the advanced or progressive stage of COPD, histological and morphological alterations have been described, for example, squamous metaplasia, excessive mucus production, emphysema and fibrosis [81].

In COPD, the recruited inflammatory cells are distinct to those in asthmatic patients, which include T suppressor/cytotoxic

lymphocytes (CD8+), macrophage, and neutrophils [81]. Like asthma, COPD is a complex inflammatory disease as it triggers the synthesis of various cytokines, which in turn, play a crucial role in the remodelling of the airway epithelium [95-97]. All these cellular events are associated with substantial decrease in lung function which eventually may cause death of the patients.

1.5 Mechanisms involved in airway remodelling and repair of respiratory epithelium wounds

It is important to define the remodelling process before explaining the molecular signalling pathways that associate with wound repair. Remodelling is a process which includes alteration in tissue structural components such as their size, mass, and number during normal growth or in response to injury and/or inflammation [81, 98].

In healthy individuals, the respiratory epithelium provides an impermeable physical barrier via different biological and chemical agents. However, as previously described in asthmatic and/or COPD patients, the epithelium is compromised and can be easily penetrated by various inhaled irritants, triggering inflammation responses and abnormal reconstitution of the epithelium and underlying tissue, particularly airway smooth muscle cells and submucosal glands.

The main characteristic manifestations of airway remodelling in asthma and COPD involve structural changes in the subepithelial components which are driven by a range of cytokines and growth factors. The main cytokine involved in these alterations is a transforming growth factor β1 (TGF-β1). It is a profibrotic cytokine mainly synthesized by eosinophils predominantly present in the airways of asthmatic patients, as well as epithelial cells, fibroblasts, and inflammatory cells [71, 99, 100]. TGF-β1 cytokine is involved in remodelling of the airways of asthmatic subjects by increasing the thickness of the reticular lamina via stimulation of the synthesis and deposition of collagen fibres, particularly collagens types I, III, and V and fibronectin [71, 78, 101]. TGF-β1 was not only found to be expressed in epithelial cells and submucosal cells in asthmatic patients but also in those affected with chronic bronchitis [99].

It has also reported that TGF-β1 can modulate the expression of metalloproteinases (MMPs), particularly MMP-2 in airway epithelial cells [102]. The MMP was found to be able to repair wounded epithelial cells *in vitro* whilst MMP inhibitors blocked the repairing. These findings may suggest that TGF-β1 regulate remodelling via controlling MMP expression *in vivo* as well.

Epidermal growth factor (EGF) is another factor which contributes to airway remodelling in asthmatic subjects via stimulation of the

migration and proliferation of epithelial cells. The specific mechanism behind EGF role in healing/remodelling of airways involves an activated interaction between EGF and its receptors in epithelial cells via 3V molecules. The 3V molecules represent an isoform of adhesive molecules CD44 that were found to be highly expressed on the damaged epithelial cells of asthmatic patients [70, 71, 103]

Puddicombe et al., have shown as well that EGF stimulated wound repair in 16HBE14o- scratched cell monolayers via activation of epidermal growth factor receptors (EGFR)[104].

Smooth muscle cell proliferation and/or hypertrophy as well as submucosa enlargement also represent important characteristic features in remodelling of the airways in asthmatic patients. A variety of studies such as computed tomography (CT) scan, radiological, and morphometric have shown that the airway smooth muscle cells and submucosal glands are functionally and morphologically altered in asthmatic patients compared with samples of control group. These changes were found to be present in both peripheral and large airways, particularly in fatal asthma [77, 80, 105-111], whereas they were detected in the peripheral airways of non-fatal asthma [109, 112]. In another separated study, Pepe et al., have shown that airway smooth muscle cells from bronchial biopsy were enlarged in severe asthmatic patients

compared to those with mild asthma [113]. Regarding cellular signalling pathways involved in airway smooth muscle cells proliferation and hypertrophy, p70S6 kinase has been found to be the main effector that led to a mitogenic effect on these cells. However, activation of p70S6k involves cellular signalling pathways that are associated with stimulation of G-protein coupled receptors (GPCRs) via inflammatory and contractile agents such as histamine, thrombin, and carbachol. It has also been found that these agents activated epidermal growth factor mediated proliferation of the human airway smooth muscle cells, this may indicate that receptor tyrosine kinase (RTKs) and GPCRs are involved in activation of p44/p42 mitogen-activated protein kinase (MAPK) [114].

Gosens et al., have also reported that the muscarinic receptor agonist, methacholine activated bovine tracheal smooth muscle cell proliferation in the presence of platelet-derived growth factor (PDGF) through muscarinic receptor, particularly the m3 subtype [115].

Exogenous acetylcholine was shown to stimulate proliferation of primary cultures of human fibroblasts and fibroblast cell lines as well through mitogenic activated protein kinase (MAPK) [116-118]. Proliferation of lung fibroblasts has been described to be implicated in airway remodelling as well.

Overall the process of airways remodelling occur as a result of abnormal interaction between the damaged epithelium and underlying tissue, particularly myofibroblasts which is called the epithelial-mesenchymal trophic unit (EMTU). This interaction was found to be normally responsible for lung development in the early stages of life [71].

Wessler and Kirkpatrick, 2008 suggested that non-neuronal acetylcholine may participate in wound repair/remodelling in the airways since its role in essential cellular functions associated with proliferation, differentiation, mucociliary clearance, secretion of mucus and water, and communication between cells has been demonstrated [11].

1.6 Acetylcholine and cholinoceptors in the lung

Acetylcholine (Ach) is a neurotransmitter synthesized and stored in neurons until it is needed for cellular functions. However, another source of Ach has been found in non-neuronal tissues. This non-neuronal Ach and cholinergic components, receptors and related enzymes have been detected in different tissues, including the respiratory epithelium

1.6.1 Neuronal origin (parasympathetic and sympathetic systems)

The cationic acetylcholine is a well-known neurotransmitter synthesized in neurons, notably in their terminal axons as shown in Figure 1.4. Ach is mainly biosynthesized in the cytosol as a result of choline and acetyl-CoA interaction following the stimulation of choline acetyltransferase (ChAT). The crucial limiting step in the biosynthesis of the acetylcholine molecule is determined by choline transporter-1 (CHL-1) as this transporter is responsible for the reuptake of choline from synaptic cleft into the cytosol where it is reused in a new synthesis cycle [119].

The biosynthesized Ach molecules are transported into small vesicles via vesicular acetylcholine transporters (VAChT), and this process is achieved by exchanging two protons between the vesicle and the cytoplasm [120]. It is estimated that each vesicle contains tens of thousands of Ach molecules which are attached to an intravesicular proteoglycans [121]. These proteoglycans regulate the detachment and release of Ach molecules with adenosine triphosphate (ATP). Following the appropriate stimuli, this will lead to neuronal depolarization. The intravesicular matrix, proteoglycan enables Ach molecules to be released by an exocytosis process and controls the liberated amount of Ach into the synaptic cleft.

The released Ach exerts its action as a result of binding to two main classes of receptors, the muscarinic and nicotinic receptors. Muscarinic receptors are either expressed on the presynaptic membrane or the postsynaptic membrane. Muscarinic receptors are G protein-coupled receptors consisting of 7 transmembrane domains, and five subtypes (M1-M5) have been identified so far [122-124].

The second class of cholinergic receptors is the nicotinic receptors. They are cation channels that allow the passage of positively charged ions through their gates. This type of receptors is widely distributed in the body and it is formed of five identical or homologous subunits. Two different models of nicotinic subunit arrangement, neuronal nicotinic receptors and muscle nicotinic receptors have been identified so far. The neuronal model was found to be formed either of only five alpha subunits or two different subunits (alpha and beta), whereas the muscle model appears to be formed of two α, one β, one δ and one γ or ε-subunit. Ten alpha subunits and four beta subunits have been detected so far and that alpha 2-10 and beta 2-4 subunits have been reported in neuronal type [125-130].

The neurotransmitter, Ach is rapidly degraded by acetylcholine esterase (AchE) into choline and acetic acid; choline is reuptaken

via choline transporters (CHT) which are expressed in the presynaptic membrane for a new cycle of Ach synthesis.

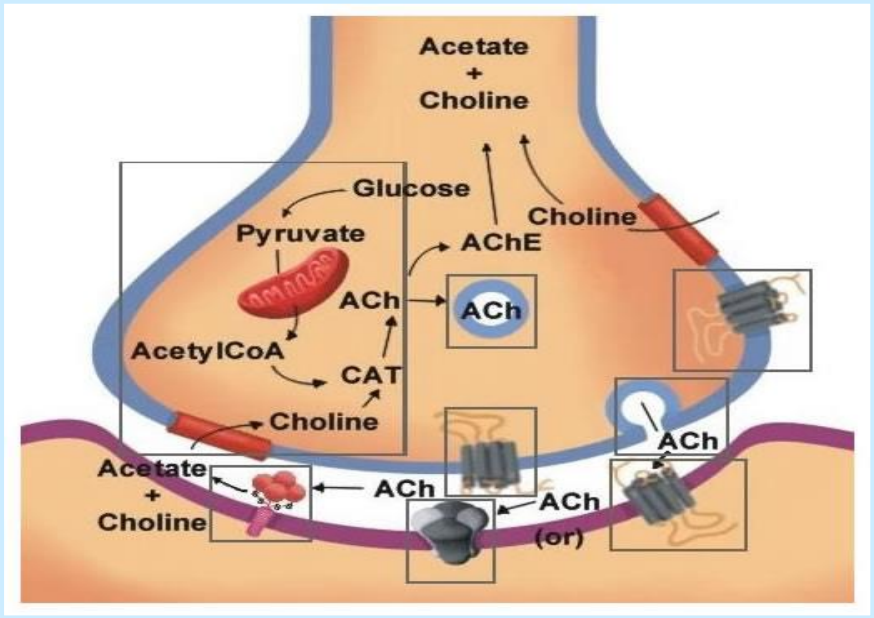

Figure 1.4: Schematic showing all steps of Ach synthesis and release from the neuron terminal.

The blue border is the presynaptic membrane of the terminal axon where Ach is synthesized in the cytosol from acetyl CoA and choline via choline acetyl transferase and, then stored in vesicles until depolarization of neurons. The red colour on the membrane represents high affinity choline transporter which reuptakes it into the cells for re-use in new Ach synthesis. The purple colour represents the postsynaptic membrane. This membrane expresses muscarinic and nicotinic receptors as well as acetylcholine esterase which cleaves Ach into acetate and choline, thus terminating its action. Taken from [131]

The tracheo-bonchial tree is mainly innervated by parasympathetic nerves which represent a main source of neuronal acetylcholine [132]. The airway smooth muscle cells [132, 133], mucus gland [134], and pulmonary vessels [135-137] are innervated with axons of postganglionic cells. The released acetylcholine was shown to be responsible for smooth muscle contraction [138, 139], mucus secretion [140-142], and dilatation of arteries and large veins

[143] in the airways through interaction with muscarinic acetylcholine receptors (mAChRs) [144-146]. All these actions were shown to be mediated via the M3 subtype receptor in different species including humans [142, 147-151]. The lungs are innervated with sympathetic nerves as well, some of which produce acetylcholine [152, 153].

1.6.2 Non-neuronal origin (respiratory epithelium)

In addition to neuronal acetylcholine which is synthesized in the neurons, it has been demonstrated that Ach can be synthesized by different types of organisms, including prokaryotic and eukaryotic cells [11, 154, 155]. The Ach has also been reported to be biosynthesized in different human tissues and organs, notably in epithelial, mesothelial, endothelial, and immune cells, and cholinacetyl transferase and cholinergic receptors have been confirmed to be expressed in those cells as well [155-158].

Numerous studies have found that Ach, muscarinic and nicotinic receptors, and ChAT, acetylcholine esterase enzyme were present in the bronchial epithelium of human and rat, particularly in goblet, ciliated and basal cells [11, 142, 157, 159-162]. Importantly, unlike neuronal Ach, the Ach in bronchial epithelial cells is released from the intracellular environment to the extracellular environment following a mechanism distinct from that of neuronal Ach release. It has been suggested that Ach in the airway epithelium and

placental cells is released into the extracellular space by active transport mediated by organic cation transporters (Figure 1.5) [161, 163, 164]. However, in addition to OCT1-3 involvement in the release of Ach, it is suggested that Ach from airway epithelium can be released by exocytosis as well.

In the airway epithelium, the released Ach acts as an auto and/or paracrine molecule, binding to cholinergic receptors, muscarinic and nicotinic receptors that are expressed on adjacent cells. It has been suggested that acetylcholine originates from bronchial epithelial cells performs essential functions that maintain cell survival such as modulation of cell attachment, particularly by tight junctions and desmosomes, migration, proliferation, differentiation, secretion as well as mucociliary clearance through enhancement of cilia beating frequency [154, 158, 161, 165-168]. The mucociliary clearance mechanism was shown to be mediated by muscarinic receptors expressed on the membrane of ciliated cells, particularly the M3 subtype receptor [166, 169-173].

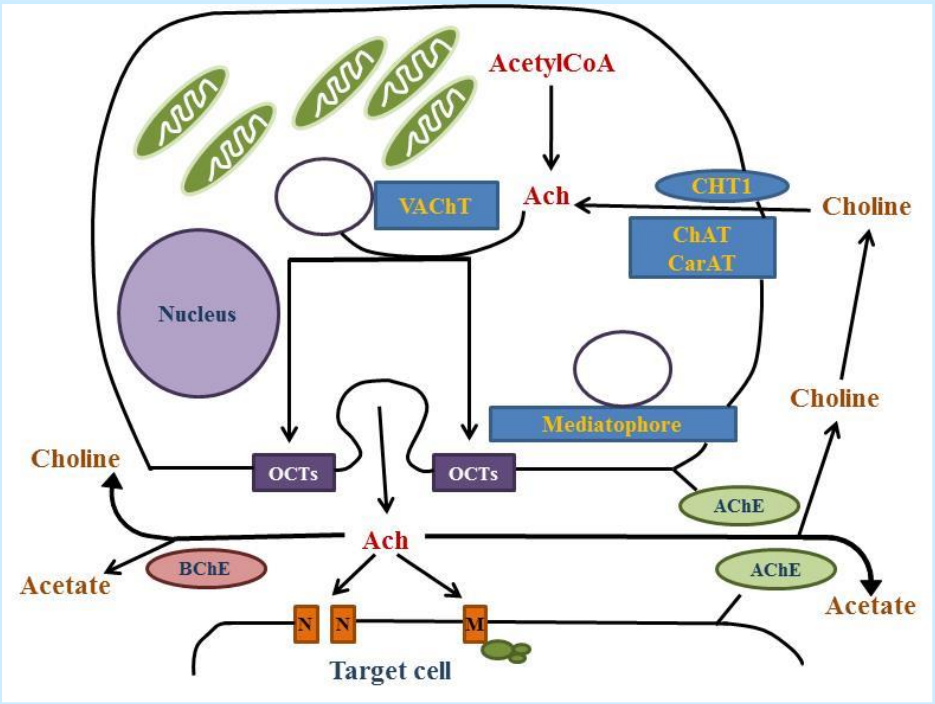

Figure 1.5: Schematic illustrating all steps involved in the synthesis, release and degradation of non-neuronal Ach.

The process of non-neuronal acetylcholine synthesis is similar to that at the neuron terminals but non-neuronal Ach is released by organic cation transporters (OCTs), particularly OCT1 and OCT2. Adapted from [161]

1.6.3 Expression of muscarinic and nicotinic receptors in the respiratory tissue

It is well known that cholinergic receptors and other related elements are abundantly expressed in the central nervous system and neuromuscular junctions. However, it has also been demonstrated that both muscarinic and nicotinic cholinergic receptors are expressed on the plasma membrane of various cellular components of the respiratory tissues; for example, smooth muscle cells, fibroblasts, immunocells and airway epithelial cells, notably ciliated, goblet and basal cells [11, 168, 174-176]. A comparative investigation using pulmonary tissue of human and

guinea pig was conducted to probe muscarinic receptors expression in the respiratory cellular components. By applying an autoradiograph technique, it was found that muscarinic subtype, M3 subtype was the only subtype expressed in smooth muscles that surround the airway ducts in humans, whilst both m2 and m3 subtypes were expressed in the smooth muscles of guinea pigs. However, a highest expression was noted for the m3 subtype. Both M1 and M3 subtypes were identified in the human bronchial submucosa, but a highest expression was reported for M3 in comparison to M1. Regarding expression in the alveolar wall, only the presence of M1 subtype receptor was confirmed in humans while no expression was reported in guinea pigs [145]. Using an alternative technique, northern blot, the expression of M2 and M3 transcripts was demonstrated in human respiratory smooth muscles, whilst only M3 mRNA was identified in the airway epithelium [177]. According to expression investigations, no presence of M4 and M5 was reported in human respiratory tissues, but the m4 subtype was found to be expressed in rabbit alveolar walls and smooth muscle [178].

The mRNA of the three muscarinic subtypes, m1-m3 was found to be expressed in murine lung slices using qPCR assay [151]. By conducting semi-quantitative PCR, Matthiesen et al., have detected the expression of muscarinic receptor subtypes in MCR-5 human

foetal lung fibroblasts and primary lung fibroblasts. Four receptor subtypes were shown to be variably expressed at the mRNA level in MCR-5 with higher expression for the M2 subtype, and no presence for M1. The presence of M2 and 3 subtypes was further confirmed at the protein level by western blot assay. Regarding the expression in the primary culture, M2 mRNA was highly expressed compared to other subtypes and no presence of M5 subtype was detected [116]. These observations were again confirmed in both the MCR-5 cell line and primary fibroblasts using PCR [118]. By applying qPCR technique, Buhling et al., have quantified transcripts of all muscarinic subtypes M1-M5 in lung tissues and lung fibroblasts. High expression level of M2 was reported in lung fibroblast compared to M3 subtype [179].

Profita et al., have reported that M1-3 were expressed in lung fibroblasts taken from COPD patients, control smokers and controls, and that M1 and M3 protein level was higher in COPD and control smoker than normal samples, whilst low level was reported for M2 in COPD subjects [180]. A further study in respect to muscarinic profile expression has shown by applying qPCR technique that the transcripts of muscarinic receptor subtypes, m1 and 3 were expressed in tracheal epithelial cells of wild mice, whereas no detection of m2 mRNA was reported [173]. Absence of m2 gene was further confirmed by immunohistochemistry. Analysis

of qPCR results also revealed insignificant levels of m4 and m5 mRNAs in the tracheal epithelial cells of the wild mice.

Concerning the distribution of nicotinic receptors in the bronchial epithelium, Zia et al., 1997 identified that alpha subunits, α-3, 4, 5, and 7 were expressed in both human and murine bronchial epithelium [181]. These functional nAChR subunits were observed in adhesive sites between the cells. The observations of this study suggest that the receptors are responsible for the modulation of cell attachment, communication as well as migration. It was also found that continuous stimulation of nAChR caused accumulation of high concentration of calcium ions in the cytosol which likely resulted in cell apoptosis. By applying the techniques, polymerase chain reaction (PCR) and *in situ* hybridization, it was confirmed that nAChRs subunits, alpha 3, alpha 5, beta 2 and beta 4 mRNAs were expressed in the human and rodent bronchial epithelium. Their structure was found to be comparable to those expressed in the skin and some neurons. Again, by using the alpha 3 blockers mecamylamine and k-bungarotoxin, it was concluded that this subunit is involved in the regulation of cell attachment and cell-cell communication [182]. Wang et al., have also shown that alpha 7 subunit mRNA and protein were expressed in the human bronchial epithelium employing PCR and western blot, respectively [183]. It is postulated that this subunit exerts some toxic effects on

bronchial epithelial cells and blood vessels as a result of nicotine/tobacco smoke stimulation of the receptors, particularly to those who are heavy smokers. The toxic effect of continuous stimulation of alpha 7 nAChR subunit via nicotine is represented in stimulation cell growth which result in development of cancer and angiogenesis [184]. Proskocil et al., have detected most of the cholinergic system components, acetylcholine and synthetic enzymes in the bronchial epithelium of neonatal and mature monkeys [185]. Cholinergic receptors, including nicotine subunits, alpha seven, alpha four and beta two have been reported. The authors also stated that these molecular structures were entirely unrelated and separated from those belonging to the neurons that innervate the lung.

Muscarinic and nicotinic receptors expression is summarized in Table 1.1.

Table 1.1: Distribution of cholinoceptors, muscarinic and nicotinic receptors in non-neuronal respiratory cells

Cell type	Muscarinic receptors	Nicotinic Receptors	Reference
Epithelial cells	M1 and M3	α1, α3, α5, α7, α9, β1, β2, β4, d, ε	[115, 174, 181-183, 185-191]
Alveolar wall (type II cells)	M1 and M3		
Airway smooth muscle	M2 and M3		[116, 174, 176, 186, 192-195]
Fibroblast (human lung)	M2>M1>M3>M4	α1, α3, α5, α6, α7, α9, β1, β2, β3, β4, δ, ε	

1.7 Organic cation transporters OCTs

Mammalian organs and tissues express a range of transporter families, one of which is known as solute link carrier family *SLC22A*. This family includes organic cation transporters (OCTs) and zwitterion or cation transporters OCTNs. Organic cation transporters include three members: OCT1-3. These are encoded from three genes, *SLC22A1-3*, while the zwitterion transporters include OCTN1 and 2 that are encoded from *SLC22A4* and 5 [1, 2]. Generally, transporter proteins can either translocate compounds that have comparable chemical structures and are thus called oligospecific, or transport a variety of substances that possess dissimilar chemical structures. This second type of transporters is

termed polyspecific transporters [3, 196]. OCT/Ns are considered to be polyspecific transporters as they are responsible for the transport of a variety of endogenous monoamine neurotransmitters across biological membrane such as dopamine, histamine or adrenaline [1-3, 196]. In addition to transportation of endogenous ligands, they influence the pharmacokinetics of a variety of drugs and xenobiotics. OCT/Ns are indeed implicated in the uptake, distribution, and excretion of a range of positively charged drugs and their metabolites [6, 9, 197-202].

1.7.1 Structure and morphology of the OCTs

SLC22A transporters, OCT1-3, and OCTN1-2 have a similar predicted structure on the intact plasma membrane as shown in Figuer 1.6, however, each protein consists of a different number of amino acids (AA). OCT1 and 2 are made of 554 and 555 amino acids, respectively [203], whereas OCT3 which is known as an extraneuronal monoamine transporter (EMT) is composed of 556 AA [204]. OCTN1 contains 551 AA and OCTN2 contains 557 AA [205, 206].

The basic structure of the OCT/Ns shows twelve transmembrane domains (TMD) and two intracellular terminals. They also possess an intracellular loop between the TMD six and seven, which contains phosphorylation sites. An extracellular loop extends between the first and second domain and possesses active sites for

N-glycosylation which are likely to be responsible for organising movement of a wide range of substances [1-3, 207, 208].

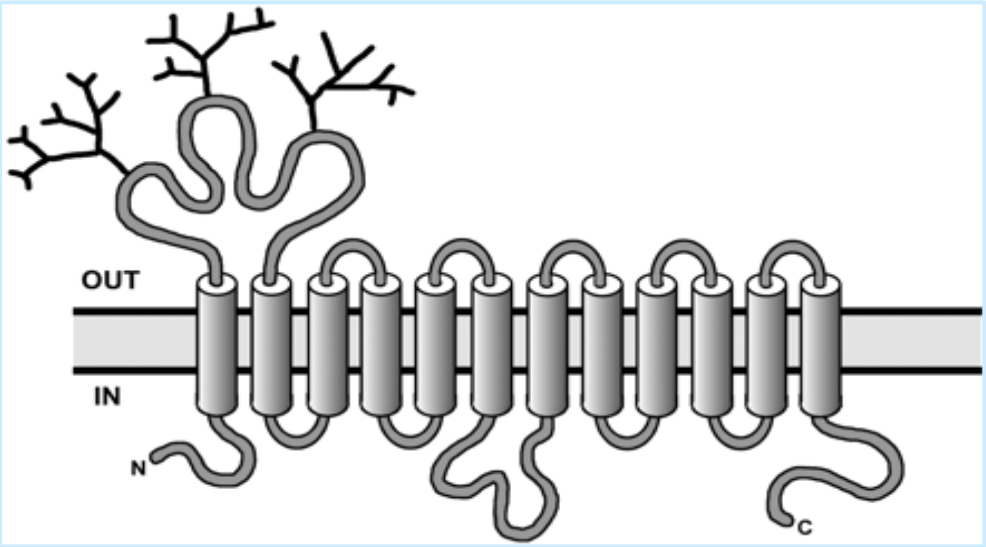

Figure 1.6: The imaginary structure of the organic cation transporters.

The structure comprises of 12 transmembrane domains (TMD), N and C intracellular terminals and a large extracellular loop between TMD 1 and 2 which contain N-glycosylation sites**.** Adapted from [198]

1.7.2 Regulation of the organic cation transporters

The regulation of OCT/Ns transport properties is complex and may involve more than one factor and varies between subtypes, species and individual tissues. It is also highly dependent on the physiological condition of the organ itself [209].

Various OCT regulatory factors have been described in humans and rats, such as protein kinases (PK), particularly PKA and PKC, and calmodulin. It has been shown that protein kinase A and C, and tyrosine kinase activation is involved in rOct1 regulation. When Protein kinase C is phosphorylated, substrate translocation is

allowed as a result of conformational changes in actively binding sites of the transporter [210]. Murine *Slc22a1* mRNA was shown to be increased in mice liver as a result of treatment with peroxisome proliferator agonist receptor (PPAR) agonist, pioglitazone [211].

Regarding hOCT1, it was found that stimulation of calmodulin (CaM), tyrosine kinase, and calcium calmodulin–dependent kinase led to activation of binding sites, followed by translocation of substrates, while PKA resulted in deactivation of hOCT1 functions [212]. This gene was also shown to be up regulated via a nuclear transcriptional factor known as hepatocyte nuclear factor-4 alpha (HNF-4α), and inactivated via the bile acid chenodeoxycholic acid [213]. By applying In Cell Western™ (ICW) assay, hOCT1 protein was found to be upregulated in bronchial epithelial Calu-3 cells monolayers cultured on membrane supports as a result of treatment with nitric oxide donor sodium nitroprusside (SNP) [214].

The expression of rat Oct2 mRNA and protein in kidney was reported to be controlled by sex hormones since rat males displayed higher expression levels than females. However, no hormonal impact was observed for rOct1 and rOct3 [215].

Stimulation of phosphatidylinositol 3-kinase (PI3K) and protein kinase A or inhibition of calmodulin (CaM) has been found to modulate hOCT2 expression in HEK-293 cells [216]. The hOCT3

protein regulation was found to be unaffected by protein kinase A and C, however, it was activated via Ca^{2+}/calmodulin pathways and deactivated with mitogen-activated protein kinase inhibitors (MAPK) [202, 217].

Regarding OCTNs, by applying microarray assay, Dransfeld et al., have shown that rat Octn1 and Octn2 mRNA were upregulated in hepatectomy during regeneration of the removed part [218]. Kato et al., 2005 have shown that OCTN2 regulation is mediated by a specific protein known as PDZK1 [219]. The latter is remarkably expressed on the plasma membrane of enterocytes and renal tubular cells. It was demonstrated that the last three amino acids of the C-terminal of the transporter are crucial for interaction with PDZK1. A couple of studies have shown that peroxisome proliferator-activated receptors α (PPARα) are involved in regulation of Octn2 in pigs and mice [220]. These receptors, PPAR were reported to be implicated in the regulation of OCTN1 and N2 proteins in humans as well. Treatment of Calu-3 cell layers grown on permeable supports with the PPARα agonist fenofibrate or the PPARγ agonist rosiglitazone resulted in increase in OCTN1 and 2 protein levels, respectively [214].

1.7.3 Expression of OCTs in the lung

Organic cation transporters are broadly distributed in absorptive, excretory and other cellular types in animals and humans, for

instance, hepatocytes, enterocytes, placenta, neurons, cardiac, and renal tissue [1, 6, 208].

Regarding OCT/Ns expression in the lung tissue, Bleasby et al., have used gene microarray to measure the expression of many transporters in a range of tissues [221]. They showed that OCT/Ns mRNA was variably expressed in lung tissue including foetal, adult, and tumor tissue. According to their investigation, OCT1 mRNA expression was reported to be higher in lung tumor compared to foetal and adult lung. The transcript levels for OCT2 were found to be similar in all tested samples and the lowest in comparison with other OCT/Ns genes. The mRNA for OCT3 was reported to be similar in foetal and cancerous lung, whilst lower in adult lung. The same levels for OCTN1 mRNA were found in adult and foetal lung, whereas lower level was identified in tumor lung. The OCTN2 mRNA was observed to be comparable in all examined samples. Overall, the study showed that OCT1, OCTN1 and OCTN2 were relatively highly expressed in the lung tissue.

Lips et al., have demonstrated that rat OCT1-3 mRNAs were expressed in tracheal and bronchial epithelial cells. The transcripts of the same genes were reported to be expressed in human tracheal and bronchial epithelium using RT-PCR technique [164]. The same researchers examined the localization of the corresponding proteins in the tracheal and bronchial epithelium of

both species as well using immunostaining technique. The protein for all rat transporters rOCT1-3 was found to be localized in the luminal membrane of ciliated cells while basal cells showed expression for OCT3 protein as well. Immunostaining in human samples showed that OCT1-3 proteins were present in the apical membrane of ciliated cells; however, weak expression was observed for OCT3. Human basal cells showed higher OCT3 protein expression than that observed in ciliated cells. This study was however restricted to OCT1-3 transporters expression. Using real time, Horvath et al., have detected low levels for mRNA of OCT1 and OCT3, whereas no expression for OCT2 mRNA was detected in the human airway epithelium [222]. Conversely, high expression of OCTN1 and 2 transcripts was identified in the airway epithelium. The expression of these genes was also measured in cystic fibrosis samples, but no difference was found. It was also found that the expression of OCT/Ns mRNA in primary cultures grown in air-liquid interface (ALI) condition was similar to freshly isolated tracheal and bronchial epithelial cells. The presence of OCTN1 and N2 proteins was further confirmed in the airway epithelium using immunohistochemistry technique. It was shown that OCTN1 protein was localized in the luminal surface of the tracheal epithelial cells, whilst low expression was identified in the alveolar epithelium. The OCTN1 protein was also found to be expressed in inflammatory cells infiltrated into the airways. Similarly to OCTN1,

OCTN2 protein expression was observed on the apical surface. Contradictory to OCTN1, OCTN2 protein level was highly identified in the alveolar epithelium. Using confocal imaging, the apical localization of OCTN1 and OCTN2 proteins was further confirmed in primary airway epithelial cells cultured in ALI condition.

Miakotina et al., have applied PCR and immunostaining techniques for detection of OCT1 and OCT2 expression at both pre and post transcriptional levels, respectively in murine lung epithelia and rodent primary alveolar epithelium type 2 [223]. The mRNA expression of OCT1 and 2 was detected in both examined samples, whereas only OCT2 protein was detected. By using immunohistochemistry, Kummer et al., have shown that OCT1 and 3 proteins were expressed in the luminal surface of murine bronchial epithelial cells, particularly ciliated cells, whereas no signal was reported for OCT2 protein [224]. They have also reported higher expression level of OCT3 protein in bronchial smooth muscle cells. By using quantitative PCR, Horvath et al., have shown that the OCT3 mRNA is highly expressed in human bronchial cells and vascular smooth muscle cells in comparison to OCT1, OCTN1 and 2, whereas no detection was reported for OCT2 [225]. They have also found that the airway epithelial cells expressed high level of OCTN1 and OCTN2 mRNA, whilst low levels were detected for OCT3 mRNA. The OCT3 protein was further

confirmed as highly expressed in bronchial smooth muscle cells compared to epithelial cells. The OCT3 protein was also detected in primary culture of human bronchial epithelium and smooth muscle cells using immunofluorescence technique.

Ishiguro et al., have reported expression levels of OCT3 mRNA in human lung and trachea as well as rat lung, trachea and alveolar type 2 cells using RT-PCR technique [226]. No expression levels for OCT2 gene was detected in lung tissue of human and rat. However, OCT1 mRNA level was identified in rat alveolar type 2 cells. By using real time PCR, lips et al., have measured alterations in Oct1-3 expression level in murine and rat lungs exposed to inhaled allergens [227]. No change in Oct1 mRNA expression was detected in allergic rats 24 hrs after exposure to allergens, whereas upregulation was observed after 48 hrs. Oct2 mRNA was found to be highly down-regulated at the points of measuring mRNA levels. RT-PCR revealed down regulation of Oct3 mRNA 48 hrs after exposure to ovalbumin. Results consistent with RT-PCR were obtained for Oct1-3 protein levels in rat bronchial epithelial cells using immunofluorescence assay 24 and 48 hrs after exposure to the challenge. No up or down regulation for Oct1-3 mRNA was reported in murine lung compared to the control samples. Mukherjee et al., have shown that normal human bronchial epithelial cells cultured at an air liquid interface for 21

days expressed mRNA of *SLC22A1*, 3-5 genes using RT-PCR [228]. In the same year, Courcot et al., have identified that the transcripts of four genes *SLC22A1*, A3-A5 were variably expressed in the normal human bronchial epithelium, healthy lung tissues, and cancerous lung tissues while no expression for *SLC22A2* mRNA was detected in any of the tested samples [229]. The levels of OCT1 mRNA were shown to be low in primary cultures and lung tissues, whereas the levels of OCTN2 mRNA were shown to be moderately expressed in all examined cells and tissues. A more recent investigation applied mass spectrometry to obtain a comprehensive overview of transporter protein expression levels in the lung [230]. It showed that OCT1, OCT2, and OCTN1 proteins were expressed in human lung tissue, tracheal epithelial cells, bronchial primary culture, and alveolar epithelial cells with a high protein expression for OCTN1. The OCT1 and OCTN1 proteins were more highly expressed in female lung tissue compared to male lung tissue. No protein expression levels were detected for OCT3 and OCTN2 in all examined samples.

1.7.4 OCTs roles in the respiratory epithelium

The expression profile of OCT/Ns in the lung tissue revealed all transporters are variably expressed in the epithelium (Figure 1.7), except that OCT2 expression in airway epithelial cells is contradictory. The respiratory epithelium modulates the absorption

of inhaled drugs used for the treatment of respiratory inflammatory diseases such as asthma and COPD [10]. Studies in airway cell lines also showed the implication of OCT/Ns in the transport of bronchodilator drugs across the cell membrane [228, 231]. In addition to their absorption functions, Kummer et al., have found that OCT1 and 2 knocked out mice exhibited high level of non-neuronal acetylcholine in the bronchial epithelium in comparison with wild type mice [224]. OCT/Ns were shown to translocate/uptake choline which is an essential molecule in cell proliferation. Wang et al., have demonstrated that OCT3, OCTN1 and N2 were partially involved in choline uptake in alveolar adenocarcinoma A549 cells [232].

The common function of organic cation/carnitine transporters OCTN1 and 2 in various organs is identified as transportation of L-carnitine with a high-affinity for OCTN2 and low-affinity for OCTN1. L-carnitine is involved in the translocation of long-chain fatty acids to the inner membrane of mitochondria for energy release [233]. Any parameter influencing their expression, particularly OCTN2 will result in carnitine systemic deficiency. The systemic deficiency of carnitine was shown to affect some organs and tissue, particularly heart, liver, and skeletal muscles. Disorder in these tissues has been manifested with symptoms such as hypoketotic hypoglycemia, hepatomegaly, hyperammonemia, and

cardiomyopathy in new borns [234, 235], and skeletal myopathy, and cardiomyopathy in childhood age [236-239], or cardiomyopathy, arrhythmias, or fatigability in adult patients [234].

Genetic defects in *SLC22A4* and *SLC22A5* genes were found to be associated with some inflammatory diseases such as rheumatoid arthritis, Crohn's disease and asthma; however the mechanisms are still unclarified. Moffatt et al., have concluded that *SLC22A5* may be an asthma susceptibility gene using a genome-wide association study in asthmatic patients [240].

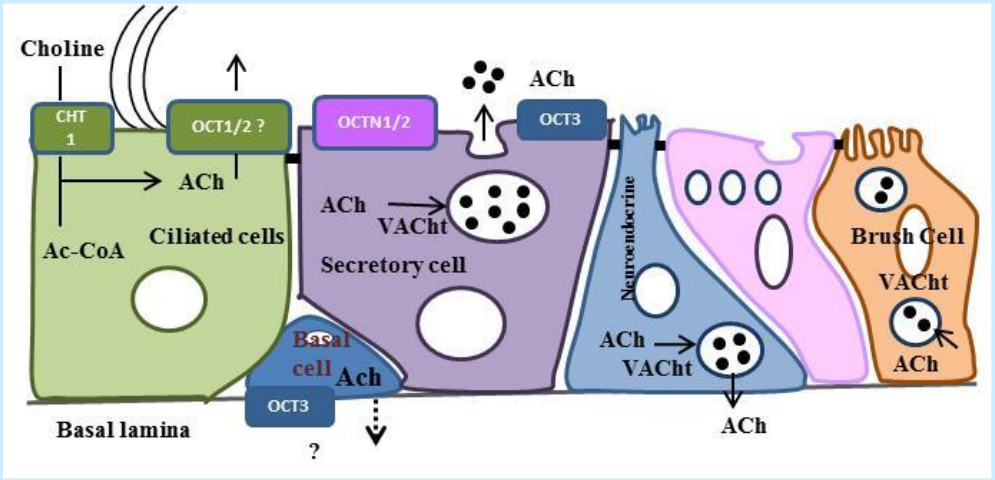

Figure 1.7: Schematic illustrating the role of organic cation transporters in the respiratory epithelium.

OCT 1 and 2 in the membrane of ciliated cells are implicated in the transport of non-neuronal Ach from the intracellular into extracellular environment where it acts as autocrine or paracrine through stimulation of two different types of receptors, muscarinic and nicotinic receptors. Other cell types also release Ach but by exocytosis not via OCTs. Basal cells may release Ach by OCT3 but this is still unproven. Adapted from [224]

1.8 Aims of the thesis

The airway wall components of asthmatic and COPD undergo structural alterations as a result of inflammatory and remodelling processes. These occur in asthmatic and COPD patients as a consequence of interaction between epithelial cells and the underlying tissue which is called the epithelial mesenchymal trophic unit (EMTU). A variety of pro-inflammatory mediators such as cytokines and growth factors are released from the damaged epithelial cells, underlying tissue and infiltrated inflammatory cells. All together, they contribute to the abnormal remodelling of the airways in asthmatic subjects.

The characteristic features of airway remodelling include hypertrophy of mucus producing cells and submucosal glands, increased thickness of the basement membrane, enlargement and proliferation of airway smooth muscle cells. Neuronal acetylcholine has been shown to cause cell proliferation and increase mucus secretion via its receptors, particularly the M3- subtype.

The airway epithelium has also been shown to produce non-neuronal acetylcholine (Ach) and express muscarinic and nicotinic receptors on the plasma membrane of ciliated, goblet and basal cells. It has also been suggested that organic cation transporters, particularly OCT1-3 are involved in the release of non-neuronal Ach

from airway epithelial cells while the OCTN2 gene has been associated with asthma.

It has been suggested that the non-neuronal Ach released from the airway epithelium via the OCTs may regulate cellular functions such as cell proliferation. Therefore, we aimed to examine whether these proteins, particularly OCT1 is involved in airway cell proliferation.

The A549 cells were used as a main model in the current study. The expression profile of OCT/Ns in the cells was first examined by normal PCR, using the Calu-3 and Caco-2 cell lines as positive controls. The expression levels of OCT1 and OCTN2 gene were then quantified at both pre and post transcriptional levels using qPCR and ICW technique, respectively. This was followed by probing the effect of a variety of OCT/Ns inhibitors on cell viability and cell proliferation. Finally, the OCT1 gene was transiently knocked down using siRNA for better understanding its role in A549 cell proliferation.

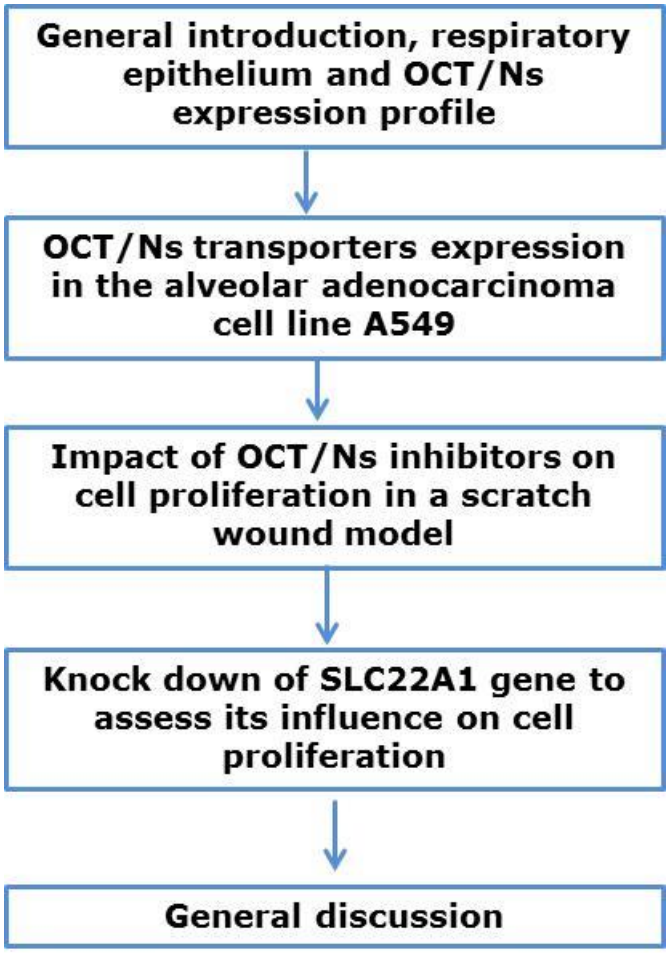

Figure 1.8: Schematic presenting the plan of the thesis manuscript

Chapter 2 Expression of *SLC22A1-5* genes in the alveolar adenocarcinoma cell line A549

2.1 Introduction

2.1.1 Respiratory epithelial cell lines

As a need for alternative models for human airway epithelial primary cultures, a number of cell lines from different part of the airway epithelium have been introduced. Human respiratory epithelium primary cultures derive from samples collected from different regions of the tracheo-bronchial tract. They have properties similar to those of the normal respiratory epithelium such as formation of cilia and tight junctions, and high values of transepithelial electrical resistance (TEER). Accordingly, they are preferable for studying the physiology of the airway epithelium [241]. However, using primary cultures in research is limited because of some drawbacks, including difficulty in obtaining many samples from donors and, huge variations between different individuals, and between passages of the same samples which causes inconsistencies in the data. In addition, primary cells cannot be grown for long period since they lose their native properties upon passaging. Finally, a specific serum-free medium is needed to culture primary epithelium which increases maintenance costs [242, 243]. For these reason a few cell lines have been used in respiratory research. The most frequently used cell lines are either

derived from the bronchial (Calu-3, 16HBE14o-, and BEAS-2B) or alveolar (A549) area.

The Calu-3 cell line derives from bronchial adenocarcinoma cells from a Caucasian male who was a 25-year old [244]. It has been demonstrated that Calu-3 cells have characteristics comparable to those of bronchial primary cultures. For example, they exhibit high values of transepithelial electrical resistance (TEER), polarized monolayers with differentiated ciliated and mucus producing cells, and form tight junctions when they are grown at an air liquid interface (ALI) [245-250]. These permit employing Calu-3 layers for testing absorption/influx of a wide variety of substances. Calu-3 cells have also been used to study epithelial disturbances in cystic fibrosis due to the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) on their cell membrane [251].

The 16HBE14o- cell line is originally a bronchial primary culture which was transformed by a simian virus SV40 large T-antigen to achieve immortalization [252]. Similarly to the Calu-3 cell line, these cells are able to form tight junctions as well as polarized and differentiated monolayers exhibiting cilia but only when the cells were cultured in submerged conditions [253]. For unknown reasons, ALI cultured 16HBE14o- cells exhibited changes in phenotype and morphology. It has however been demonstrated

that 16HBE14o- cells provide reliable estimations of the permeability or influx of certain tested compounds [254].

The BEAS-2B is an immortalized cell line that was introduced by transforming native bronchial epithelial cells via insertion of a combination of two viruses, adenovirus 12 and simian virus 40 [255]. These cells have been broadly used for examining the mechanistic functions of the epithelium, particularly those related to their role in inflammatory responses and cytokines synthesis [256]. It has also been used for probing the epithelium response to the impact of tobacco smoke as well as various environmental particles [257]. These cells have shown their inability in forming tight junctions and for this reason, have not been used for studying compound permeability [250].

The A549 cell line is an alveolar adenocarcinoma cell line which was derived from alveolar type 2 cells of a 59-year old man [258]. A549 cells are characterized by a phenotype and biological functions similar to those of native alveolar type 2, and can be applied to investigate the pathways involved in synthesis and releasing of the unsaturated fatty acid, phospholipid [226, 259, 260]. They have also been employed to test peptides and proteins permeability [261, 262], inflammatory responses to nitric oxide and mould [263, 264], the impact of oxidative stress produced by cigarette smoke as well as the damaging effects of airborne

particulate matter on DNA [265, 266]. The A549 cell line has also been used to study choline transport via organic cation transporters [232].

Unlike Calu-3 and 16HBE14o- cells, A549 cells are highly permeable to various compounds since no TJs have been detected in their intercellular junctions. Thus, its application is very limited for the study of drug transport/influx [242].

2.1.2 Expression of *SLC22A1-5* in respiratory cell lines

Organic cation transporters expression OCT1-3, OCTN1-2 at pre and post transcriptional level in lung tissues and primary cultures has been described in (1.7.3). However, this chapter will emphasize on their expression in various lung cell lines including bronchial cell lines (Calu-3, BEAS-2B, and 16HBE14o-) and the alveolar cell line A549. Traditional PCR, real time PCR, immunohistochemistry, In Cell Western assay, Western blot analysis, and mass spectrometry have been used for detecting expression levels of OCT/Ns mRNAs and proteins in lung derived cell lines so far table 2.1.

Alveolar adenocarcinoma cell line A549 was shown to express mRNA of OCT1, OCT3, OCTN1 and 2, whereas no mRNA for OCT2 was detected using normal PCR [229, 232, 267]. These cells were also shown to express OCT1, OCT3, OCTN1 and 2 applying

Western blot and mass spectrometry [230, 232]. However, based on Salomon et al 2012 these cells were shown to express the protein of the five transporters, OCT1-3 and OCTN1-2 using Western blot analysis. In respect to the bronchial cell lines, the undifferentiated Calu-3 cells were found to express transcripts of OCT1, OCT3, OCTN1 and 2 applying traditional PCR [228, 267, 268]. None of the investigations detected OCT2 mRNA in Calu-3 cells. After 15 days of culturing, Calu-3 cells expressed mRNA of OCT1, OCTN1 and 2 but did not express the transcript of OCT2 and OCT3 [267]. Expression at the post transcriptional level was detected for OCT1, 3, OCTN1 and 2 in differentiated Calu-3 applying immunohistochemistry and In Cell Western techniques [214, 228]. The undifferentiated Calu-3 cultured in submerged conditions were also shown to express OCT1, OCT3, OCTN1 and 2 proteins using Western blot and mass spectrometry [269, 270]. The second bronchial cell line 16HBE14o- was found to express the mRNA of OCT1, OCTN1 and 2 on day 15 and 18 of culturing using conventional PCR [267]. In a separate study, these cells were shown to express OCT1, OCT3, OCTN1 and 2 using qPCR [229]. The post transcriptional level of OCT1, OCT3, OCTN1 and 2 transporters was detected in 16HBE14o- by Western blot [269]. The BEAS-2B cells were shown to express transcripts of OCT1, OCTN1 and 2 using PCR [267]. These cells also showed expression of the mRNA of all transporters except OCT2 applying qPCR [229,

271]. The protein of all transporters was detected in these cells [270].

Table 2.1: OCT/Ns transporter expression in A549 cells using different techniques in published studies and the current study.

OCT/Ns	Current study	Endter study [267]	Salomon study[269]	Courcot study [229]	Sakamoto study [270]
OCT1	+	+	+	++	+
OCT2	-	-	++	-	-
OCT3	+++	+++	+++	+++	+++
OCTN1	+	++	++	++	++
OCTN2	++	+	+	+++	-

Plus symbol denotes to expression of the transporter, minus for absence of the transporter. In the current study, PCR was used for detection all members, while qPCR and ICW assays were used for detection OCT1 and OCTN2 transporters. Endter study based on normal PCR assay and Courcot based on qPCR for detection all transporters, while Salomon and Sakamoto studies based on western blot and mass spectrometry, respectively.

2.1.3 Muscarinic and nicotinic receptor expression in respiratory epithelial cell lines

The expression of acetyl choline, acetylcholine transferase, muscarinic and nicotinic receptors in various airway wall components and primary cultures has been described in (1.6.3).

Regarding the expression of acetylcholine related elements in airway derived cell lines, by applying RT-PCR Plummer et al., have

shown that mRNA of α7 nicotinic acetylcholine receptors was expressed in seven small cell lung cancer (SCLC) cell lines including WBA, NCI-H69, NCI-H82, NCI-H146, NCI-H187, NCI-H209, NCI-H526, primary culture of peripheral airways, and in two pulmonary adenocarcinoma [189]. High expression levels of α7 mRNA was detected for NCI-H82 compared to primary cultures using nuclease protection assay. Western blot analysis revealed expression of α7 protein in the six of the SCLC cell lines. The mRNA of this nicotinic subunit was also expressed in A549 cells and three other squamous cell lines. The α7 subunit protein was also shown to be expressed in primary cultures, A549 cells and NCI-H226 cells. This study suggested that α7 nAChR may be involved in inducing various cancer types associated with tobacco smoke.

By applying real time PCR and microarray assays different expression patterns for nicotinic acetylcholine receptors (nAChR) subunits were detected in non-small cell lines and bronchial epithelial cell lines as a result of nicotine exposure. It was concluded that nicotinic receptors subunits are implicated in inducing cell proliferation and can be a target for lung cancer therapy [272]. Carlisle et al., have examined a variety of cancerous lung cell lines including A549 cells for nicotinic acetylcholine receptor subunits expression [186]. Immunoblot

assay showed that α1, α5, β1, β2, and δ nAChR subunits were expressed in A549 cells.

In respect to muscarinic receptors expression, the qPCR assay revealed that M1-M5 transcripts were expressed in A549 cells with abundant expression for the M3 subtype. The investigation showed that M5 subtype was expressed in A549 cells [179]. Profita et al., have shown that transcripts of muscarinic receptor M1-M3 subunits were expressed in the bronchial cell line 16HBE14o-, with higher expression level for the M3 subtype followed by M1 and M2 subtypes using real time PCR [180]. The same expression level for M1-M3 subunits was further confirmed by flow cytometric assay, i.e., M3>M1>M2. Protein expression levels for the three muscarinic subunits were in agreement with the flow cytometric and qPCR assay results. Xu et al., have found low level of mRNA of M1-M3 subtypes in untreated A549 cells applying qPCR assay, however, their mRNA expression levels were modulated via TNF-α [273]. TNF-α induced A549 cells showed increased levels in mRNA for M3 and M2, whereas M1 was decreased.

In this chapter, OCT/Ns transporter expression at both pre and post transcriptional levels was confirmed in the alveolar adenocarcinoma cell line A549. Selection of this cell line was based on a predicted expression of OCT/Ns, in particular OCT1 and OCTN2. In addition, this cell line has been shown to be transfected

easily compared to bronchial cell lines such as Calu-3 cells. This is because these cells are loosely attached to the neighbouring cells and are not specialized in the production of mucus that may affect or prevent the incorporation of nanoparticles siRNA/vehicle complexes into the cytosol. Furthermore, A549 cells had been shown to express cholinergic components such as Ach, muscarinic and nicotinic receptors. The expression of cholinergic elements was also essential to examine the proposed hypothesis.

2.2 Materials and methods

2.2.1 Cell culture maintenance

The A549 cell line was used as the main model to conduct most of the experiments. However, both the bronchial adenocarcinoma Calu-3 and intestinal adenocarcinoma Caco-2 were used as positive controls in this chapter.

The A549 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). It was applied between passages 20-35. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% foetal bovine serum (FBS), 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 20 mM of L-glutamine. All these materials were from Sigma-Aldrich (Poole, UK) and were filtered before adding them into the medium to avoid contamination.

The Calu-3 cell line was obtained from ATCC. Calu-3 cells were used between passages 30-40. They were cultured in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 which was added with10% foetal bovine serum (FBS), 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 20 mM of L-glutamine, and 1% (v/v) non-essential amino acid.

The Caco-2 cell line was obtained from European Collection of Cell Culture (ECCC, Salisbury, UK). Caco-2 cells were used between passages 10-15 as a positive control since all members of OCTs were expressed on the cell membrane of these cells [269]. The cells were grown in the same medium as used for culturing A549 cells.

Cells were maintained at 37 °C with 5% CO_2 and humidity. Approximately 10-12 ml of specific fresh medium was added every two to three days and cells were trypsinized when the confluence reached approximately 85% or more. Then the cells were seeded again in new 75 cm^2 flasks (Costar Corning, Corning, UK) with a split ratio of 1:5 for A549 and Caco-2 or 1:3 for Calu-3. Cells were tested for Mycoplasma contamination every 2-3 months. In addition, the stock was regularly frozen in liquid nitrogen after preparing a cell suspension in medium containing 10% of dimethyl sulphoxide (DMSO). Cells were first kept in the -80 °C freezer for 2 days and then transferred into liquid nitrogen.

2.2.2 Testing of different types of serum

Growing of the A549 cells was satisfactory in serum purchased from Sigma (Lot no 040M3398). However, the bronchial epithelial cell line, Calu-3 which was employed as a positive control unexpectedly exhibited slow growing. Therefore, there was a need to select an appropriate type of serum that would provide cells with essential components for acceptable growing. Two types of serum were purchased, one from Sigma with Lot No. 070M3399, and the second from Biochrom with Lot No. 0378A. These sera were tested with the three different cell lines, A549, Calu-3 and Caco-2. Growing of the involved cell lines in the current investigation was monitored by different methods, including examining cells cultured in a 75 cm^2 flasks by optical microscope. In addition, Calu-3 and Caco-2 growing was monitored on Transwell® supports by measuring trans-epithelial electrical resistance (described in details in 2.2.4). Furthermore, the impact of serum type on gene expression at both, pre and post-transcriptional levels was investigated.

2.2.3 Cell culture conditions

The cell lines were cultivated on different tissue culture plates and in various conditions. A549 cell line was cultured in submerged condition on a 24-well plate or 12-well plate at different density depending on the experiment (Costar Corning, UK).

The Calu-3 cells were cultured at density of 10^5 cells/well onto 0.4 µm pore size, 1.13 cm^2 surface area polyester Transwell® cell culture inserts (CoStar Corning, Corning, UK). To obtain differentiated monolayers of Calu-3, air liquid interface (ALI) condition was provided by pipetting 0.5 ml of cell suspension on the membrane support, followed by adding 1.5 ml of medium into the plate wells. After 24 hrs of culturing the Calu-3 cells, the media was first aspirated from the basolateral chamber then from the Transwell® insert and 0.5 ml of fresh medium was added into the wells only in order to create an ALI condition (Figure 2.1). The medium was replaced every 2-3 days until the cells were harvested when layers were 21-day old.

The intestinal adenocarcinoma Caco-2 cells were cultured at density of 2 x 10^5/well onto Transwell® inserts. In contrast to Calu-3 cells, Caco-2 cells were kept in submerged conditions, 0.5 ml medium in the supports and 1.5 ml in the plate wells. The medium was replaced every 48 hrs.

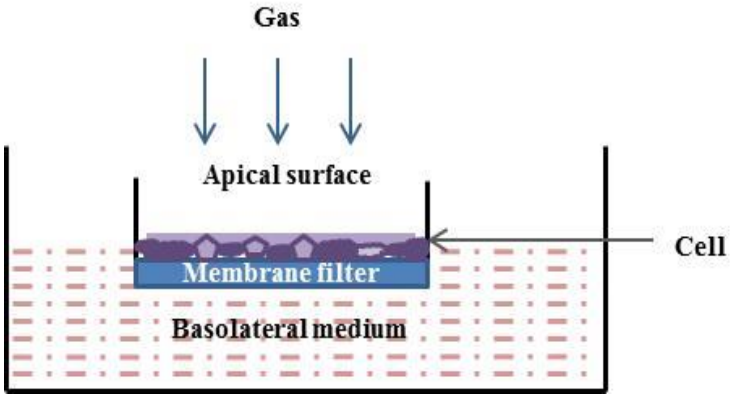

Figure 2.1: Air liquid interface conditions for Calu-3 cells.

The purple colour represents the monolayer which is supported by the Transwell® membrane. Cells are in contact with air as no medium is added on the upper surface of the monolayer, and the medium is only provided in the bottom of the well plate. Image was adapted from [274]

2.2.4 Trans-epithelial electrical resistance measurement for Calu-3 and Caco-2 cell lines

The formation of tight junctions by Calu-3 and Caco-2 cultured on Transwells was monitored by measuring the trans-epithelial resistance (TEER). TEER was measured at different days of culturing by using an epithelial voltammeter (EVOM) with its accessory chopstick STX2 electrodes (World precision Instrument, Stevenage, UK). Because Calu-3 cells were cultivated in ALI condition, 0.5 ml and 1ml of medium needed to be added into the inserts and well chambers, respectively. Then the plate was placed at 37 °C for 30 min before TEER was measured. After recording the reading of TEER for all wells, cells were kept again in ALI condition until the next reading. Cell layers with reading values of TEER above 600 Ω cm^2 were harvested on day 21 for experimental purpose, while those recorded less than 600 Ω cm^2 were omitted.

Regarding Caco-2, wells that recorded less than 300 Ω cm^2 were omitted. On day 21 both inserts and plate wells were washed with PBS and the cells were harvested for RNA extraction.

2.2.5 Gene expression

Investigation of gene expression was the first objective before starting examination of the function of the genes of interest. This was conducted by applying semi-quantitative or normal polymerase chain reaction (PCR) and quantitative real time polymerase chain reaction (QRT-PCR).

2.2.5.1 RNA extraction

The alveolar adenocarcinoma cell line A549 was cultured onto a 24-well plate at a density of 25-30 x 10^3 cells/well using normal medium containing 10% FBS. A549 cells were then harvested on third day of culturing and RNA was extracted using GeneElute™ Total RNA Miniprep Kit (RNT70, Sigma).

However, Calu-3 and Caco-2 were cultured on Transwell® well supports and harvested at day 21 of culturing. Briefly, the extraction involved releasing RNA from the cells using lysis solution and 2-mercapto ethanol in blue columns, binding RNA to the red column, washing to dispose of the contaminants and finally eluting purified RNA. The extraction was completed using a refrigerator centrifuge, (5430R, Eppendrof, Germany). To obtain total RNA free

of genomic DNA contamination, the kit On-Column DNase I digestion (Sigma) was applied before the final step of extraction.

The extracted RNA was either kept at -80 °C since it is unstable and can be easily degraded or it was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA). The optical ratio for the samples at 260:280 was 2.0-2.1. Some techniques can be applied to determine the quality of the total RNA and its reliability to be used for the next step. For the current investigation gel electrophoresis was run by using 1% agarose in 1x Tris acetic acid EDTA to ensure reliable quality and undigested amount of the total RNA which reflects the amount of mRNA. To obtain linear bands of RNA, buffer containing ammonia was added in order to denature the RNA structure. This involved placing the samples at 64 °C for 3-5 minutes then loading them into the gel electrophoresis. The required amount of RNA to be loaded was 600 ng/µl, and a 1 kb ladder was run alongside the samples. The images of visualised gels were obtained using an image reader LAS-4000, (Fujifilm, Japan) (Figure 2.2). The integrity and quality of total RNA can be measured using alternative methods; however, gel electrophoresis was used in this study as it is an inexpensive method.

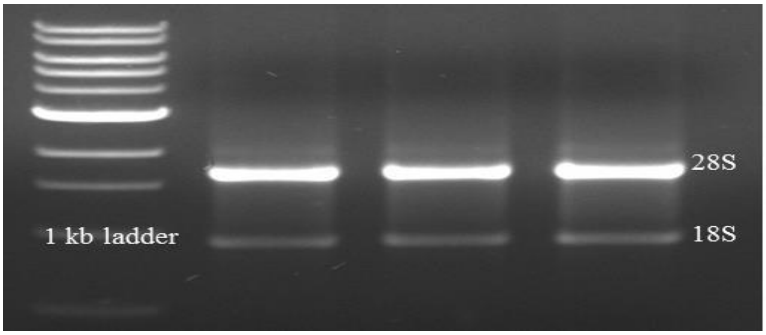

Figure 2.2: Gel electrophoresis of the total RNA.

The total RNA includes two types of bands, 18S and 28S, 18S is faint and the least bright one, while the bright bands represent 28S. Total RNA generally consists of 80-85% ribosomal RNA (rRNA), 10-15% transfer RNA (tRNA), and the least amount is 5% for mRNA. Appearance of undigested bands reveals the integrity of extracted RNA. 1 kb ladder was run alongside the gel which is represented on the left side of the gel.

2.2.5.2 Reverse Transcription polymerase chain reaction RT-PCR

Measuring the level and the expression of mRNA transcripts of the genes of interest involved transcribing RNA to complementary deoxyribonucleic acid (cDNA). This can be achieved using various types of kits. In the current study, Enhanced Avian HS RT-PCR Kit (HSRT100, Sigma) was used to achieve RT.

PCR primers were validated for five members of organic cation transporters, OCT1-3, OCTN1-2 using Calu-3 and Caco-2 as positive controls. These primers were previously applied by Dr.Manali Mukherjee. The validation involved applying Enhanced Avian HS RT-PCR Kit (HSRT100, Sigma) for RT and PCR amplification, and REDTaq® ReadyMix™PCR Mix with $MgCl_2$ (Sigma) was used for the gene amplification.

RT was carried out by applying a 2 step reaction according to the manufacturer's instructions. The first step involved adding RNA template, 200 ng, 1 µl of each deoxynucleotide mix and random nanomers/anchored oligo(dT) 23 or specific antisense primer and PCR water up to 10 µl. The preparation was mixed and centrifuged then incubated at 70 °C for 10 minutes using an Eppendorf thermocycler AG, 22331 (Hamburg, Germany). This incubation period is optional and the purpose of it is to denature RNA secondary structure, however it was applied for all samples. Thereafter, the tubes were placed on ice and additional reagents were added for the second step reaction, 6 µl PCR water reagent, 2 µl of buffer for RT, 1 µl of each Enhanced AMV-RT and RNase inhibitor to end up with 20 µl. Then the reaction of 20 µl was continued by incubation at 46 °C for 50 minutes. However, using random nanomers to prime mRNA involved extra incubation of the samples at 25 ºC for 15 min. At this point of reaction the cDNA was formed and ready to be used for amplification by either traditional PCR or Real time polymerase chain reaction. Minus RT and no template control were run alongside the samples to ascertain that the samples were free of genomic contamination. After examining all of random nanomers, anchored oligo(dT)23, and specific antisense primer, anchored oligo(dT)23 was found to be the best one for priming RNA to cDNA.

The RT kit was applied again for the PCR or amplifying the genes of interest. The volume of the PCR reaction components was 50 µl included: 40 µl water PCR reaction, 5 µl 10x Accu Taq buffer, 1 µl of both deoxynucleotide mix and PCR primers, 2 µl of cDNA and finally 1 µl of JumpStar Accu Taq LA DNA polymerase. The primer sequences for forward and reverse of genes are listed in Table 2.2.

The cycle's options for the run were as the following:

1- **Initial activation:** 94 °C for 5 min
2- **Denaturation:** 94 °C for 30 seconds
3- **Annealing temperature:** 50-55 °C annealing temperature for 45 seconds
4- **Extension:** 72 °C for 1 min
5- **Final extension:** 72 °C for five min

The PCR products were then run by gel electrophoresis on a 1% agarose gel, and then visualized using LAS-4000 image reader, but no bands were observed.

For this reason, a different kit was used to conduct PCR, REDTaq® ReadyMix™PCR Mix with $MgCl_2$ (Sigma-Aldrich, USA). The final volume of the reaction was 20 µl comprising of these reagents, 10 µl of REDTaq Readymix, 2 µl of primer the gene of interest (forward and reverse, shown in Table 2.2 mixed in a 5 µM concentration, 1 µl of cDNA and finally 7/8 µl of HyClone®,

HyPure™ Molecular Biology Grade water, Nuclease free, (Ogan, Utah, USA). Pipetting was carried out while the tubes were placed on ice. Finally, all PCR tubes were mixed and centrifuged then placed into the well of the thermo cycler. The same previous cycle's options were used with the second kit.

At the end of the reaction, the products were checked for their size by running gel electrophoresis, using 1% TBE and visualized with ethidium bromide 10 µl/100 ml of buffer. A ladder of 100 bp DNA marker was run alongside the samples. No template control (NTC) and no RT control were run as well to verify that the reaction was free of contamination. Various loading dyes were tested for the best band intensity, ficoll-200, glycerol, sucrose and 6x DNA loading dye. It was found that 6x DNA loading dye was the best in providing obvious and clear bands.

Table 2.2: PCR primers details

Gene	Forward and Reverse (5'-3')	TM	Annealing temperature	Size in bp
GAPDH	**F**:ACAGTCAGCCGCATCTTC **R**:GCCCAATACGACCAAATCC	**57.39/ 56.36**	**50**	**101**
SLC22A1	**F:**TGAAGGACGCCGAGAACC **R:**AGGAAGAATACAGAGAAGTG AAGG	**59.35/ 57.71**	**55**	**188**
SLC22A2	**F:**AAGTTGCCTATACAGTTGG **R:**GCGATGTGCTTAATGATTC	**52.18/ 52.49**	**50**	**196**
SLC22A3	**F:**CCACTCCACCATCGTCAG **R:**ACACCAAGGCAGGATAGC	**57.08/ 56.6**	**55**	**168**
SLC22A4	**F:**TGTCATCACCCGTAGTTG **R:**ACATACCATTGAAGCCATTG	**53.9/ 53.91**	**50**	**156**
SLC22A5	**F:**TTACTTCATCCGAGACTGG **R:**CTGCTTCTTGGAACTTAGG	**53.59/ 52.97**	**50**	**229**

Names of genes and the sequence of both forward and reverse primers, melting temperature, annealing temperature and product size are mentioned.

2.2.5.3 Purification of the PCR products

Purification of the PCR products for all members of *SLC22A* family A1-5 was the step which followed PCR reaction. The main aim of this step was to clarify the products of all substances that may affect sequencing. These include primers, incorporated nucleotides, and salts such as magnesium chloride MgCl2. The steps of purification involved adding 100 µl of amplified products into 1.5 ml tube, then 500 µl of GENECLEAN® Turbo for PCR salt solution (MP Biomedicals, Ohio, USA) was added into the same tube. After

mixing PCR/salt solution, the mixture was transferred into Cartridge tubes then they were centrifuged for 5 seconds. Thereafter, Cartridge tubes were washed with 500 µl of PCR wash solution and centrifuged again for 5 seconds. The eluting liquid was disposed of after each centrifugation, and the tubes were centrifuged for an additional 4 minutes to remove the rest of the wash solution as the latter contains ethanol. Finally, 30 µl of PCR eluting solution onto GLASSMILK® -embedded membrane was added for eluting amplified products. The filtered liquid at this point contained DNA which was purified of the PCR reaction kit reagents and ready to be sent for sequencing.

2.2.5.4 Sequencing of DNA

The main objective of sequencing *SLC22A1-5* and HKG (*GAPDH*) was to confirm that the selected primers were successfully designed and amplified the genes of interest. This was carried out using The BigDye® Terminator Cycle Sequencing Kit (Applied Biosystem). The principle of reaction involves adding cDNA template, one of the primer (forward or reverse), buffer, dNTPs, fluorescently labelled dideoxynucleotides ddNTPs (ddGTP, ddATP, ddTTP and ddCTP), and finally polymerase in a single tube. The reaction includes three steps, denaturation, hybridization and elongation, and it was run on Applied Biosystems® 3130xL Genetic

Analyzer and the terminator dye applied was v3.1 (ThermoFisher Scientific).

The results from sequencing were analysed using different softwares.BLAST from *the website (http://www.ncbi.nlm.nih.gov/), was employed to confirm the identity* between the sequence and the original gene sequence. The second software, Chromas 2.1.1 (Applied Biosystems and Amersham MegaBace) was used to obtain the figures which represent the sequence of nucleotides of the amplified products.

2.2.5.5 Validation of the primers for quantitative real time polymerase chain reaction qRT-PCR

Various sets of qPCR primer were tested for quantification of the *SLC22A1* and *A5* genes. In addition, HKG, *GAPDH* and *18S* were quantified and used for normalization of the gene of interest. The primers for real time PCR were first purchased from Bio-Rad. Generally, these primers have the same annealing temperature of 60 ◦C and are already validated by the company. No sequences of the four primers were provided, however, each primer had a specific ID as shown in Table 2.3.

For validation of the qPCR primers and gene quantification, the brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent

technology, CA, USA) was employed to screen the level of mRNA of the genes of interest.

The volume of qPCR reaction was 20 µl consisting of 10 µl SYBR green, (mutant *Taq* DNA polymerase, dNTPs, Mg^{2+}, a buffer specially formulated for fast cycling, and the double-stranded DNA-binding dye SYBR Green I for detection), 1 µl of qPCR mixed primers (Forward + Reverse) at 4 µM as final concentration, 1 µl of cDNA and 8 µl of nuclease free water/diethylpyrocarbonate (DEPC water). The thermal profile of the reaction was 3 minutes at 95 °C for one cycle as an initial activation, and 40 cycles of 20 seconds at 95 °C and 22 seconds at 60 °C as shown in Figure 2.3. No temperature and time was applied for extension, however, the last cycle was set up to obtain a dissociation or melting curve to confirm that SYBR green amplified one product, as reflected by only one peak obtained.

The reaction was run on Mx3005P Real-Time PCR Detection System (Stratagene) and MxPro QPCR Software version 4.10 was used to generate the efficiencies of reactions, amplification plots, standard curves and dissociation curves (Figure 2.3).

Table 2.3: qPCR primers for SYBR assay.

Gene	Alternative name	Product size (BP)	ID related to Bio-Rad
SLC22A1	Organic cation transporter (OCT1)	100	qHsaCID0009240
SLC22A5	Organic cation transporter (OCTN2)	107	qHsaCID0012205
GAPDH	Glyceraldehyde3-phosphate dehydrogenase	117	qHsaCED0038674
18S	Ribosomal	67	qHsaCED0037454

All primers have the same annealing temperature of 60 ºC and they are designed to span intron junction to avoid amplification of genome.

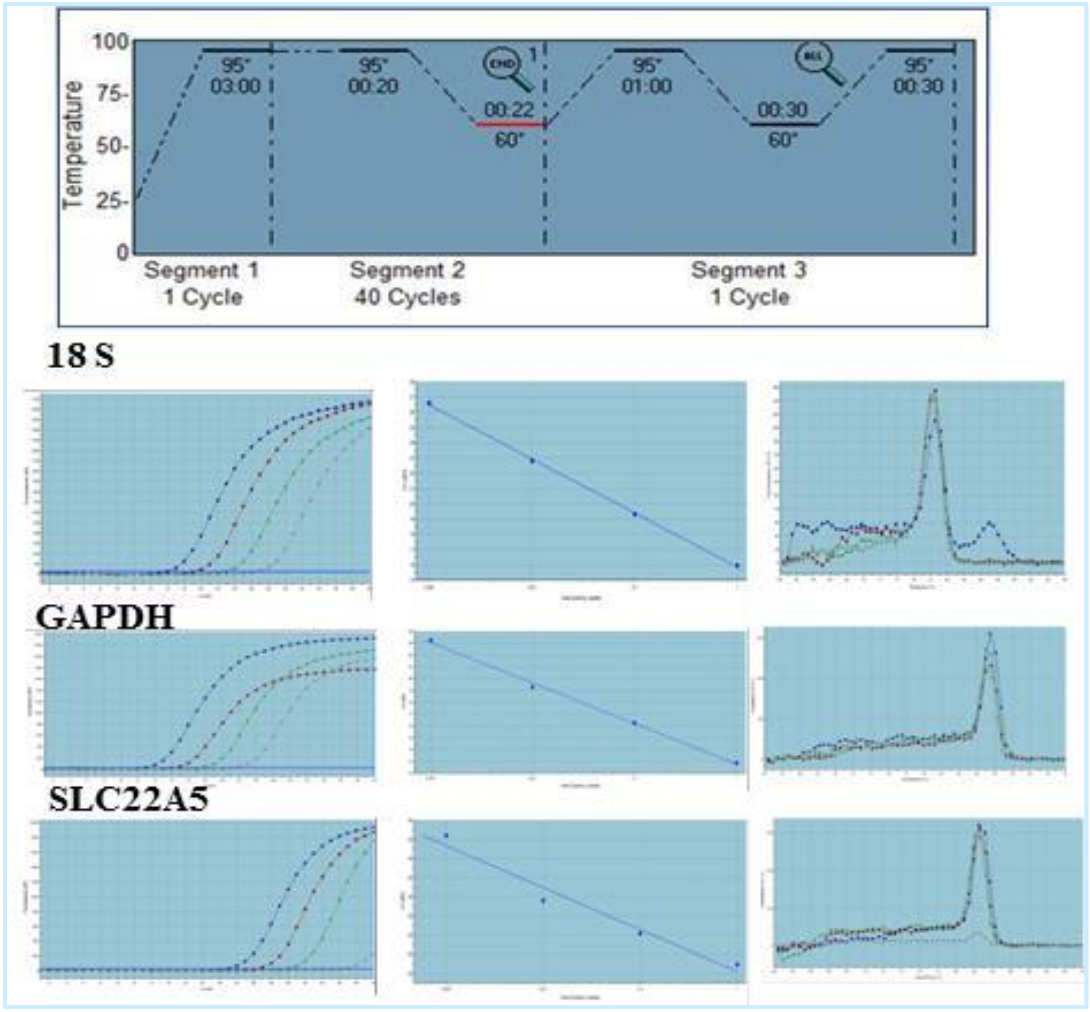

Figure 2.3: Thermal profile, amplification plots, standard curves, and melting curves for 3 genes.

The primers of HKGs, *18s* and *GAPDH*, and *SLC22A2* were obtained from Bio-Rad. The primers efficiency was obtained by achieving serial dilution of the primers. Ten-fold dilution was applied for all examined genes and the reaction was run depending on the described thermal profile. The amplification plot figures for all genes are shown on the left panel, the middle panel represents the standard curves, and the right panel shows the melting curves.

Regarding quantification of the *SLC22A1* gene, the melting curve revealed amplification of another material and this probably was a primer dimer. This can misestimate quantification of the gene. For this reason another set of primers (Bio-Rad, USA) or the same primers used for PCR reaction (Table 2.2) was used again for qPCR

reaction. In addition, a new set of primers for *GAPDH* was tested again and applied for normalization of the *SLC22A1* gene.

For the new set of primers, a different thermal profile was applied. This included three minutes at 95 °C for the first cycle or activation step, 20 seconds at 95 °C, 45 seconds at 60 °C, and 30 seconds at 72 °C for 40 cycles. The run also includes melting or dissociation curve.

The efficiency of the new primers was obtained by carrying out serial dilutions as well. The amplification plots and standard curve were obtained by the same software as described above (Figure 2.4).

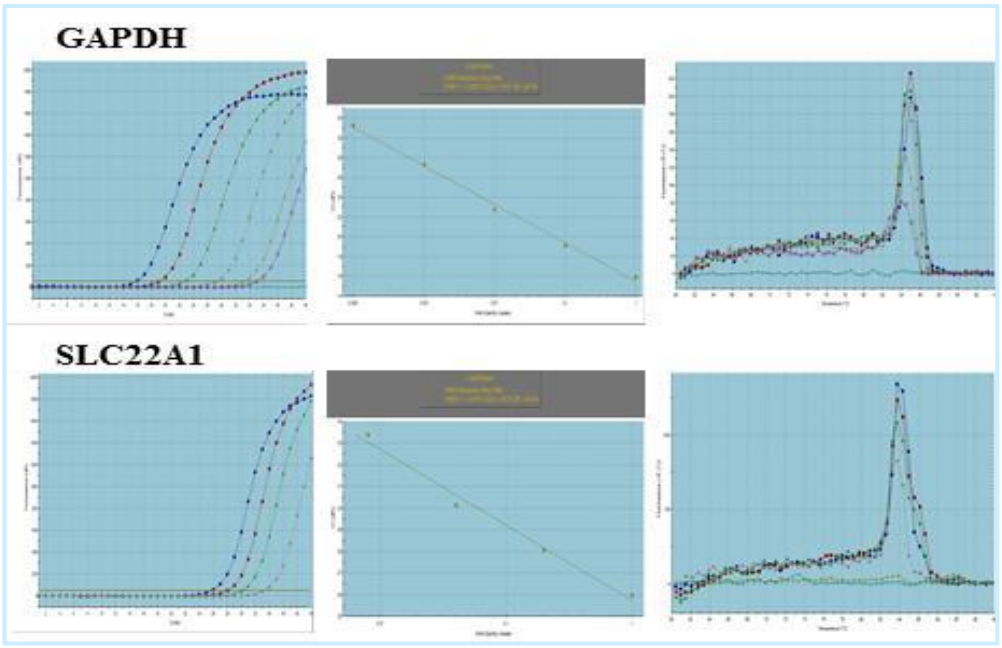

Figure 2.4: Amplification plots, standard curves, and melting curves for 2 genes

The primers of HKGs, *GAPDH* and *SLC22A1* were the same as those used for the normal PCR reaction. The primers efficiency was obtained by performing serial dilution of the primers. Ten-fold dilution was applied for *GAPDH*, and five-fold serial dilution for *SLC22A1*. The left panel represents the amplification plot figures for the two genes, the middle one represents the standard curves, and the right side shows the melting curves.

Relative gene expression was calculated by the ΔΔ method:

1- Relative quantification = $2^{-\Delta\Delta ct}$

2- ΔCt = C_t GOI – C_t of reference gene

3- Fold change = $E^{\Delta ct}$ GOI/$E^{\Delta ct}$ reference gene

Where GOI stands for gene of interest, E is the efficiency of reaction. C_t is the threshold cycle that gives detectable fluorescence signal above background. *GAPDH* was used as the reference gene to normalize OCT expression level.

2.2.6 Protein quantification by In-Cell Western™ Assay

2.2.6.1 In Cell Western™ assays

In Cell Western assay (ICW) is one of the assays that can be applied to relatively quantify protein expression of the gene of interest by employing an immunocytochemistry (ICC) technique. ICW assay has been widely used for various purposes such as analysis of protein phosphorylation, gene expression, and cell surface proteins quantification.

The main steps of ICC involve an antigen-antibody reaction [275]. Firstly, a primary antibody (Ab) is employed to detect the protein of interest. The next step of the reaction involves tracing the primary Ab by adding a fluorescently labelled Ab which binds to the primary Ab. Finally, the level of the expressed protein can be quantified by measuring the intensity of the fluorescently labelled secondary Abs as shown in Figure 2.5. Various methods are available for protein level detection. One of which is the Western Blot (WB) technique. WB can be carried out by following different steps; separation of proteins depending on their molecular weight by running polyacrylamide gels, transferring into nitrocellulose membranes, blocking, adding primary Abs, washing, and incubation with fluorescently labelled secondary Abs, and finally scanning [276]. However, we have applied ICW for quantifying the level of OCT1 and OCTN2 proteins.

For ICW, monolayers have to be fixed first on a 384-well or a 96-well plate. This assay can detect two types of primary Abs simultaneously as the equipment LI-COR, an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE, USA) detects two wavelengths which are close to spectrum, 700 nm and 800 nm. This allows normalizing the examined target gene between all examined wells. This assay can be applied to relatively detect protein expression and changes in protein levels after treating the cells with inhibitors or stimulators since it yields significant throughput and sensitivity.

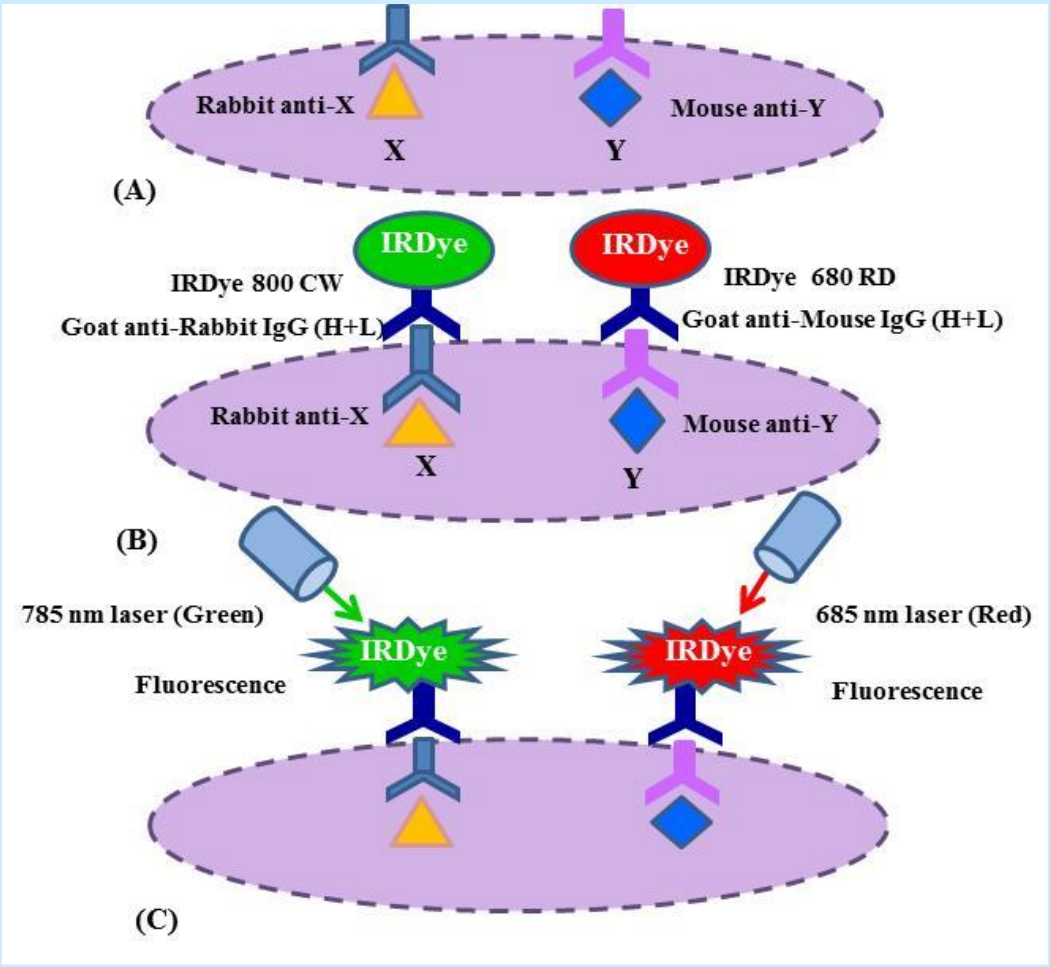

Figure 2.5: ICW assay principles.

(A) Represents the interaction between antigens, x and y for the gene of interest and the normalizer, respectively with the primary antibody (blue and purple). (B) Shows the interaction between primary Abs and IRDye fluorescence labelled secondary antibodies (green and red). (C) Detection of fluorescence by applying two different wavelengths by LI-COR Odyssey imaging system, 785 nm and 685 nm for protein of interest and reference protein, respectively. The fluorescence green or 785 nm wavelength is for detection of OCT1 or OCTN2 proteins, while, the fluorescence red is for the reference gene, *GAPDH*.

2.2.6.2 Primary and secondary antibodies employed

Protein level of OCT1 and OCTN2, and housekeeping gene, *GAPDH* as normalizer was detected and quantified on fixed A549 monolayers using three primary antibodies. These included rabbit anti OCT1, cat# OCT1-1-A, rabbit anti OCTN2, cat# 1-A (Alpha

Diagnostic Intl. Inc.). The monoclonal anti-*GAPDH* antibody produced in mouse was applied to target the protein expression for the reference gene, (Sigma-Aldrich, USA). The secondary antibodies used in the current investigation were a goat anti-rabbit IGg (IRDye 800® CW conjugated) for *SLC22A1* and *A5*, and a goat anti-mouse IGg (IRDye 680® CW conjugated) for detection of *GAPDH*. They were purchased from LI-COR Biosciences UK Ltd (Cambridge, UK).

2.2.6.3 Visualization options

The tool, LI-COR Odyssey infrared imaging system was applied to scan plates treated with the IRDye fluorescently labelled secondary Abs with two distinct wavelengths, 680 nm and 800 nm. Setting of the software, image studio version 3.1, involved applying a 169 µm resolution, lowest quality, 2.5 and 2 as the intensity of the 685 nm red signal and the 800 nm green signal, respectively and an offset of 3. The data, images and values of intensity for both wavelengths were collected using the software, and then were transferred to an excel sheet.

2.2.6.4 Analysis of data

ICW results were collected by the LI-COR Odyssey software. The green signal represents the fluorescently labelled secondary antibody, goat-anti rabbit 800 nm which reports the intensity for

OCT protein, while the red colour refers to the signal reported from the goat-anti mouse secondary antibody (680 nm) which reflects the intensity of housekeeping gene *GAPDH*. The experiments were designed to include two types of controls. The first one was added only the fluorescently labelled secondary Abs (blank) in order to assess unspecific binding of fluorescent Abs. The second control (background) was to add only the GAPDH primary Ab, and the fluorescently labelled secondary Abs. This was applied to calculate the percentage of protein expression above the background. Normalization of wells was conducted by calculating the ratio between 800 nm and 685 nm values for each well after subtraction of the corresponding values obtained for blank controls. The expression of proteins was calculated as a percentage above the background as shown in the equation below.

Background control (C) = 800/700

Percentage of protein expression above the background = [(800/700) of basal or treated–C) / C] x 100

2.2.6.5 Validation of primary antibody dilutions

Cultured adenocarcinoma A549 cells seeded at a density of 25-30 x 10^3/well were fixed with 3.7% paraformaldehyde (PFD) when they were confluent. The monolayers were first washed with iced PBS, and then 500 µl of PFD was added per a well for twenty minutes. Thereafter, the fixative liquid was gently aspirated and cells were

washed again, then 0.5 ml of PBS was added onto the monolayers and the plate was either kept at 4 °C or it was processed immediately. The important steps after fixation involved cell permeabilization with a solution consisting of 1% BSA, 0.15% Triton-x100 in PBS, 4x for 5 minutes at room temperature with gentle shaking, then they were blocked with human serum type AB, male, (Sigma-Aldrich) 1:25 in PBS for 1 hr at room temperature with gentle agitation. Primary Abs were prepared at different ratio or dilutions, 1:100, 1:200, 1:400, and 1:800 for OCT1 protein, and 1:300, 1:400, 1:600, and 1:800 in 0.1% BSA in PBS for OCTN2 protein, while the concentration of *GAPDH* antibody was kept constant as 1:600 for all wells except for the blank controls (no primary Abs were added). 200 µl of each dilution was added onto the wells, and then the plate was placed at 4 °C overnight with gentle shaking. Before processing or adding the secondary Abs, all wells were washed with a solution containing 0.05% Tween-20 and 0.1% BSA in PBS, 3x for 10 minutes. Secondary Abs were diluted as 1:1000 in 0.1% BSA in PBS, and 200 µl was added/well and the plate was placed at 37 °C for 30 min under shaking to provide an equal distribution across the monolayers. Again these were washed with the same solution, three times for 10 min, and 2x for 5 min in order to remove unbound Abs and other substances that may affect scanning. The last step involved quick washing of the plate before scanning using

the Odyssey® Infrared Imaging System, LI-COR Biosciences (NE, USA). The fluorescence intensity of secondary Abs for prepared dilutions was plotted as shown in Figure 2.7 and Figure 2.9. Images for OCT1 and OCTN2 primary Abs dilutions were obtained from the software as shown in Figure 2.6 and Figure 2.8.

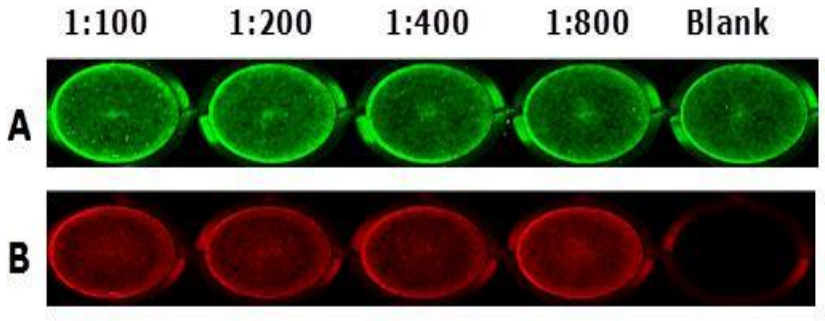

Figure 2.6: Validation of the primary antibody concentration for OCT1

(A) Green signal of fluorescently labelled secondary antibody for *SLC22A1*, goat-anti rabbit 800 nm using different dilutions, 1: 100, 200, 400, and 800, (B) red signal for fluorescently labelled *GAPDH* secondary antibody, goat-anti mouse 680 nm. *GAPDH* primary Ab was added to all wells at a 1:600 dilution except for the blanks (no primary antibody was added), but the two fluorescently labelled secondary Abs were added to all wells.

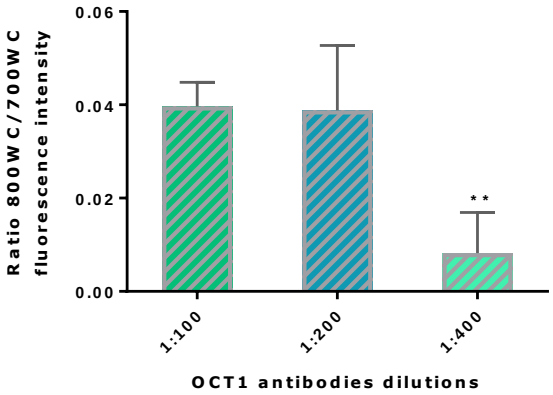

Figure 2.7: Fluorescence intensity as a function of OCT1 primary Ab dilutions.

The symbol (*) indicates significant decrease in intensity of the ratio 1:400 compared to 1:100 and 1:200 dilutions using one way ANOVA test followed by post-hoc Tukey, $P < 0.05$, the experiment was conducted once in 4 wells. The dilution 1:200 was selected to assess OCT1 protein expression.

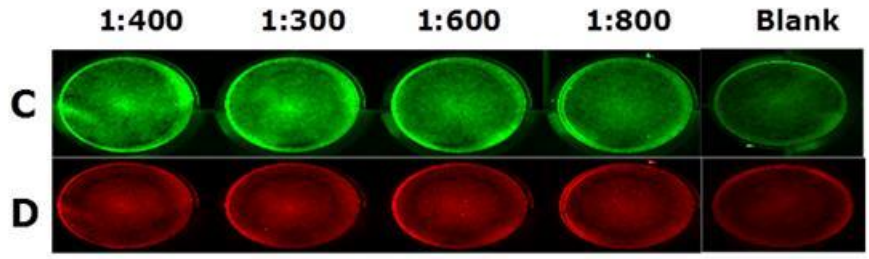

Figure 2.8: Validation of primary antibody concentration for OCTN2 transporter.

(C) Green signal of fluorescently labelled secondary antibody for *SLC22A5* (OCTN2) using different dilutions, 1: 300, 400, 600, and 800, (D) red signal for *GAPDH* fluorescently labelled secondary antibody. *GAPDH* primary antibody was added to all wells at a dilution of 1:600 except for the blank controls (no primary antibody was added). The two fluorescently labelled secondary Abs, goat- anti mouse 680 nm for red signal and goat anti-rabbit 800 nm for green were added to each wells.

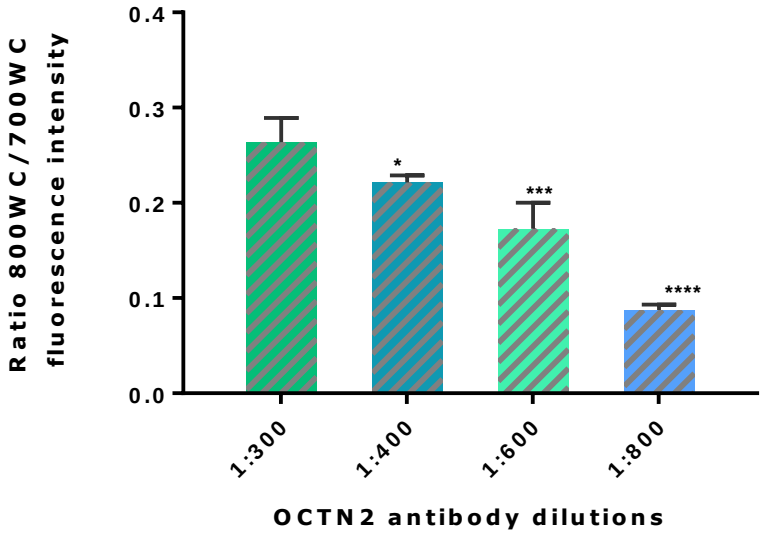

Figure 2.9: Fluorescence intensity as a function of OCTN2 primary Ab dilutions.

The symbol (*) indicates significant decrease in ratio of dilutions 1:400, 1:600 and 1:800 compared to 1:300 dilution using one way ANOVA test followed by post-hoc Tukey, $P < 0.05$,the experiment was conducted once in 4 wells. The least difference was found between 1:300 and 1:400 dilutions, therefor, 1:400 dilution was selected to be used for OCTN2 protein quantification.

2.2.6.6 *SLC22A1* and *A5* protein expression in A549 cell line

SLC22A1 and *A5* protein levels were quantified in fixed A549 monolayers after validation of the primary Abs for both genes. The cells were cultured at a density of approximately 30 x 10^3/well on a 24-well plate in presence of two various batches of Sigma serum. After reaching confluency within 3-4 days they were fixed and prepared for ICW assay as previously mentioned. *GAPDH* primary Ab was employed for normalization of the cell number between treated wells. The data were collected in triplicate or quadruplicate and analysed by the software image studio version 3.1.

2.2.7 Statistics

Each set of experiment was conducted on 3 or 4 or 12 wells per a group, and the values represent mean ± standard deviation (SD) or standard error of mean (SEM) for repeated experiments. Different passage numbers were used for repeated experiments. The results were analysed by either student's–t test for comparing two groups or one way analysis of variance (ANOVA) test, followed by a Tukey post-hoc test for comparing more than two groups. Differences between groups were considered to be significant at $p<0.05$. All data were analysed statistically using Graphpad prism software version 6.0 (San Diego, California, USA, (www.graphpad.com). The figures were also obtained using the same software.

2.3 Results

2.3.1 Effect of the serum on Calu-3 and Caco-2 cell growth

Sera from two companies, Sigma and Biochrom were examined for validity in growing both Calu-3 cells and Caco-2 cells. Growing of the two cell lines was monitored in a 75 cm^2 flasks and on the Transwell® supports by optical microscopy and measuring TEER values, respectively. Cells showed significant growing and spreading out with Sigma serum in the flask and on the Transwell® supports. The wells revealed gradual increase in the TEER value from day 9 until day 21 as shown in Figure 2.10. However, slow

growing of the cells in the flask was observed with Biochrom serum compared with the Sigma one. When the cells were seeded on the Transwell® supports, TEER values were less than those reported at the same day for their counterparts grown with Sigma serum. Calu-3 cells did not spread to from a confluent monolayer; instead, they grew as clumps or multiple cell layers. Regarding Caco-2 cells, growth in the flask was normal and no delay in reaching confluence was observed for both types of serum. Although their growing on Transwell® supports was significant, TEER values obtained with Biochrom serum were less than those measured when cells were grown with Sigma serum (Figure 2.11).

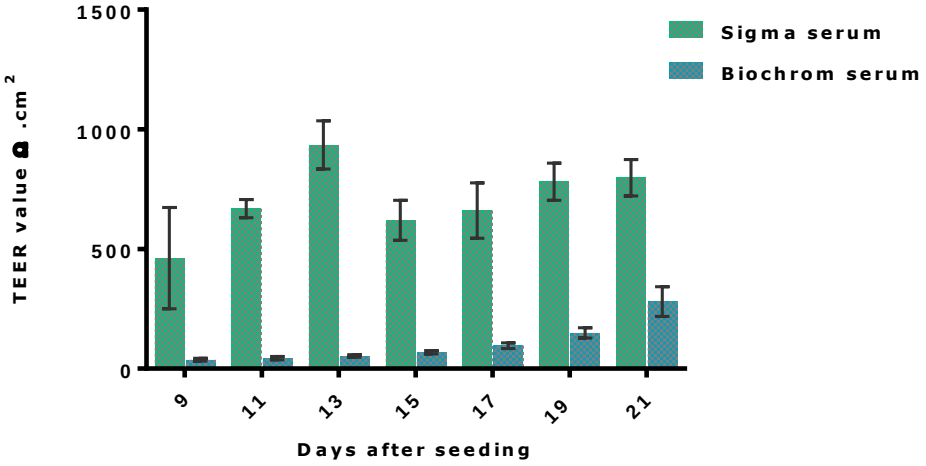

Figure 2.10: TEER values for Calu-3 layers cultured on Transwell® supports in presence of two types of serum.

Data presented as mean ± SD, the experiment was conducted once in 12 wells.

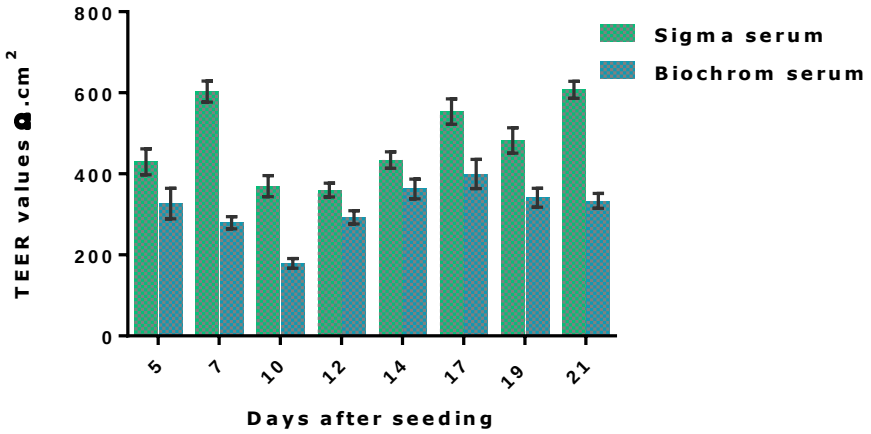

Figure 2.11: TEER values for Caco-2 layers cultured on Transwell® supports in presence of two types of serum.

Data presented as mean ± SD, the experiment was conducted once in 12 wells.

2.3.2 Semi-quantitative PCR

OCT/Ns gene expression on A549 cells, Calu-3 cells, and Caco-2 cells was assessed using semi-quantitative PCR. From the visualized images, it was confirmed that mRNA of all members of *SLC22A* family was expressed in Caco-2 cells cultured with the two different types of serum as shown in Figure 2.12 A and B, while four of the members were observed in Calu-3 cells. Indeed, no band was detected for OCT2 as shown in Figure 2.12 C.

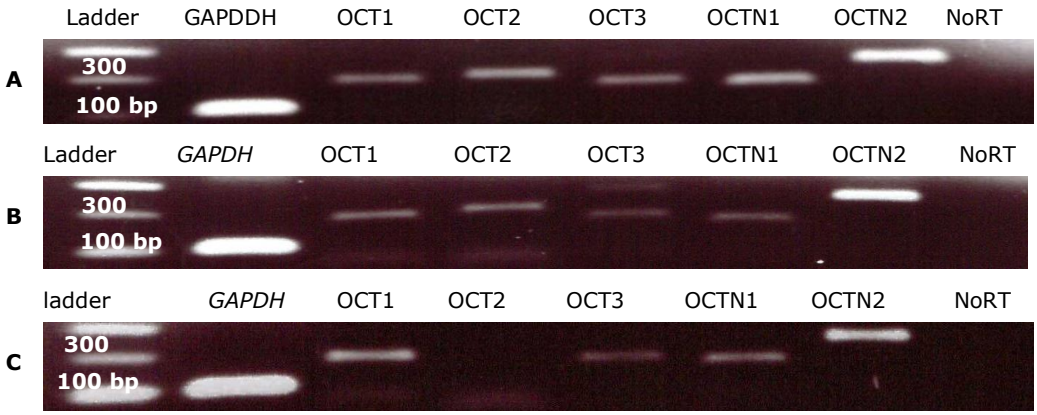

Figure 2.12: PCR gel electrophoresis for amplified products of six genes for Caco-2 cell line cultured with two different types of sera and Calu-3 cells cultured with the Sigma serum.

(A) Caco-2 cells with Sigma serum (B) Caco-2 cells with Biochrom serum, and (C) Calu-3 cells cultured with the new batch of Sigma serum. The size bp for all amplicons was as expected. The gel was run for three different passages

Regarding A549 cells, bands were detected for OCT1, OCT3, OCTN1 and OCTN2 transporters, while OCT2 was not expressed as shown in Figure 2.13 D, E, and F. No differences in the band presence or brightness were observed for cells grown in different sera.

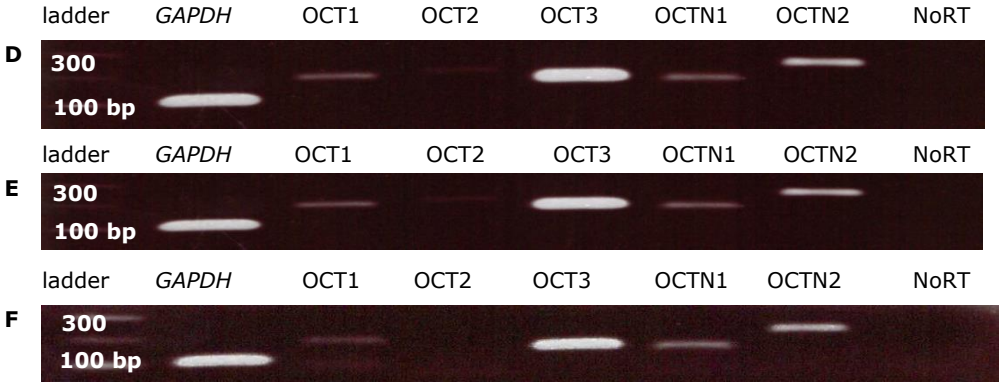

Figure 2.13: PCR electrophoresis gel for amplified products of six genes for A549 cells cultured with three different types of serum.

(D) New serum from Sigma, (E) Biochrom serum, and (F) an old serum batch from Sigma. NTC was run alongside all samples. The size of all amplified products was as expected. The gel was run for three different passages

2.3.3 Sequencing

As described before, all amplified products for all genes were purified and sent for sequencing. The amplicons resulting from sequencing were blasted against the original genes, using the web site (http://www.ncbi.nlm.nih.gov/). The Chromas software was employed to generate figures for all amplicons resulting from sequencing as shown in Figure 2.14. The amplicons aligned with the original genes, and this indicates that the primers used for amplified the genes of interest.

OCT1

Score	Expect	Identities	Gaps	Strand
263 bits(142)	2e-67()	145/146(99%)	1/146(0%)	Plus/Plus

```
Query  22          AACACGATTTACCTTAAGGTCCAACCTCAGAACCCTCGGGCACCTGAGAGAGATGTTTT  80
                   |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  158049357   AACACGATTTACCTTAAGGTCCAAACCTCAGAACCCTCGGGCACCTGAGAGAGATGTTTT  158049416

Query  81          GCGGCGATGTCGTGTTGGAGGGATGAAGATGGAGTTATCCTCTGCAGAAATTCCTAGACG  140
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158049417   GCGGCGATGTCGTGTTGGAGGGATGAAGATGGAGTTATCCTCTGCAGAAATTCCTAGACG  158049476

Query  141         CCTTCACTTCTCTGTATTCTTCCTCA  166
                   |||||||||||||||||||||||||
Sbjct  158049477   CCTTCACTTCTCTGTATTCTTCCTCA  158049502
```

OCT2

148 bits(80)	2e-33()	80/80(100%)	0/80(0%)	Plus/Minus

```
Query  93          GGTGCATACCTGAGTCTCCCAGGTGGCTGATCTCCCAGAATAAGAATGCTGAAGCCATGA  152
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160668331   GGTGCATACCTGAGTCTCCCAGGTGGCTGATCTCCCAGAATAAGAATGCTGAAGCCATGA  160668272

Query  153         GAATCATTAAGCACATCGCA  172
                   ||||||||||||||||||||
Sbjct  160668271   GAATCATTAAGCACATCGCA  160668252
```

OCT3

163 bits(88)	6e-38()	91/92(99%)	1/92(1%)	Plus/Plus

```
Query  12          GTC-ATGCGTGGATGCTGGACCTCACCCAAGCCATCCTGAACCTCGGCTTCCTGACTGGA  70
                   ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158293634   GTCAATGCGTGGATGCTGGACCTCACCCAAGCCATCCTGAACCTCGGCTTCCTGACTGGA  158293693

Query  71          GCATTCACCTTAGGCTATGCAGCAGACAGGTA  102
                   ||||||||||||||||||||||||||||||||
Sbjct  158293694   GCATTCACCTTAGGCTATGCAGCAGACAGGTA  158293725
```

OCTN1

211 bits(114)	2e-52()	122/125(98%)	3/125(2%)	Plus/Plus

```
Query  8           GCA-TGGG-AGCATGCGGGA-TACGACGAGGTGATCGCCTTCCTGGGCGAGTGGGGGCCC  64
                   ||| |||| |||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  126822559   GCAGTGGGAAGCATGCGGGACTACGACGAGGTGATCGCCTTCCTGGGCGAGTGGGGGCCC  126822618

Query  65          TTCCAGCGCCTCATCTTCTTCCTGCTCAGCGCCAGCATCATCCCCAATGGCTTCAATGGT  124
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126822619   TTCCAGCGCCTCATCTTCTTCCTGCTCAGCGCCAGCATCATCCCCAATGGCTTCAATGGT  126822678

Query  125         ATGTC  129
                   |||||
Sbjct  126822679   ATGTC  126822683
```

OCTN2

237 bits(128)	5e-60()	128/128(100%)	0/128(0%)	Plus/Plus

```
Query  47          GGTTCATCCCTGAGTCCCCCCGATGGCTCATCTCTCAGGGACGATTTGAAGAGGCAGAGG  106
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126914962   GGTTCATCCCTGAGTCCCCCCGATGGCTCATCTCTCAGGGACGATTTGAAGAGGCAGAGG  126915021

Query  107         TGATCATCCGCAAGGCTGCCAAAGCCAATGGGATTGTTGTGCCTTCCACTATCTTTGACC  166
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126915022   TGATCATCCGCAAGGCTGCCAAAGCCAATGGGATTGTTGTGCCTTCCACTATCTTTGACC  126915081

Query  167         CGAGTGAG  174
                   ||||||||
Sbjct  126915082   CGAGTGAG  126915089
```

Figure 2.14: Identity between sequence of amplicons and the original gene sequence.

Query is referred to amplified amplicon and subject represents the sequence available in database. Expect (E) describes the random background noise.

2.3.4 Quantitative polymerase chain reaction

The level of the *SLC22A1* mRNA was quantified in A549 cells and Calu-3 cells by applying real time polymerase chain reaction. Regardless of the type of serum, the level of *SLC22A1* mRNA was found to be significantly more highly expressed in Calu-3 than in A549 cells as shown in Figure 2.15 A. The level of *SLC22A1* mRNA in A549 was not significantly different when cells were cultured in the two different batches of Sigma serum

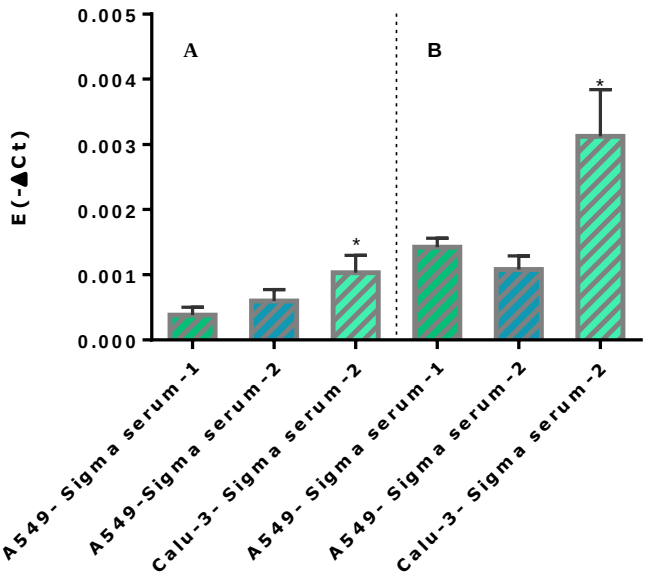

Figure 2.15: Expression level of *SLC22A1* (A) and *SLC22A5* (B) mRNA in A549 and Calu-3 cells as measured by qPCR.

A549 cells were grown in submerged conditions on 24 well plates using an old (Sigma serum-1) and new batch (Sigma serum-2) of serum, while Calu-3 cells were cultured in ALI conditions for 21 days on Transwell® inserts in presence of the new batch of serum(Sigma serum-2). Data are presented as mean ± SEM, (replicate, n=3, n represent no of experiment). The primers used for quantification of the gene are shown in Table 2.2 and Table 2.3. The gene expression was normalized to the *GAPDH* housekeeping gene. * indicates significant difference in the OCT1 mRNA level compared to that in A549 treated with Sigma serum-1.

2.3.5 OCT1 and OCTN2 protein expression in A549 cells

The protein levels of the two members of organic cation transporter family, OCT1 and OCTN2 was quantified in fixed monolayers of the A549 cell line. Both OCT1 and OCTN2 proteins were shown to be expressed in the A549 cell line. No significant difference in OCT1 or OCTN2 protein expression was detected when cells were cultured with the two different batches of serum (Figure 2.17). The A549 cells cultured with the two batches were imaged as well (Figure 2.16)

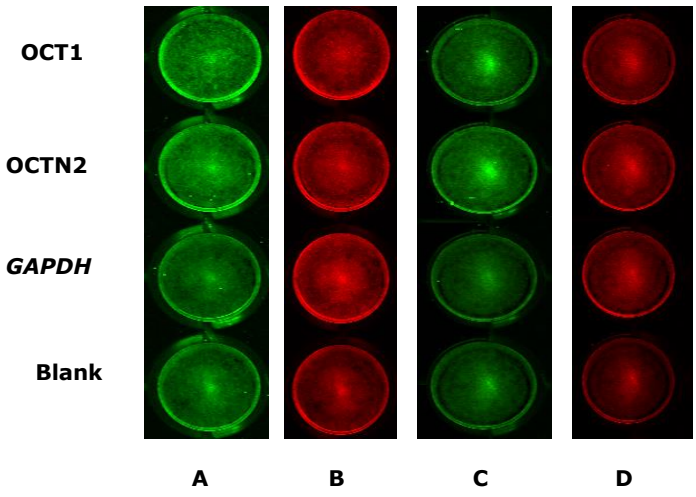

Figure 2.16: ICW scan images of A549 cultured in Sigma serum batches 1 and 2.

(A and B) represent images for Sigma serum batch 1, and (C and D) refer to Sigma serum batch 2. (A and C) show the intensity of the goat-anti rabbit, IRDye labelled secondary Ab which reflects OCT protein expression, while (B and D) represent the signal for *GAPDH*, as show via the intensity of the goat-anti mouse, IRDye labelled secondary Ab.

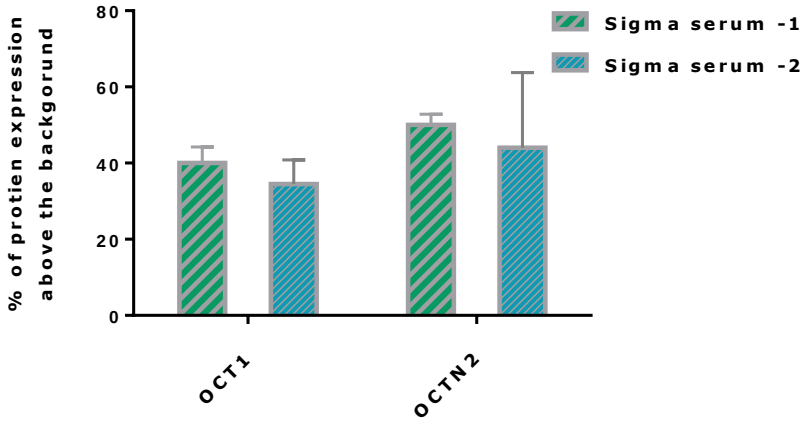

Figure 2.17: Percentage of OCT1 and N2 protein expression above the background level in A549 cell line cultured with the two batches of Sigma serum.

Data are presented as means ± SED, (triplicate, n=2, n represents no of experiment). No significant difference was observed between the two batches of Sigma serum using (Student's t test, p≥ 0.05). All expression experiments were carried out using Sigma serum-2.

2.4 Discussion

2.4.1 Effect of the serum on Calu-3 and Caco-2 growth

Two types of serum were tested on the bronchial cell line, Calu-3, and the intestinal cell line, Caco-2. Both of the cell lines were cultured on Transwell® supports for 21 days and the formation of tight monolayers was monitored by measuring TEER. This was conducted as a result of poor proliferation of Calu-3 cell line with a previous batch of Sigma serum. Reporting TEER values was commenced when the monolayers had formed, and thereafter it was measured every three/four days.

Regarding Calu-3 monolayers, the reported TEER values were higher for wells supplied with Sigma serum compared to their

counterparts cultured with Biochrom serum. As it is known that TEER values reflect acceptable connectivity and firm attachment between adjacent cells, this suggests that the quality of Sigma serum is more convenient for the cell requirement and proliferation. However, Calu-3 cells cultured with Biochrom serum did not form monolayers even after 21 days of culturing. This data presumably indicates that the two types of serum vary in their compositions, which in turn, has various effects on cell growing and formation of monolayers.

Concerning Caco-2 cells, it was shown that TEER values for this cell line cultivated with Sigma serum were higher compared to those obtained with the Biochrom serum. This again confirmed that the quality of Sigma serum was more beneficial than the one from Biochrom. However, it should be mentioned that one batch of Biochrom serum was tested, and untested batches might be as beneficial as Sigma one.

In terms of serum impact on cell proliferation, it has been demonstrated that serum can act either as a stimulator or as an inhibitor simultaneously. These two contradictory effects and complicated responses result from substances that can cause cell duplication or cease their proliferation. Some of the substances which are available in foetal bovine serum include several of biological origin, for example, proteins, lipids, hormones, growth

factors, vitamins, in particular lipid soluble vitamins, spreading factors, inhibition factors, and factors that boost attachment of cells on plastic plates or flasks.

Different batches of serum can also cause variable behaviour in cell growing depending on some parameters such as the method serum was produced or the nutrient given to the pregnant cows whose foetuses were used to collect serum [277].

2.4.2 Expression of mRNA

The intestinal adenocarcinoma cell line Caco-2 was used as a positive control to validate primers as they are known for their OCT/Ns expression [267, 269]. By applying normal PCR we have confirmed that the five members of OCT/Ns family were expressed in the differentiated Caco-2 cells treated with two types of sera, Sigma and Biochrom.

We have found that, in Calu-3 and A549 cells, the mRNA for four genes *SLC22A1*, *A3-A5* was observed in gel electrophoresis image, whilst no expression for *SLC22A2* was detected. Real time PCR also confirmed that mRNA of OCT1 and OCTN2 genes was expressed in both cell lines. However, OCT1 mRNA was found to be highly expressed in differentiated Calu-3 cells compared to A549 cells. Similarly to OCT1, OCTN2 mRNA was highly present in the bronchial cell line compared to the alveolar cell line.

In terms of OCT/Ns expression in the differentiated Calu-3 cells, our findings were in agreement with results obtained from the undifferentiated Calu-3 on day 8 and 15 of culturing using PCR [267]. Again, our results were similar to those showed in 21-day old Calu-3 cell layers [228]. However, the same group showed absence of OCT2 and OCT3 in submerged Calu-3 and in a 14-day old Calu-3, suggesting that culture condition and length of culturing has an obvious effect on gene expression level. Higher level of OCTN2 compared to OCT1 transporter in Calu-3 using qPCR was also similar to that obtained [278]. A more recent study has demonstrated that the level of OCT1 and OCT3 mRNA was expressed in these cells using qPCR [279].

In respect to OCT/Ns expression in A549 cells, our results were identical to those obtained using normal PCR [267]. Similarly to our findings, A549 cells were shown to express mRNA of OCT3, OCTN1 and 2 using PCR [232]. In contrast to our result, the same study did not detect OCT1 in these cells. This is probably because the F12K medium was used for culturing A549 cells, whereas we used DMEM. The passage number of the cells might also have affected expression but was not mentioned in the study. The A549 cells were found to express OCT1 and OCT3 using traditional PCR [226]. The A549 cells were shown to express low level of OCT1 mRNA compared to other lung cancerous cell lines using qPCR

[280]. Again, our observations were in agreement to those obtained using the same technique [229]. By applying qPCR, a more recent study has shown that A549 cells expressed mRNA of OCT1 and OCT3 transporters, with higher level for OCT3 [279]. Our results in respect to OCT1 mRNA expression were in agreement with most of expression studies, and low level of this member was identified using qPCR technique.

2.4.3 Protein expression

Regarding quantification of protein level, we have relatively quantified OCT1 and OCTN2 protein level on A549 fixed monolayers. This was carried out by performing ICW technique which was previously described. We have selected these two proteins based on the fact OCT1 protein is involved in the translocation of acetylcholine synthesize in airway epithelial cells, which is suggested to be implicated in airway remodelling and the OCTN2 gene was found to be involved in asthma susceptibility [233].

Variable Abs dilutions were used for quantification of OCT1 and OCTN2 proteins 1:200 and 1:400, respectively. A549 cells cultured in different Sigma serum batch numbers showed expression of both proteins and no difference in protein expression levels were reported. Our data in terms of OCT/Ns protein expression in A549 cells were similar to those obtained applying western blot [232,

269]. Similarly to our result, More et al., have shown that OCT1 protein was expressed in A549 using western blot [280]. In our work, using more diluted Ab for OCTN2 protein suggests higher level for this transporter compared to OCT1 protein. Again, by using mass spectrometry, Sakamoto et al., observations in A549 cells in respect of OCT1 expression were in agreement with our result [270]. However, this study did not detect expression of OCTN2 protein in A549 cells. This is probably because no validation of the probes was conducted.

2.5 Conclusion

Alveolar A549 cells were shown to express mRNA of OCT1, OCT3, and OCTN1-2. Additionally, protein expression levels for OCT1 and OCTN2 transporters were detected and quantified via ICW assay. Based on expression OCT1 and OCTN2 as well as cholinergic system components including Ach, its synthesizing enzyme, muscarinic and nicotinic receptors, the A549 cells were selected to carry on the study. The next objective was probing OCT/Ns involvement in cell proliferation in the presence of well-known OCT/Ns inhibitors.

Chapter 3 Impact of Organic Cation Transporter Inhibitors on cell proliferation

3.1 Introduction

Organic cation transporters perform an essential function in transporting a variety of positively charged endogenous substances and xenobiotic compounds through cell membranes. The transportation of these molecules is mediated by one or more members of the *SLC22A* family [6, 208]. Acetylcholine is one of the endogenous substances that was shown to be transported via OCTs, particularly OCT1 [163, 164], and was suggested to contribute to cell proliferation in the respiratory epithelium [168].

OCT/Ns structure was previously described in Figure 1.6. It is crucial to know which domains that form their common structure are involved in carrying the molecules through biological membranes in a bidirectional movement. A few studies emphasized on probing which domains/amino acids are involved in transporting cations or forming binding sites by introducing mutation or replacement of certain amino acids. These investigations have collectively demonstrated that seven amino acids are implicated in the binding region in rOct1 [281-283]. Three of these are located in the fourth transmembrane helix domain: Trp218, Tyr222, and Thr226 [283] ; another three are existent in the tenth domain:

Ala443, Leu447, and Gln448 [282], and the final amino acid is located in the eleventh domain, Asp475. Together, these amino acids form a large pocket by assembling in close space where the substrates/inhibitors bind, and either translocate/inhibit the movement of molecules from the extracellular environment into the intracellular space and vice versa. From these observations, it has been demonstrated that amino acids are involved in the selection of substrates and/or inhibitors by the transporter based on their affinity for these residues.

OCT/Ns have been transfected into various models such as oocytes *Xenopus laevis*, the human embryonic kidney (HEK293), Hela cells (human cervical tumour), Chines hamster ovary (CHO), and Human retinal pigment epithelial (HRPE) cell lines in order to determine the functional properties of these transporters. In addition, human tissues and primary cultures have been employed. The experimental conditions were variable in terms of exposure time or applied temperature, but the objective of the studies was probing the activity of these proteins in the cell type/tissue under investigation.

The most frequently applied cations in the uptake investigations are 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+), 1-methyl-4-phenylpyridinium (MPP^+), and Tetraethylammonium (TEA). These model cations have been shown

to be substrates for more than one member of the *SLC22A* family. For example, ASP^+ was identified as a substrate of hOCT1 [212], hOCT2 [284], hOCT3 [285], hOCTN1 and 2 [222, 285]. However, Grigat et al., have shown that this was not a substrate of OCTN2 [286]. TEA cation was found to be transported via hOCT1 [287], hOCT2 [203], and hOCT3 [288]. TEA was also shown to be a substrate of OCTN1 [289, 290], and OCTN2 [291]. MPP^+ was shown to be transported via hOCT1-3 [203, 287, 288].

Using model cations in transfected cells, several drugs have been found to cause inhibitory effects on at least two or more of the OCT/Ns proteins such as quinine, corticosterone, salbutamol, and ipratropium [7, 163, 208, 231, 291-294]. These chemicals can be substrates and inhibitors in a concentration-dependent manner or only inhibitor without being transported. For example, corticosterone is well known to cause inhibition of OCT1-3 proteins in humans and rats, its affinity for OCT3 is higher than OCT2 and OCT1 [208, 295, 296].

Based on previous studies, we have selected some inhibitors of the OCT/Ns transporters to screen their effects on cell proliferation and wound closure using wounded layers of alveolar adenocarcinoma cell line A549. Epithelial wound repair either of the bronchial epithelium or alveolar epithelium is a complex process and involves a variety of growth factors, cytokines, chemokines, interleukins,

prostaglandins as well as the extracellular matrix. The soluble molecules were shown to be secreted via fibroblasts, capillary endothelial cells, macrophages, and airway epithelial cells both from damaged and adjacent cells. These mediators were identified as important regulators of cellular repair events such as spreading, migration and proliferation. These crucial events in the airway epithelium wound repair were found to be mediated via specific cellular signalling pathways such as sonic hedgehog, Rho GTPases, mitogenic activated protein kinase pathways, STAT3, and Wnt [297]. The impact of these molecules and the induced signalling pathways has been widely investigated in respiratory primary cultures and cell lines from humans and animals.

Mechanically induced wound in rat primary alveolar type 2 cell were shown to be primarily repaired/closed as a result of two main events, cellular spreading and migration. These two fundamental processes were induced via cytokine IL-1β in the presence of mediators such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) [298, 299]. IL-1β cytokine was also identified as a stimulator of hepatocyte growth factor from cultured fibroblasts via a cyclooxygenase (COX-2) dependent mechanism [300]. In addition to the role of IL-1β in respiratory wound repair, it has been reported that some of the growth factors such as hepatocyte growth factor (HGF) and keratinocyte growth factor

(KGF) were involved in wound repair and closure [301, 302]. HGF was shown to cause proliferation in rat alveolar type 2 cells in a concentration-dependent manner [303], as well as *in vivo* [304]. Lee et al., have found that adding HGF to human bronchial epithelial cells resulted in increase in COX-2 mRNA via mediating p42/p44 MAPK, Akt and beta-catenin pathways, and inhibition of COX-2 was reported to cause decrease in cell growth [305]. In addition to HGF role in cell growth, Myerburg et al., have found that differentiation of human bronchial epithelial cells cultured with fibroblasts was attributed to stimulation of HGF which is mainly secreted by the fibroblast [306]. The KGF was also shown to exhibit proliferation effect in alveolar type 2 in lungs of adult rats [307-309]. Additionally to mitogenic activity on alveolar type two cells, it has been demonstrated that KGF stimulated cell migration via promoting special protrusions known as lamellipodia toward migration, and reducing the rigidity of the cytoskeleton [310], as well as cell adhesion via affecting the Rho A pathway [311].

Inflammatory mediators such as chemokines, eicosanoid and interleukins have also been shown to play an important role in respiratory epithelial wound repair. For example, chemokine receptor-3 (CCR3) was found to be highly expressed in asthmatic patients, and adding CCR3 ligands into BEAS-2B, 16HBE14o- cell lines and primary HBE induced wound repair [312]. Eicosanoid

such as prostaglandin 2 (PGE2) was found to participate in wound repair in the respiratory epithelium from human and feline, particularly in the early stage of wound, suggesting involvement of these molecules in spreading and migration. Interleukins have also been found to be implicated in lung wound repair, particularly IL-1 beta [313], IL-4 [314], and IL-13 [315] using primary HBE and 16HBE14o- cells.

In addition to growth factors and mediators, integrins, matrix and glycoproteins have also been shown to participate in wound repair. Integrins are cellular surface receptors that interact with the extracellular matrix and mainly consist of two subunits alpha and beta. These receptors play an important role in cell adhesion and migration during wound closure. Antibodies blocking alpha2-, alpha3-, and alpha 6-integrins were shown to decrease 16HBE14o- cell spreading and migration on collagen type five and that beta 1-integrin was required for cells to move on laminin type 1 or 2 [316].

Transforming growth factor beta (TGF-β), is another mediator which has been shown to be involved in lung wound healing mechanism in a complex and controversial way. For example, some studies showed its implication in cell migration using different models such as rabbit tracheal epithelium [317], 16HBE14o- [318], A549 cells and primary mouse alveolar type 2

cells [319]. Conversely, other investigations demonstrated that TFG-β exhibited an inhibitory influence on cell migration employing bovine bronchial epithelial cells [320], and primary airway epithelium [321]. This contrasting impact may be attributed to different examined cells.

Another important factor that participates in wound repair is matrix metalloproteinases (MMP), particularly type 1, 7 and 9. The MMP is a family of enzymes that modulate cell attachment via affecting the extracellular matrix. All of MMP types have been shown to participate in airway wound repair through inducing cell migration [322-324].

In addition to the implication of lung components in lung wound repair, another source unrelated to lung was identified to participate in airway epithelial wound repair, bone marrow-derived stem cell (BMSC). The BMSCs were found to participate in the healing of damaged epithelial cells [325], and showed their ability to differentiate into airway epithelial cells [326-328].

Overall respiratory wound repair/healing is a complex process that involves a variety of molecules and signalling pathways and is still incompletely understood. In this chapter we aimed to use a range of inhibitors to probe whether OCT/Ns proteins have a role in

respiratory epithelial wound healing using alveolar adenocarcinoma cells A549.

3.2 Materials and methods

3.2.1 Validation of cell proliferation and viability assay PrestoBlue

The PrestoBlue™ (PB) assay (Invitrogen, Life Technologies LTD) is one of the frequently applied assays for examining cell viability and proliferation *in vitro*. PB reagent consists of a blue-coloured non-fluorescent compound, known as resazurin. This compound is present in an oxidized form in the reagent. Viable cells have the capacity to reduce and convert resazurin into a pink fluorescent compound (resorufin) by the activity of metabolic enzymes in their cytosol [329-331]. The intensity of the fluorescent resorufin can be measured, and this reflects the quantity of viable cells.

To generate a standard curve for the PB assay, the A549 cells were cultured at various densities 5×10^3 –1.6×10^6 into a 12-well plate (CoStar Corning, Corning, UK) in triplicate. They were grown in Dulbecco's modified Eagle's medium as described in chapter two (2.2.1). The cells were placed at 37 °C for 6-8 hours to allow them to attach. PB was prepared at a 1:9 ratio with serum-free medium, and kept unexposed to light due to the reagent being light sensitive. Old media was aspirated and 0.5 ml of the reagent was

added into each well and the plate was placed into the incubator at 37 °C for 15 minutes. 100 µl of supernatant was transferred into a black 96-well plate (Costar), protected from light. Finally, the intensity of the reduced form of the reagent was read on a Tecan Infinite® M200 plate reader (Männedorf, Switzerland) at Ex560 nm/Em590 nm. The data was collected and arbitrary fluorescent units (AFU) were plotted against cell number. The relationship was linear with a R^2 value > 0.99 as shown in Figure **3.1**. This standard curve was used to estimate cell numbers under experimental conditions. The values of collected data were subtracted of the background before being used.

The PB assay was applied on scratched and unscratched monolayers of A549 to probe the effect of OCT/Ns inhibitors on cell proliferation and cell viability, respectively.

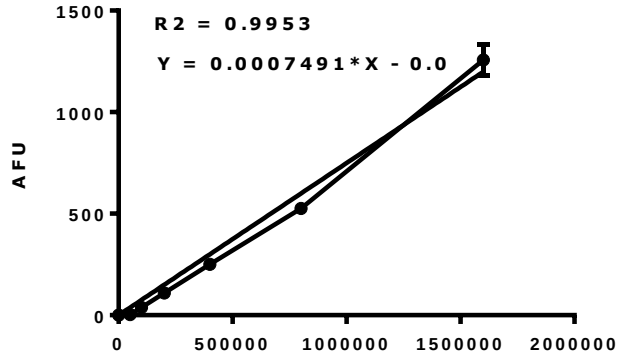

Figure 3.1: **Linear regression between A549 cell number and arbitrary fluorescent units.**
Each point represents mean of 3 wells/cell density ± SD.

3.2.2 Wounding assay on monolayers

Wounding of cell monolayers can be performed either by exposing them to chemicals or by scratching the monolayers using a sterile pipette tip or syringe.

Scratching the monolayers has been carried out by various researchers employing different cell types, either primary or cell lines, for example, primary corneal epithelial cells, the Madin-Darby Canine Kidney (MDCK) cell line and the 3T3 fibroblast cell line [332-340]. Different multi-well plates such as 6, 24, and 384-well plates as well as coverslips have been tested. All these studies focused on understanding cell signalling pathways that govern or coordinate cell migration or proliferation.

The scratch assay in the current study was applied to examine the role of OCT/Ns on cell proliferation. A 12-well plate was cultured with A549 cells at a density of $\leq 2x10^5$ per well. After 48 hrs of seeding, the confluency had reached approximately 95% and the monolayers were ready to be scratched, employing a-10 µl sterile pipette tip. Three scratches were made on the monolayers whilst they were still submerged with medium. After completing the scratches in all wells, the medium was aspirated and cells were thoroughly washed with PBS in order to dispose of cellular debris. Then, fresh medium was applied. The healing/wound closure was

monitored over 48 hrs via either PB assay or time lapse microscopy, in presence or not of inhibitors.

3.2.3 Time lapse microscopy

The scratched monolayers were prepared as previously described in (3.2.2). The time lapse microscope was applied to monitor healing of the scraped area through visualization of migration and proliferation of A549 cells. The Velocity software Version 5.4 was set up on power 5 to capture an image of the wounded confluent layers by the microscope camera every 2 hrs for 48 hrs (Hamamatus 1394 ORCA-285, Japan).

The scratch on untreated or control monolayers was repaired in 36 hrs as shown in Figure 3.9. The time lapse microscopy was also used to monitor wound repair in scratched A549 treated with TEA at 5 mM

3.2.4 Organic cation transporters inhibitors

3.2.4.1 Tetraethylammonium TEA

Tetraethylammonium TEA or $Et4N^+$, is a quaternary ammonium cation consisting of a central nitrogen atom and four ethyl groups (Figure 3.2). The positively charged compound, TEA has been widely employed to study OCT/Ns. It is well known that TEA is a substrate for rOCT1 and 2, hOCT1 and 2, and human extraneuronal monoamine transporter (hEMT) proteins [4, 341,

342], as well as human, rat and mice OCTN2 transfected in human retinal pigment epithelial cells [343]. However, Tamai et al., have shown that TEA has a low affinity for OCTN1 compared to other proteins [344].

In addition to being a substrate, TEA has an inhibitory effect on OCT/Ns as well. It has been demonstrated that the uptake of labelled TEA was inhibited by unlabelled TEA in Hela cells transfected with hOCT1, hOCT2, and rOCT1 proteins [345]. Lee et al., have shown that TEA led to significant inhibition of ethidium uptake in HEK293 cells overexpressing hOCT1, with different IC50 for the three expressed proteins [292]. It has been shown that L-[^{3}H] carnitine uptake in HEK293 transfected with hOCTN2 was significantly inhibited in the presence of 0.5 mM TEA [291]. TEA was applied in our experiments at 5 mM in an attempt to examine the effect of OCT/Ns on cell proliferation. This concentration was shown to inhibit all members of the transporters [208]. It is important to mention that it also causes inhibition of potassium ion channels either from the external or internal surface of the cell membrane, where the K^{+} ion channels are located [346-350].

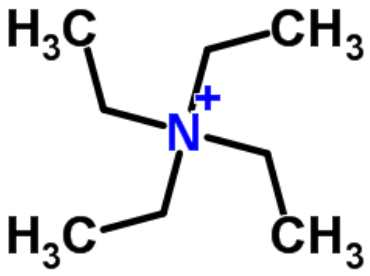

Figure 3.2: Chemical structure of tetraethylammonium ($C_8H_{20}N+$)

3.2.4.2 Quinine

Quinine was extracted from a tree bark, which is called cinchona (quina-quina) and has a chemical structure as shown in Figure 3.3. This drug has been employed for the treatment of malaria (anti-malaria) since the seventeenth century [351, 352].

Quinine is a positively charged drug, which can be either transported by OCTs or cause inhibition of transportation of other cations depending on its concentration. For example, quinine has been employed in transportation related-research to identify the functional characteristics of OCT/Ns [163, 291, 292, 341, 353]. It was reported that quinine is also involved in potassium ion channels inactivation [163]. In the present study, quinine was applied at three different concentrations: 0.01, 0.1, and 0.3 mM.

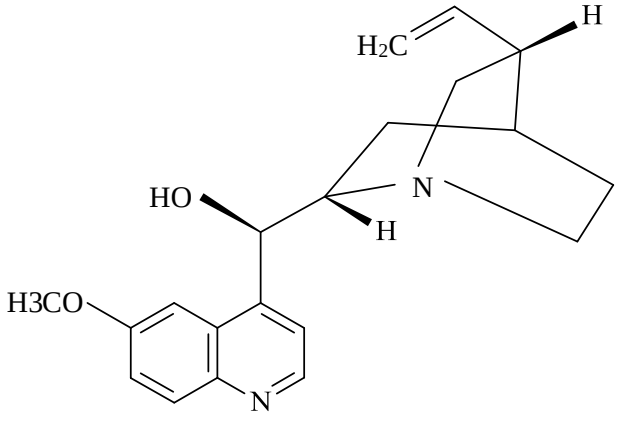

Figure 3.3: Chemical structure of quinine ($C_{20}H_{24}N_2O_2$)

3.2.4.3 Corticosterone

Corticosterone is a glucocorticoid hormone, mainly produced in adrenal gland in different species. Corticosterone has been reported to be synthesised as a result of adrenocorticotopic hormone (ACTH) signals. ACTH regulates the synthesis of a specific protein, which in turn, modulates the production of corticosterone hormone [354].

Natural or synthetic corticosterone is a neutral compound and passively permeates through biological membranes [355]. However, it has been shown to have an inhibitory impact on OCT1, 2, and 3, employing different cell lines and tissues derived from rodents and humans [4, 163, 197, 293, 295, 296, 355-357]. Wessler *et al*., have found that 0.1 mM of corticosterone led to a significant decrease in Ach release from human placenta as a result

of inhibition of OCT1-3, with 2 μM as an IC50. It has been identified that corticosterone affinity for hOCT3 was higher than OCT1 and 2, and that the obtained IC50 values for hOCT1, 2, and 3 were approximately 10, 30, and 0.1 μM, respectively [196]. The chemical structure of corticosterone is shown in Figure 3.4.

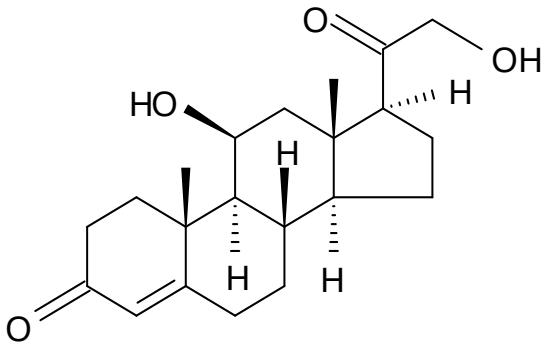

Figure 3.4: Chemical structure of corticosterone ($C_{21}H_{30}O_4$)

3.2.4.4 Salbutamol

Salbutamol (also known as albuterol) is a short-acting β2-adrenoceptor agonist, which is commonly used for the treatment of COPD, asthma, and bronchitis. Salbutamol antagonises bronchoconstriction by binding to its receptors, β2-adrenoceptor resulting in relaxation and dilation of the bronchioles diameter.

Salbutamol is mostly administered by inhalation and is absorbed through the airway epithelium before exerting its bronchodilatation effect. The drug is positively charged at physiological pH, and the

involvement of transporters in its translocation from the extracellular space into the cytosol of the epithelial cells was predicted. There has been substantial debating regarding the mechanism of permeability of the drug across biological membranes. Ehrhardt et al., have shown that salbutamol is actively translocated across bronchial epithelial cells *in vitro*, but did not determine which transporter was involved [358]. It has been found that carnitine delayed pulmonary uptake of salbutamol, suggesting that salbutamol is probably a substrate for OCTN2 [359]. Recently, Salomon *et al*., have shown using HEK-293 overexpressing hOCT1-3 transporters; that salbutamol is a substrate for OCTs transporters, with higher affinity for hOCT1 than hOCT3, but low affinity for hOCT2 [360].

In addition to being a substrate for some OCT/Ns proteins, it has been demonstrated that the OCT substrate ASP^+ uptake in bronchial epithelial cells was inhibited by salbutamol, suggesting that the drug is likely to interact with solute link carrier family members [222]. Manali *et al*., have demonstrated that ASP^+ influx into Calu-3 cell layers was significantly decreased at 500 μM of salbutamol, anticipating its capacity to inhibit one or some members of OCT/Ns proteins [228]. Again, Salomon *et al*., have found that the drug at 500 μM caused decreased ASP^+ uptake in

bronchial epithelial cells, Calu-3 and 16HBE14o- as well as A549 cells [269].

Depending on the previous studies, salbutamol was applied at 0.05, 0.1 and 0.5 mM on confluent scratched and unscratched A549 monolayers to probe its influence on cell proliferation. The chemical structure of salbutamol is shown in Figure 3.5.

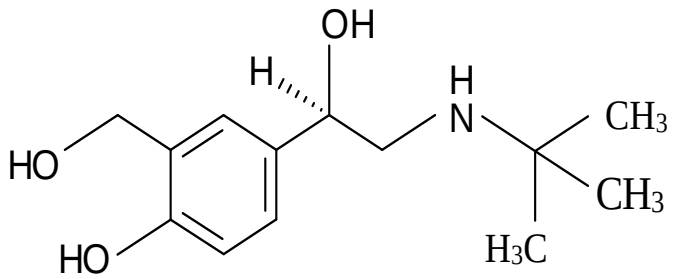

Figure 3.5: Chemical structure of salbutamol ($C_{13}H_{21}NO_3$)

3.2.4.5 Ipratropium

Ipratropium is a quaternary cationic drug, which is frequently employed in the treatment of chronic obstructive pulmonary disease (COPD) and to a lesser extent, asthma. Ipratropium exerts non-selective anti-muscarinic effects on muscarinic receptor subtypes (anti-cholinergic) M1-5; and is as a bronchodilator [361-363]. Once bound to M3 subtype receptors expressed in bronchial smooth muscle cells, it antagonises the action of

bronchoconstriction in different species including humans [148, 149, 151, 364].

It has been demonstrated that ipratropium was mainly transported via OCTN2 and to lesser extent via OCTN1 in bronchial epithelial cells BEAS-2B [365]. By using OCTs inhibitors such as cimetidine, quinidine, and corticosterone, it has been revealed that ipratropium is substrate for rat Oct1 and 2 proteins expressed in kidney tissue as well as in HEK293/rOct1 and HEK293/rOct2 [294]. The same team showed that hOCT1 and hOCT2 expressed in HEK293 cells were involved in ipratropium elimination.

In addition to being a substrate for some OCT/Ns proteins, it exerts inhibitory effect as well. It has been found that methyl-L-[^{3}H] carnitine uptake was significantly inhibited by ipratropium in Caki-1 cells, suggesting OCTN2 protein involvement [366]. Zolk et al., have shown that the anticholinergic drug at 1 mM caused significant inhibition of MPP^{+} uptake in stably expressed cells HEK293/hOCT2 [367].

Ipratropium in the current study was applied at 0.05, 0.1, and 0.5 mM to examine its impact on cell proliferation. Its chemical structure is illustrated in Figure 3.6.

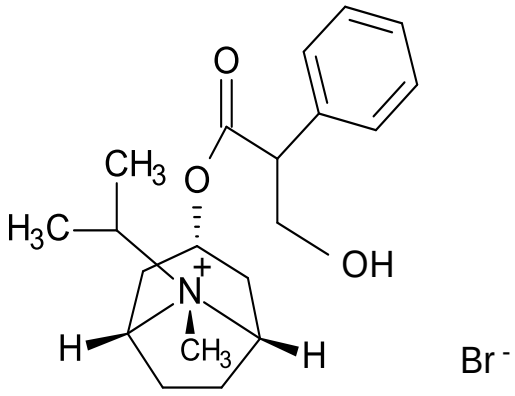

Figure 3.6: Chemical structure of ipratropium bromide ($C_{20}H_{30}BrNO_3$)

3.3 Results

3.3.1 Tetraethylammonium

TEA was applied in both scratched and unscratched monolayers in two different plates as previously described in (3.2.1). On the scratched A549 monolayers, the TEA at 5 mM resulted in significant decrease in the fluorescence intensity in comparison to the scratched control cells at 48 hrs. However, TEA exhibited inhibitory effects on unwounded confluent monolayers since a significant decrease in the fluorescence intensity of the treated group in comparison to the untreated cells was observed at 48 hrs. For this reason, the result of wounded monolayers as shown in Figure 3.7 cannot be taken into consideration, and TEA cannot be used to achieve our aim.

The time lapse microscopy images showed complete wound repair for control group at 36 hrs of scratching, while in TEA treated monolayers the scratch was still observed even after 48 hrs of wounding as shown in Figure 3.9.

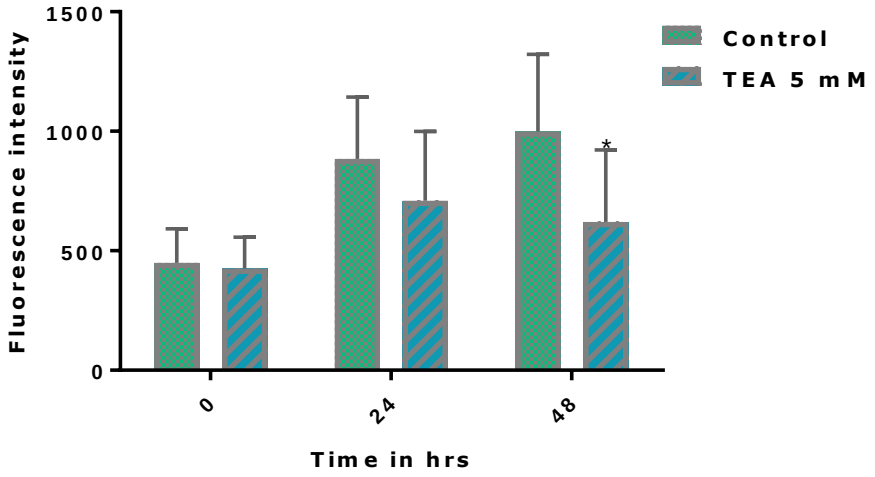

Figure 3.7: Effect of tetraethylammonium (TEA) on cell proliferation in A549 wounded monolayers.

The data are mean ± SEM (triplicate, n=3, n represents no of experiments).* indicates significant decrease in the fluorescence intensity of TEA treated group compared to control (Student t-test, $p < 0.05$).

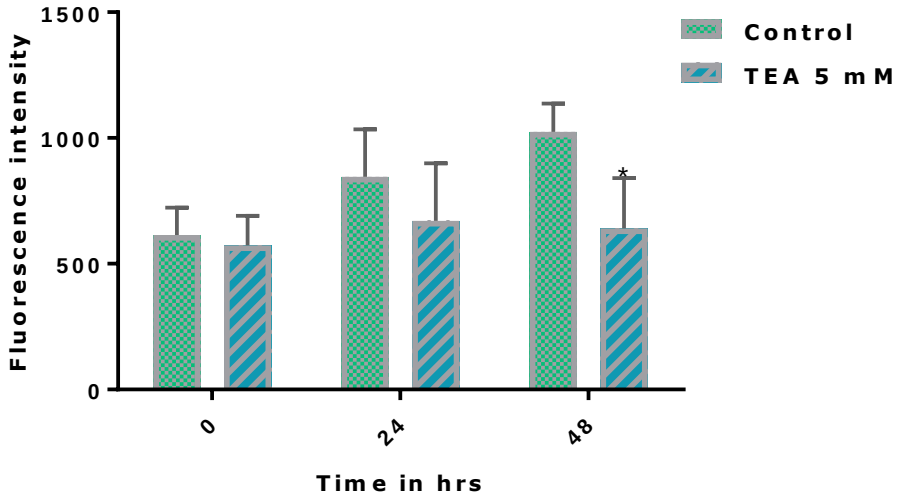

Figure 3.8: Effect of tetraethylammonium in cell viability of A549 confluent unwounded monolayers.

The data represents mean ± SEM (triplicate, n=3, n represents no of experiments). * indicates significant decrease in the fluorescence intensity of TEA treated group compared to control group at the same time point (Student T-test, $p < 0.05$).

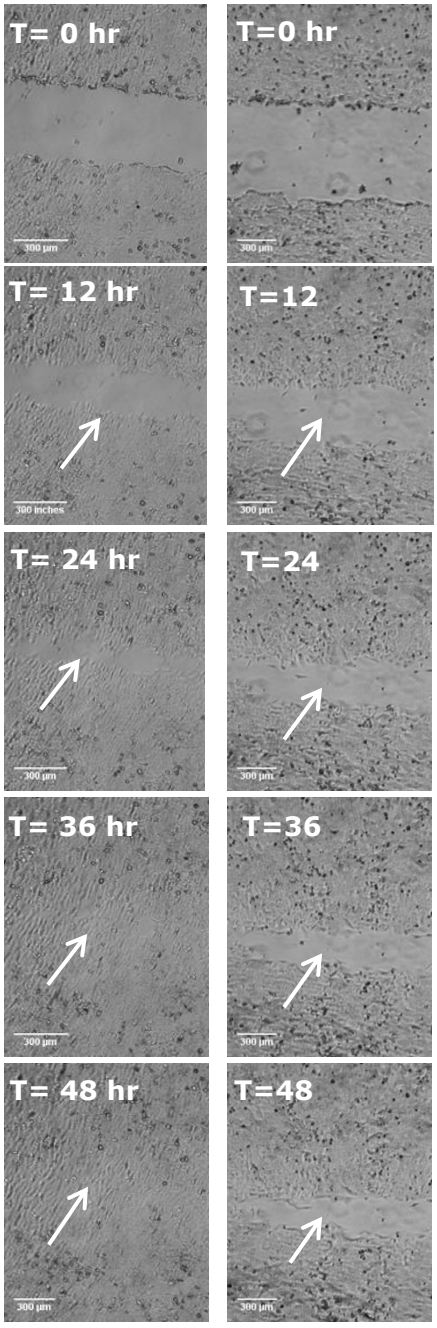

Figure 3.9: Wound repair in control and TEA treated scratched A549 monolayers.

Left panel represents untreated control monolayers, and right panel represents TEA added monolayers. Control group showed wound repair at 36 hrs of wounding, while the scratch was still observed after 48 hrs in the TEA treated layers. The scale bar represents 300 µm and the white arrow indicates the scratch area.

3.3.2 Quinine

On wounded layers, all quinine concentrations resulted in a significant decrease in FI compared to control 24 hrs after treatment, whilst the highest concentration led to a significant decrease in FI compared to other treated groups and control after 48 hrs of exposure as revealed in Figure 3.10. Quinine did not cause inhibitory effect on unwounded monolayers at 0.01 mM during the period of the experiment. However, the 0.1 and 0.3 mM doses showed inhibitory effect/decrease enzyme metabolic activity compared to control and the lower concentration as shown in Figure 3.11. Due to this effect of quinine, it was considered to be an unreliable OCT/Ns inhibitor to probe their function on cell proliferation.

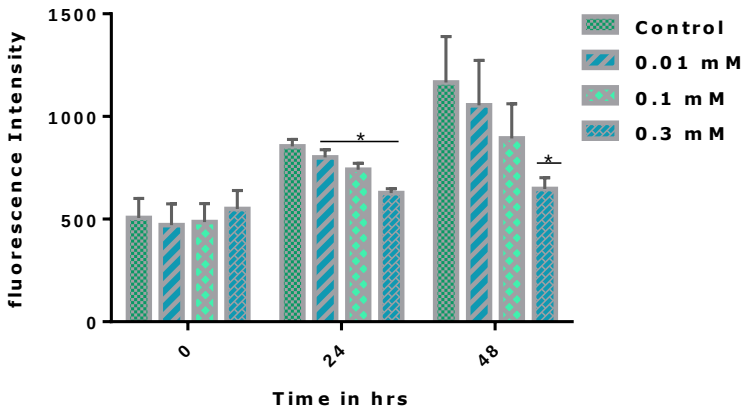

Figure 3.10: Effect of quinine on cell proliferation in A549 wounded monolayers.

The data represent mean ± SD (triplicate, n=2, n represent no of experiments). *indicates significant decrease in fluorescence intensity as compared to control (One way ANOVA, followed by Tukey test, $p < 0.05$)

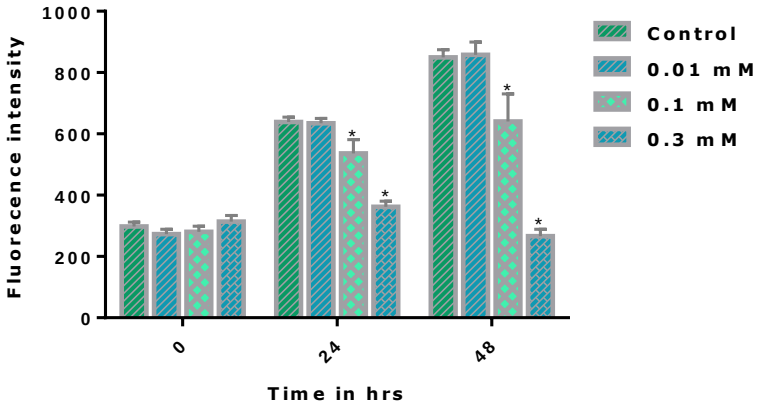

Figure 3.11: Effect of quinine on cell viability in A549 <u>unwounded</u> monolayers.

The data represent mean ± SD (triplicate, n=1, n represents no of experiments). *indicates significant decrease in the fluorescence intensity as compared to control (One way ANOVA, followed by Tukey test, $p < 0.05$)

3.3.3 Corticosterone

All three applied corticosterone concentrations, 0.05, 0.1, and 0.5 mM, resulted in a significant decrease in FI values compared to control group 48 hrs post-treatment in the wounded monolayers as shown in Figure 3.12. Similar results were observed for unwounded layers after two days of adding the compound as shown in Figure 3.13. Due to the decrease in mitochondrial metabolic activity on unscratched confluent monolayers, corticosterone was unsuitable for carrying on our experiments

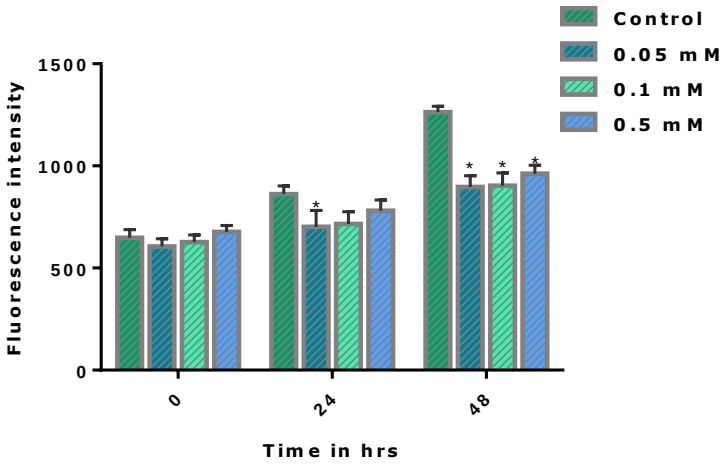

Figure 3.12: Effect of corticosterone on cell proliferation in A549 wounded monolayers.

The data represents mean ± SD (triplicate, n=1, n represents no of experiments). * indicates significant decrease in the fluorescence intensity for corticosterone treated groups compared to control (One way ANOVA, followed by Tukey test, p <0.05)

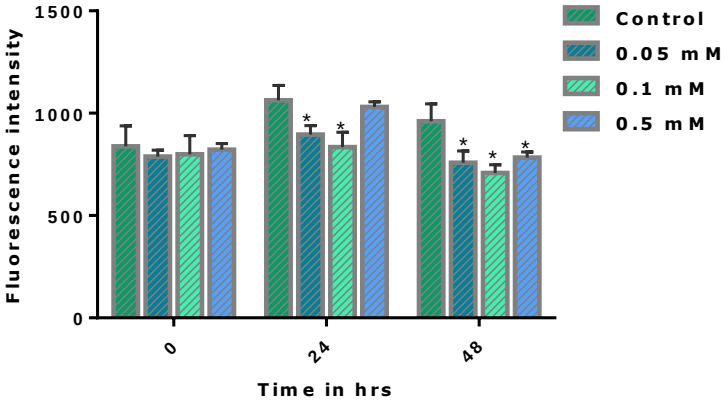

Figure 3.13: Effect of corticosterone on cell viability in A549 unwounded monolayers.

The data represents mean ± SD (triplicate, n=1, n represents no of experiments). *indicates significant decrease in the fluorescence intensity of corticosterone treated groups compared to control (One way ANOVA, followed by Tukey test, p <0.05).

3.3.4 Salbutamol

No significant differences in FI values were reported using three different concentration of salbutamol 0.05, 0.1 and 0.5 mM on

A549 wounded layers. Similar impact on unwounded layers was observed during the experiment as shown in Figure 3.14 and Figure 3.15.

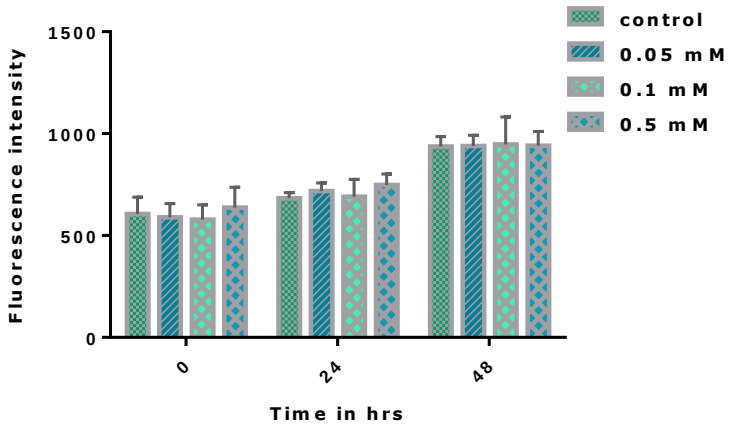

Figure 3.14: Effect of salbutamol on cell proliferation in A549 wounded monolayers.

The data is mean ± SD (triplicate, n=2, n represents no of experiments). No significant differences were observed between control and all salbutamol concentrations (One way ANOVA, followed by Tukey test, $p \geq 0.05$)

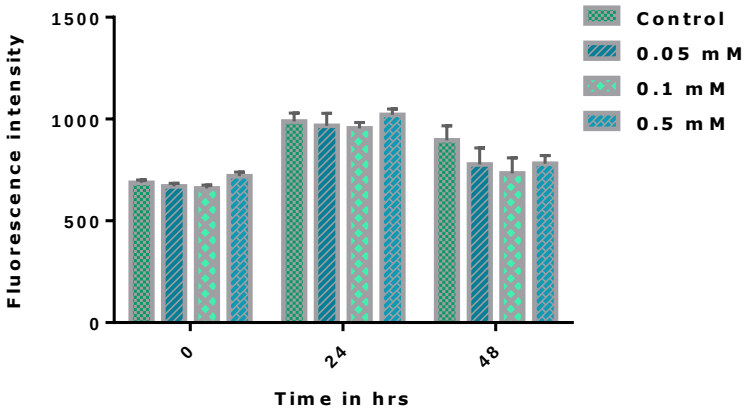

Figure 3.15: Effect of salbutamol on cell viability in A549 unwounded monolayers.

The data is mean ± SD (triplicate, n=2, n represents no of experiments). No significant differences were observed between control and all salbutamol concentrations (One way ANOVA, followed by Tukey test, $p \geq 0.05$)

3.3.5 Ipratropium

Three concentrations of ipratropium, 0.05, 0.1, and 0.5 mM were applied on unscratched and scratched monolayers. The highest concentration of 0.5 mM caused significant decrease in FI values compared to other concentrations and control group on wounded monolayers at 48 hrs of treatment as shown in Figure 3.16. No significant influence of the drug was observed on unscratched monolayers as compared to the control group at any concentration tested (Figure 3.17).

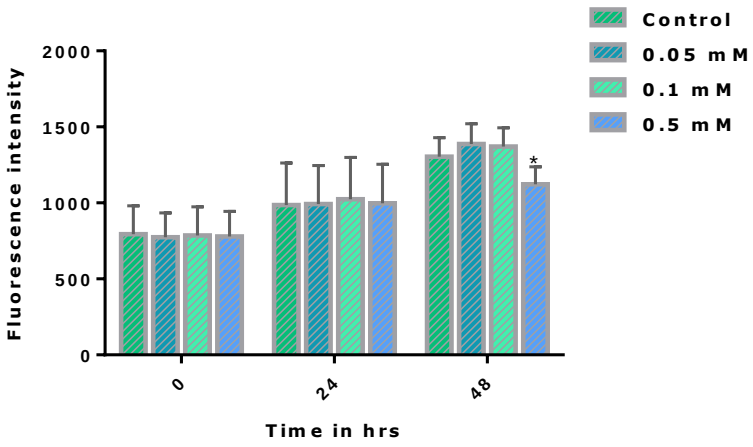

Figure 3.16: Effect of ipratropium bromide on cell proliferation in A549 wounded monolayers.

The data represents means ± SEM (triplicate, n=3, n represents no of experiments). * indicate significant decrease in the fluorescence intensity of ipratropium treated groups compared to control (One way ANOVA, followed by Tukey test, $p < 0.05$)

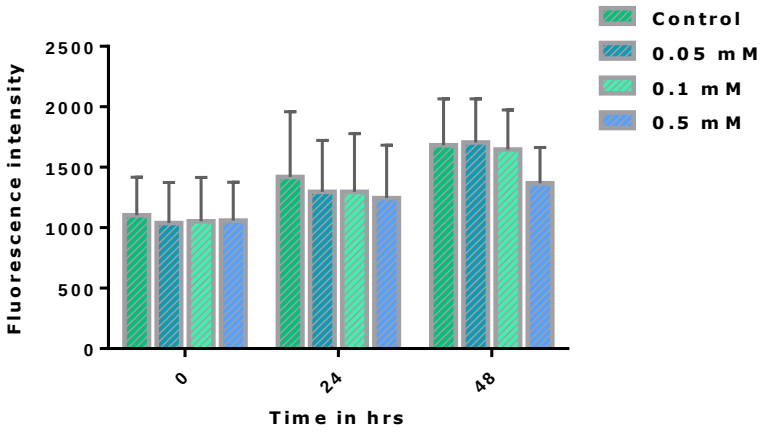

Figure 3.17: Effect of ipratropium bormide on cell vaibility in A549 unwounded monolayers.

The data represents mean ± SEM (triplicate, n=3, n represents no of experiments). No significant differences were observed between groups at (One way ANOVA, followed by Tukey test, $p \geq 0.05$).

3.4 Discussion

In this chapter, we have applied different organic cation transporter inhibitors on confluent wounded and unwounded A549 monolayers for a consecutive 48 hrs. Depending on previous uptake studies, some of these inhibitors are known to inhibit one or some of members of the *SLC22A* family. We have applied PrestoBlue (PB) assay to examine the influence of these inhibitors on both cell viability and cell proliferation in confluent and scratched A549 cells, respectively. The PB reagent has been widely applied by researchers in determination of cytotoxicity and proliferation.

Istivan et al., have used PB reagent to monitor cellular viability and toxicity of normal and cancer cell lines exposed to virus analogue

peptide at different time points of treatment [368]. The PB assay was also applied to examine viability of primary cortical neurons from mice exposed to substances that generate reactive oxygen species (ROS) such as hydrogen peroxide and cobalt chloride [369]. Lall et al., have found that PB reagent was shown to be a more reliable reagent in examining cell growing and viability compared to other similar commercially available reagents, including AlamarBlue, INT (p-iodonitrotetrazolium violet) and XTT (sodium 3'-[1-(phenyl amino-carbonyl) -3 ,4- tetrazolium]- bis-[4- methoxy-6-nitro] benzene sulfonic acid hydrate) using different strains of organisms and cell lines such as Hela cells and A431 cells [370]. It has also been shown that the PB assay was more reliable than MTT assay in assessing the viability of human corneal epithelial cells [371]. Depending on the findings of previous studies, the PB reagent is suggested to be reliable in evaluation of cell viability following exposure to different chemical compounds.

3.4.1 The effect of TEA

TEA was applied to investigate OCT/Ns function on cell proliferation. The TEA is a well-known inhibitor for OCTs proteins.

TEA led to decrease in fluorescence intensity of treated group compared to control on unwounded A549 monolayers after 48 hrs of exposure. The conditions of our experiments are completely different from others previously conducted. Indeed, in our

experiments, the monolayers were exposed to TEA at 5 mM for 48 consecutive hours rather than for a short period of time. In addition of having inhibitory effects on OCTs members, TEA has been shown to block potassium ion channels, preventing K^+ influx into the intracellular environment [346-348, 372]. Neylon has shown that potassium ion channel activity plays an essential role in the regulation of calcium influx. The accumulative intracellular calcium ions regulate cellular signalling pathways such as contractile activity and vascular smooth muscle cells proliferation [373]. Accordingly, inhibition of these channels resulted in prevention of vascular smooth muscle cells growth and proliferation. Pardo has described that some members of K^+ channels exhibited a crucial role in initiation of the G1 phase of cell cycle division [374]. The involvement of potassium ion channels in cell proliferation has been widely shown in a variety of tumour cells/cell lines. Persistence inhibition of potassium ion on A549 cells might cause inhibition in cell proliferation which resulted in decrease in fluorescent intensity.

In addition to inhibitory effects of TEA on OCT transporters and potassium channels, Wang *et al*., have shown that TEA caused inhibition of choline uptake to approximately 34% of control in cancerous cell lines, A549 and SPC-A [232]. It was found that choline is an essential substance for cell growth and that the rate

of choline requirement increased and proportionated with proliferative activity in cancerous cell lines compared to fibroblast cells [375]. It is difficult to determine whether the decrease in metabolic enzyme activity we observed was due to an inhibition of the organic cation transporters or blocking influx of potassium ions or choline transporter. Because of decreasing cell viability on unscratched monolayer compared to untreated layers, we were unable to carry on probing the effect of OCT/Ns on cell proliferation using this inhibitor. In order to discriminate between proliferation inhibitory effect and cytotoxicity of tested compounds, one of the methods that reflects cytotoxicity due to exposing to chemicals is examining morphological changes of tissue culture via microscope such as shrinking, detach of cells and hydrolysis (debris). This parameter was performed for testing a variety of anti-tumour and antibiotic compounds on Hela cells, a pH changes of medium was also considered to be as an indicator of cytotoxicity [376]. In the current study, the monolayers were examined using microscopic before and after applying PB assay, no detach and hydrolysis was observed. TEA may cause inhibition on A549 cell proliferation as a result of affecting K^+ and choline transportation. Increase concentration of cytoplasmic enzymes in growth media represents a remarkable feature of damage or injury of plasma membrane of treated cells with different compounds [377]. In addition to previous assays, a number of assays are available for measuring

cell toxicity as well such as sulforhodamine B (SRB) and neutral red uptake assay [378, 379]. The SRB assay measures cell density based on quantification of cellular protein content, while neutral uptake assay is dependent on the ability of viable cell to bind with neutral red dye in lysosome. In the current investigation we only used PB assay for detection the effect of the selected OCT/Ns inhibitor.

3.4.2 Quinine impact

Three different concentrations were employed, 0.01, 0.1, and 0.3 mM on the unwounded and wounded monolayers for 48 hrs. The lowest concentration did not show any inhibitory effect on both unscratched and scratched layers. However, 0.1 and 0.3 mM were observed to cause a significant decrease in FI compared to untreated group. In previously conducted studies, cell lines such as *Xenopus* oocytes were exposed to quinine for a short period. One of these has demonstrated that 0.3 mM of the drug caused 99% inhibition of acetylcholine release in placenta through inhibition of hOCT1-3 [163].

None of the uptake studies have applied quinine for a continuous 48 hrs. Fatherazi and Cook has shown that quinine caused blocking of some types of K^+ ion channels, calcium ion activated potassium channel [380].Quinine was shown to block potassium efflux in pancreatic beta cells from pigs and rats [381, 382]. It has

been found that quinine inhibited chloride transport and disturb integrity of Calu-3 and human airway primary culture monolayer cultured at ALI condition [383]. The effect of blocking potassium ion channels has been previously explained in (3.4.1). Disturbance in ions transportation may affect metabolic activity of cells and attenuate their ability to reduce the reagent without affecting cell proliferation. In this chapter, we rely only on measuring metabolic activity which was insufficient to determine whether cell proliferation was inhibited or there is decrease in metabolic activity. The PB assay had to be carried out with an alternative assay to draw a clear understanding.

As for TEA, it is not clear whether quinine inhibitory effect on OCTs family members or potassium ion channel inhibition is responsible for the decrease in cell viability on A549 cells. Similarly, this prevented us from carrying on the experiment with this drug.

3.4.3 The influence of Corticosterone

All three corticosterone concentrations 0.05, 0.1, and 0.5 mM were found to cause significant decrease in cell viability on unwounded monolayers after 48 hrs of exposure.

Regarding our investigation of OCTs expression profile in A549 cells, OCT2 was not recognised, while OCT1 and OCT3 were confirmed to be expressed using normal PCR. The mRNA of OCT1

was further confirmed employing real time PCR. Post-transcriptional levels of OCT1 were reported to be expressed applying ICW technique. Regarding previous studies, it has been shown that glucocorticoids induced lymphoma cell death as a result of metabolic alterations [384]. These alterations subsequently caused activation in endonuclease which in turn resulted in chromatin degradation and cell death. Dani et al., have shown that dexamethasone caused neuronal cell death in mice culture (*in vitro*) after 24 hrs of exposure [385]. Corticosterone was found to exhibit anti-mitogenic effect in mouse adrenal tumor cell line Y-l in dose dependent manner applying [^{3}H]thymidine incorporation assay [386]. Inhibitory effect of corticosterone in cell proliferation was again demonstrated in fetal rat adrenals and decrease in cytochrome P450 mRNA expression was reported as well [387]. Wiegers et al., have shown that corticosterone affected splenic T-cell proliferation depending on cell density and period of treatment [388]. Shikatani et al., have also found that 3D endothelial cell treated with corticosterone for 48 hrs were shown to reduce their proliferation [389]. The previous study also found that corticosterone reduced production of MMP-2 in both monolayer and 3D endothelial cells. Treatment of chicken chondrocytes with corticosterone for 48 hrs resulted in decrease in their viability and this was accompanied with decrease alkaline phosphatase activity and collagen type X expression [390]. The observations of these studies in different cell cultures strongly demonstrate that corticosterone inhibits cell proliferation which in turn will decrease cell number. In our study,

corticosterone may result in decrease in cell proliferation which caused reduce in FI of treated groups compared to control group.

3.4.4 The impact of salbutamol

Salbutamol at 0.05, 0.1, and 0.5 mM was applied on unscratched and scratched monolayers of A549 cells for two days. Salbutamol did not affect cell viability. No effect was observed either on wound layers during the period of treatment.

Previous uptake studies on different airway epithelial models have shown that salbutamol at 0.5 mM caused inhibition of at least two members of OCT/Ns proteins, particularly OCT1 and OCTN2 [222, 228, 269, 360]. Salbutamol is a beta-adrenoceptors agonist which binds to and stimulates this type of receptors and exhibit functions related to receptors activated. Expression of β-adrenoceptor (βARs) has been studied in airway epithelial cells in human and other species including cats and dogs. By applying autoradiography technique, human airway epithelial cells were shown to express βARs in luminal membrane surface and that submucosal gland were shown to express β1ARs and β2ARs [391, 392]. These results were further confirmed by applying *in situ* hybridization [393]. Feline tracheal epithelial cells, particularly ciliated cells were shown to express βARs using immune detection of cyclic adenosine monophosphate (cAMP) [394]. Mucus and serous cells were also shown to express adrenergic receptor as well. A further study

suggested that dog epithelial ciliated cells may express these receptors [395]. Activation of these receptors was shown to be correlated with activation of alpha subunit of an adenyl cyclase stimulatory G-protein (G_s) [396]. Activation of these receptors via beta agonists has been identified to be involved in various epithelial cell functions including mucociliary, mucus secretion, ion transport and protection [397, 398]. In addition, β2-agonists were found to accelerate alveolar fluid clearance in normal rat lungs as a result of increasing cAMP and decreasing pulmonary oedema in acidic-induced lung injury due to decreasing vascular permeability [399, 400]. Furthermore, these receptors were shown to be implicated in wound repair as well via increasing intracellular cAMP and activation of PKA. The cAMP and PKA have been identified to be involved in cell migration and wound repair. For example, the beta agonist isoproterenol was found to enhance cell migration and repair of mechanically and enzymatically induced wound in bovine bronchial epithelial cells cultured in submerged conditions [401]. Interestingly, and far from the inhibitory effect of salbutamol on OCT/Ns, salbutamol was found to enhance wound repair in wounded A549 cells through stimulation of cell spreading/migration [402].

3.4.5 Ipratropium effect

We have tested ipratropium at three concentrations, 0.05, 0.1, and 0.5 mM on unscratched and scratched A549 cells for 48 hrs. None of the concentrations caused a decrease in cell viability when applied on confluent monolayers. However, 0.5 mM was shown to cause a significant decrease in FI measured with scratched monolayers after 48 hrs of exposure. Unfortunately, there is no relevant experiment to compare our result with.

Uptake studies have shown that ipratropium exhibited inhibitory influence on OCT2 [365, 367], and OCTN2 [366]. However, OCT2 was not detected in A549 cells (Chapter 2). In addition of having OCTN2 inhibitory effect, ipratropium is an anti-muscarinic drug, acting in particular on the M3 receptor. Previously, this subtype was shown to indirectly influence bovine tracheal smooth muscle cell proliferation in association with platelet-derived growth factor (PDGF) [115], and human airway smooth muscle cell proliferation in the presence of epidermal growth factor [403]. Expression of muscarinic receptor subtypes M1-M5 has been identified in A549 by applying qPCR [179], with higher expression for the M3 subtype. Proliferative effect induced by activation of muscarinic subtypes has been shown to be synergistic. Stimulation of M2 and M3 resulted in activation of mitogen-activated protein kinase/extracellular signal–regulated kinase1/2pathway [404-406],

which has been shown to be implicated in cell proliferation in different cell types [407-409]. Anticholinergic tiotropium was shown to exhibit anti-mitogenic effects in a concentration-dependent manner in primary human fibroblast and myofibroblasts via binding to muscarinic receptors M1-M3 [117]. Therefore, ipratropium at 0.5 mM might have caused decrease in cell proliferation as a result of antagonizing muscarinic receptors rather than through OCTN2 inhibition.

3.5 Conclusion

Some of the applied inhibitors have shown decrease in enzyme metabolic activity on unscratched monolayers, particularly, TEA, quinine, and corticosterone. For this reason, employing these compounds to achieve our aim was unreliable. Salbutamol did not have any effect on both unwounded and wounded layers, although uptake related studies have shown its inhibitory effect on the OCT1 and OCTN2 proteins. Ipratropium affected cell proliferation at a high concentration but it is not clear whether the effect resulted from antagonising the muscarinic receptors or inhibition of OCTN2.

Due to the difficulties in studying OCTs role in cell proliferation using inhibitors, we decided to knock down OCT1 and OCTN2 genes in order to probe their influence on cell proliferation separately.

Chapter 4 Knock down of *SLC22A1* and *A5* genes via small interfering RNA

4.1 Introduction

Because of the difficulty in determining *SLC22A1* and *A5* gene role in cell proliferation using a variety of inhibitors, we have selected an alternative technique to examine these gene functions in A549 cells.

RNA interfering (RNAi) is one of the crucial techniques frequently applied to probe gene functions. RNAi is a natural process which results in specific gene silencing in a diversity of organisms including, types of worm and plant cells [410-414]. It has been found that the specificity of gene silencing is caused by a specific length of double strand RNA of about 22 nucleotides [415-418]. This specific sequence of nucleotides assembles with other components to form RNA-induced silencing complexes (RISC), which in turn, degrade certain mRNAs according to the match sequence of the guide strand [417]. The molecules of mRNA are mainly transcribed from a specific sequence of DNA known as a gene via RNA polymerase II. The mature molecules of mRNA bind to ribosomes for synthesis of a protein corresponding to a particular gene by translating its coding sequence in association with transfer RNA (tRNA) [419].

RISC components include an enzyme which is known as Dicer. This enzyme is a member of the RNase III family which separates the short double strand RNA sequence, thus liberating each strand [420]. Dicer is characterized by a special region for binding different components, one of which is the Argonaut family, particularly the protein member-2. Argonaut-2 protein is an important component of the RISC complex that prevents gene expression [421-424]. Altogether, the guide strand, Dicer and argonaute-2 protein cooperate in RISC complex to destroy particular mRNAs as shown in Figure 4.1, thus preventing expression of a certain gene at the post transcriptional level. In addition to the explained RISC components, in human cells it has been found that RNA helicase A (RHA) is implicated in destroying a specific mRNA [425].

Although RNAi is a natural process, it can be achieved synthetically using two tools, either small interfering RNA (siRNA) or short hairpin (shRNA) [426]. Small interfering RNA technique involves incorporating short double strand RNA within a delivery vehicle. Using a vehicle is a crucial step that allows the successful transfer of nucleic acids (NA) into the cytosol. A delivery vehicle is necessary firstly, because of the negative charge of the phosphate groups in the NA, and secondly, for protection of the nucleic acid from degradation by nucleases [427-430]. Diverse vehicles have

been used either *in vitro* or *in vivo*, including liposomes [431, 432], lipid-based reagents [433-435], polymers [436-438], peptide-based vehicles [439, 440], and virus-based vectors [441]. Although there are more than 90 papers published regarding siRNA in mammalian cells, many challenges remain that make applying RNAi for the treatment of diseases such as cancer and viral infections in humans unsafe. This is particularly because of the drawbacks of delivery vehicles and siRNA double strands. Generally, the siRNA and their delivery systems induce cytotoxicity and immune responses [427, 428, 430, 442-444]. However, the non-viral transfection reagents were reported to be less toxic and less prone to induce immune responses. In addition, preparing these vehicles is easier than viral based vectors [429]. The drawback of non-viral reagents is producing low transfection efficiency compared to the viral methods.

The positively charged vehicles form stabilized complexes with negatively charged siRNAs that can be taken into subcellular spaces via endocytosis [442]. As a result of incorporation between the released siRNAs and the RISC, transient knock down of the gene of interest will be achieved in a specific and selective way.

In respect of shRNA, the fundamental advantage of shRNA on siRNA is producing a long term gene silencing ranging from weeks to months as a result of incorporation of viral DNA to the genome

of exposed cells. Inducing gene silencing for a long term is needed to treat chronic conditions, however, this may also cause disadvantages such as inducing immune response and affecting miRNA pathway, which in turn can cause toxic effects to the cells [430]. This effect is not observed with the siRNA transfection method.

siRNAs have been widely applied in scientific research for probing a variety of gene functions. In this chapter, we knocked down *SLC22A1* and *A5* gene using siRNA method as an attempt to probe their involvement in cell proliferation and wound repair in A549 cells as a result of translocation of Ach from the intracellular space into the extracellular environment. Knock down of these genes was previously conducted by Dr. Manali Mukherjee using the same model [445].

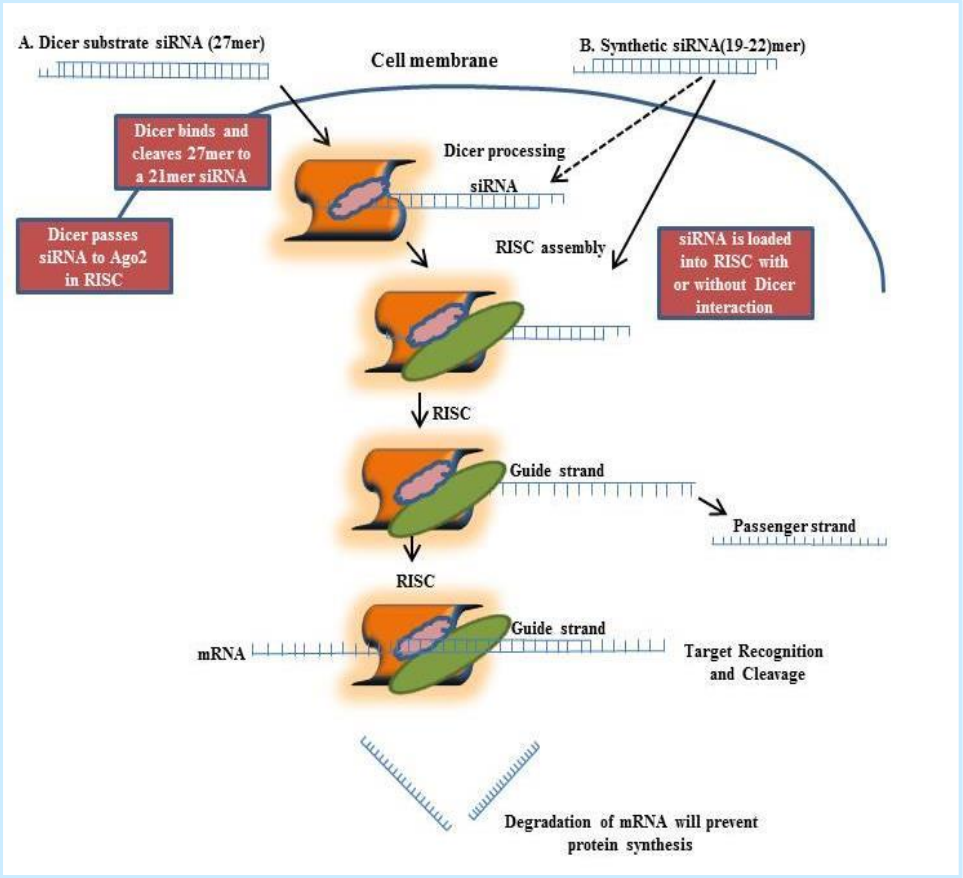

Figure 4.1: Schematic illustrating the processing of synthetic double strand RNA.

The synthetic double strand RNA is processed through collaboration of more than one molecules resulting in damaging of a certain mRNAs depending on the sequence of a guide strand, thus preventing translation into a specific protein.

4.2 Materials and methods

4.2.1 Materials

Different transfection reagents were tested for their knock down efficiency of two genes, *SLC22A1* and *A5*. N-TER™ peptide-based Nanoparticle siRNA Transfection System (Sigma) and Fugene®HD lipid-based vehicle (Roche) were applied to transfect A549 cells with two targets of *SLC22A1* siRNA and one target of *SLC22A5* as shown in Table 4.1. Universal Mission® negative siRNA (#SIC001,

Sigma) was used as a scrambled control. The siRNAs were diluted to 5 μM and preserved at -20 ºC. N-TER™ reagent was kept at -20 ºC, whilst Fugene®HD vehicle was kept at 4 ºC. The final concentration used with N-TER vehicle was 20 nM.

A new lipid-base vehicle, INTERFERin® (Polyplus-transfection) was also examined for its knock down efficiency with three new targets of *SLC22A1* siRNA A, B, and C (OriGene's 27mer siRNA) as shown in Table 4.2. Universal scrambled negative control from OriGene MRP (SR30004) was applied with INTERFERin® reagent. The siRNA was preserved at -20 ºC and was thawed on ice during experiments, whereas INTERFERin® reagent was kept at 4 ºC.

Table 4.1: *SLC22A1* and *5* siRNA sequences used with N-TER™ and Fugene® HD reagents

Name	Mission® siRNA product code	Accession number	Molecular weight
SLC22A1	SASI-Hs01-00172601	NM-003057	13304
SLC22A1	SASI-Hs01-00172604	NM-003057	13304
SLC22A5	SASI_Hs02_00334973	NM_003060	13319

Table 4.2: *SLC22A1* siRNA sequences used with INTERFERin® reagent

***SLC22A1* (Human) siRNA sequence**
SR304459A -rGrGrArGrCrUrGrArArCrUrArUrArCrArGrUrGrCrCrArGGC
SR304459B -rGrCrArArUrArArArGrArUrArArUrGrGrArCrCrArCrArUCG
SR304459C -rGrGrGrArArUrCrArCrCrArUrUrGrCrArArUrArCrArArATG

4.2.2 Transient knockdown protocols for N-TER peptide/ Fugene HD

The A549 cells were cultured on 24 well plates at a density of ≤ $3x10^4$/well with medium containing 10% serum but free of antibiotics. The cells were transfected when they had reached approximately 50-70% confluency.

For the N-TER peptide transfection reagent 13 µl of siRNA (5µM) were added into a sterile tube containing 37 µl of siRNA buffer. 8 µl of N-TER peptide was added into 42 µl of nucleases free water in another separated tube. These siRNAs and N-TER containing tubes were left for 5 min at room temperature, and then they were mixed (100µl) and left at RT for 20 min to allow formation of the NTER/siRNA complexes. 92 µl of the complex preparation was pipetted and added into 2.908 ml of serum containing medium, and then 600 µl of medium containing particles was added per well where the final siRNA concentration was 20 nM. The 24-well plates were incubated at 37 °C. The transfection medium was replaced by

medium containing serum and free of antibiotics after 4-6 hrs, and 24 hrs post knockdown the cells were harvested and the total RNA was extracted as described in 2.2.5.1

For the protocol involving the Fugene reagent, 100 µl of the transfection mixture was prepared by mixing 6 µl of the reagent, 1 µl of siRNA (100µM) and 93 µl of serum and anti-biotic free medium. The complexes were left to form for 15 min at RT, and then 33 µl of the complex preparation was added per well containing 267 µl of serum containing medium. Finally the plate was incubated at 37 °C, and after 4-6 hrs, the media was replaced by medium containing 10% serum but free of antibiotics. Harvesting of transfected cells for RNA extraction was conducted after 24 hrs of transfection.

Three types of controls were designed to be run alongside treated/transfected cells: negative control (scrambled control), vehicle only control, and untreated cells control. The negative control applied with these reagents was Universal Mission® negative siRNA (#SIC001, Sigma).

4.2.3 Transient knockdown protocol for INTERFERin®

Cells were seeded on 24-well plates at approximately ≤ $2.5x10^4$ cells/well with medium containing 10% serum but free of

antibiotics. The transfection was conducted when the confluency had reached approximately 40%.

Different volumes of the INTERFERin® reagent were used depending on the final concentration of siRNA duplexes. Two and three µl of the reagent were used with low concentrations of 1 and 5 nM, respectively, while 4 µl was applied for a high siRNA concentration of 20 nM. The 100 µl transfection mixture was made up of 2/3/4 µl of the reagent, 1.2 µl of different concentrations of siRNA, and 96.8/95.8/94.8 µl of serum and antibiotic free media. Then it was incubated at RT for 15 min before being added into 500 µl of serum containing medium. The 24-well plates were maintained at 37 °C for 10-12 hrs, and then medium was replaced with fresh medium. The cells were harvested at two time points, 24 and 48 hrs post transfection.

Three types of controls were designed to run alongside treated/transfected cells, negative control (scrambled control), vehicle only control, and untreated cells control.

4.2.4 Measurement of *SLC22A1* and *A5* mRNA levels by qPCR

The A549 cells transfected with N-TER peptide and Fugene HD transfection reagents were harvested 24 hrs post knock down, while they were harvested 24 and 48 hrs post transfection with the

INTERFERin® transfection reagent. Thereafter, total RNA was extracted as previously described in Chapter two (2.2.5.1), and transcribed into cDNA as explained in (2.2.5.2). Finally, the cDNA of transfected A549 cells was employed for qPCR as earlier described in (2.2.5.5). The sequence of applied primers is listed in Table 2.3 and Table 2.2.

4.2.5 Measurements of OCT1 protein levels by ICW

OCT1 protein level was relatively quantified via ICW technique in A549 cells transfected with the OCT1 siRNA-A and B. The protocol for protein quantification by ICW was previously validated and applied for quantification of normal level of OCT1 protein (See Chapter two 2.2.6.5)

Three different concentrations of siRNA were applied, 1, 5, and 20 nM with INTERFERin® transfection reagent. The lower concentration of siRNA was used once, whilst 5 and 20 nM were repeated trice. The fixed A549 cells were either processed immediately for OCT1 quantification or placed at 4 °C where they were left submerged with PBS to prevent drying of the fixed monolayers. After confirming the consistency of cell number between wells via *GAPDH* antibody signals, the percentage of protein expression was calculated as following:

Percentage of protein expression = [(800/700) of treated cells] / [(800/700) of control] x 100

4.3 Validation of the CyQUANT® NF cell proliferation assay

CyQUANT® NF (Invitrogen™) cell proliferation assay is based on measuring the cellular DNA content via a fluorescent binding dye. The commercial kit consists of CyQUANT NF dye reagent (Component A), a dye delivery reagent (Component B), and Hank's balanced salt solution 5X (HBSS) buffer (Component C). Applying the dye delivery reagent was optional, therefore, this was omitted and only the two other reagents were used. A standard curve was generated using seeding densities of A549 cells on a 24-well plate before employing this reagent on treated cells (Figure 4.2). The validated protocol for this assay involved preparing 11 mL of 1X HBSS buffer by diluting 2.2 mL of 5X HBSS buffer (Component C) with 8.8 mL of deionized water, then preparing 1X dye binding solution by adding 22 µL of CyQUANT® NF dye reagent (Component A) to 11 mL of 1X HBSS buffer. Post 8-9 hrs of seeding, the medium was removed and 300 µl of the reagent was added/well, and then the plate was incubated at 37 °C for 60 min. The 24-well plate cultured with A549 cells was placed in a plate reader (TECAN, infinite®M200) (Männedrof, Switzerland) to measure the fluorescence intensity of the DNA-binding dye.

Excitation and emission were set at 485 nm and 530 nm, respectively.

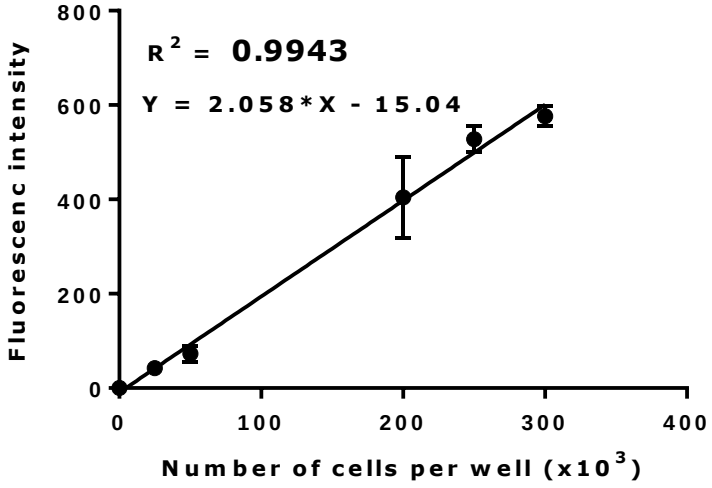

Figure 4.2: Standard curve of Cyquant cell proliferation assay in A549 cells.
Each point represents mean of triplicate

4.3.1 Cell proliferation assays on transfected A549 cells

Both Cyquant and PrestoBlue assays were performed in A549 cells transiently knocked down with OCT1 siRNA at 20 nM to examine whether the *SLC22A1* gene is involved in cell proliferation. The assays were carried out on unwounded cell layers 48 hrs post transfection.

The Cyquant and PrestoBlue assays were performed as described in 4.3 or 3.2.1, respectively. However, in this experiment, a different volume lauof the PrestoBlue reagent was added per well since a 24-well plate was used for transfection of A549 cells. 200 µl of the PB reagent was pipetted and released into a well and then

the plate was incubated at 37 °C for 15 min. 100 µl of the supernatant was transferred into a black 96-well plate (Costar) and read using the plate reader.

4.4 Results

4.4.1 *SLC22A1* and 5 mRNA level in A549 cells transfected with N-TER reagent

The mRNA levels of *SLC22A1* and *A5* genes were quantitatively measured. No significant down regulation of *SLC22A1* and *A5* was obtained using N-TER peptide transfection reagent at 20 nM siRNAs (Figure 4.3).

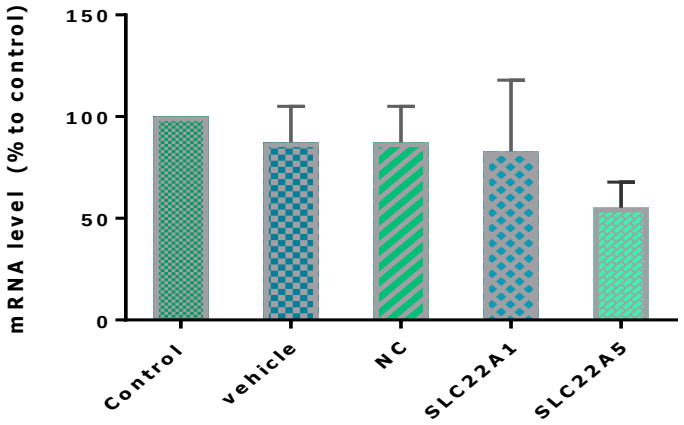

Figure 4.3: *SLC22A1* and *A5* mRNA levels in A549 cells transfected with N-TER reagent at 20 nM siRNA.

A549 cells were transfected with OCT1-siRNA-1 SASI-Hs01-00172601**,** and OCTN2 siRNA SASI_Hs02_00334973**.** Values represent mean ± SD (triplicate, n=1, n represent no of experiment). No significant differences were observed between control and all treated groups (One way ANOVA, followed by Tukey test, $p \geq 0.05$). NC stands for negative control.

4.4.2 *SLC22A1* mRNA level in A549 cells transfected with N-TER and Fugene transfection reagents

In this experiment, primers were those previously used by Dr. Manali Mukherjee. No significant down regulation of OCT1 mRNA was measured using two different OCT1 siRNA when either N-TER or Fugene were used as the transfection reagent as shown in Figure 4.4.

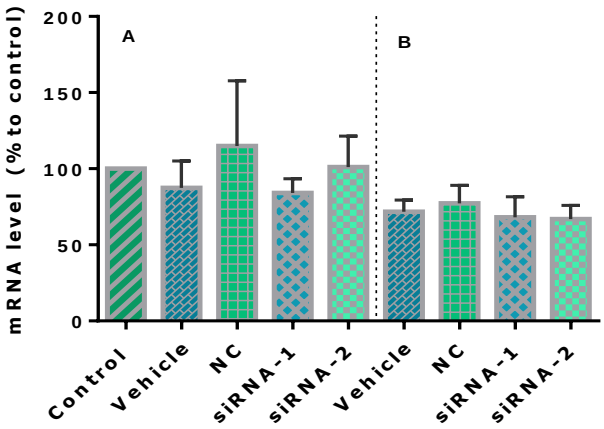

Figure 4.4: *SLC22A1* mRNA level in A549 cells transfected with (A) N-TER and (B) FuGENE ® HD reagent at 20 nM siRNA.

A549 cells were transfected with siRNA-1 and 2 as described in Table 4.1. Values represent mean ± SD (triplicate, n=1, n represent no of experiment). No significant differences were observed between control and all treated groups (One way ANOVA, followed by Tukey test, $p \geq 0.05$). NC stands for negative control.

4.4.3 *SLC22A1* mRNA level in A549 cells transfected with INTERFERin transfection reagent

INTERFERin reagent was alternatively used for knocking down *SLC22A1* mRNA level. The gene level in A549 cells transfected with INTERFERin was significantly down regulated 24 hrs post transfection compared to vehicle using 1 nM of siRNA-B, whilst

siRNA-A did not cause significant down regulation of the gene transcripts. However the same concentration of siRNA-A caused significant down regulation in the gene transcripts compared to other groups 48 hrs post transfection (Figure 4.5), while no effect was observed for siRNA-B at the same time point post transfection. Surprisingly, siRNA-C resulted in up regulation of the siRNA level. For this reason, siRNA-C was omitted and the knock down was conducted with higher concentrations of siRNAs A and B. The siRNA-A and B at 5 nM had no gene silencing effect 24 hrs post transfection, while both caused significant down regulation of *SLC22A1* mRNA 48 hrs post transfection as denoted in Figure 4.6. Interestingly, no down regulation of gene was observed at a higher dose of 20 nM of both siRNA- A and B 24 and 48 hrs post transfection as shown in Figure 4.7.

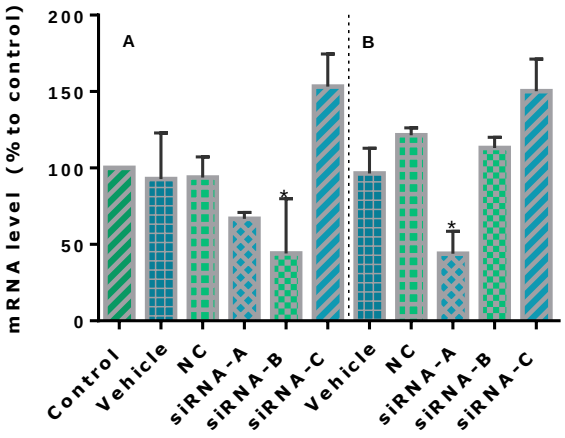

Figure 4.5: *SLC22A1* mRNA level in A549 cells transfected with INTERFERin® at 1 nM siRNA.

Cells were transfected with OCT1 siRNA-A-B-C (Table 4.2) at 1 nM as a final concentration. * indicates significant (One way ANOVA, followed by Tukey test, $p < 0.05$), values represent mean ± SD, (triplicate, n=1, n represents no of experiment). Panel (A) represents 24 hrs post transfection, and (B) 48 hrs post transfection. NC stands for negative control.

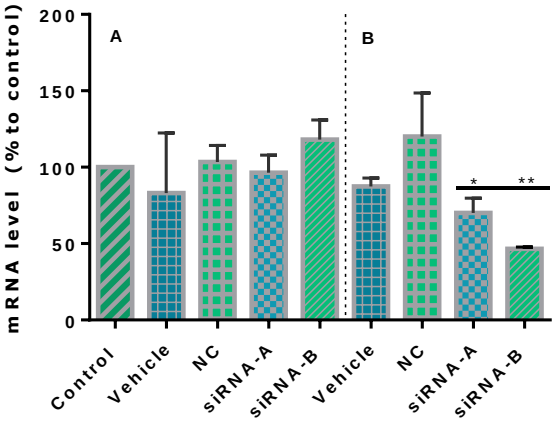

Figure 4.6: *SLC22A1* level in A549 cells transfected with INTERFERin® at 5 nM siRNA.

Cells were transfected with OCT1 siRNA-A and B at 5 nM as a final concentration. * indicates significant (One way ANOVA, followed by Tukey test, $p < 0.05$), values represent mean ± SD, (triplicate, n=1, n represents no of experiment). Panel (A) represents 24 hrs post transfection, and (B) 48 hrs post transfection. NC stands for negative control.

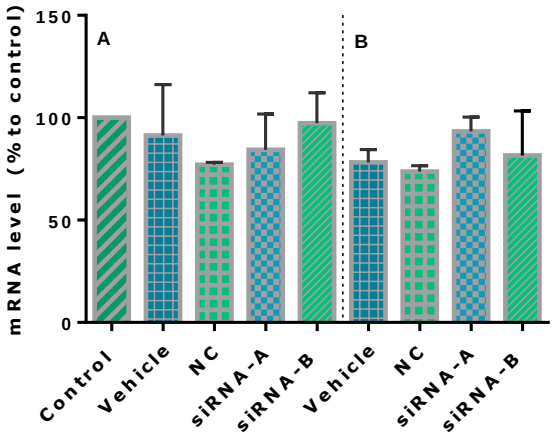

Figure 4.7: *SLC22A1* mRNA level in A549 cells transfected with INTERFERin® at 20 nM siRNA.

Cells were transfected with OCT1 siRNA-A and B at 20 nM as a final concentration. No differences between groups were observed (One way ANOVA, followed by Tukey test, $p \geq 0.05$). Values represent mean ± SD, (triplicate, n=1, n represent no of experiment). Panel (A) represents 24 hrs post transfection, and (B) 48 hrs post transfection. NC stands for negative control.

4.4.4 Protein expression in transfected cells

The OCT1 protein was relatively quantified applying ICW technique in A549 cells transfected with siRNA-A and B at 1, 5 and 20 nM 48 hrs post transfection. At 1 nM OCT1 siRNA-B resulted in a significant 28±11% decrease in OCT1 protein expression compared to control group as shown in Figure 4.8. No down regulation in OCT1 protein was observed for OCT1 siRNA-A. Using higher doses of 5 and 20 nM siRNA- A and B caused significant down regulation in OCT1 protein compared to control and vehicle. The percentage of down regulated protein caused by 5 nM was 44 ± 18% for OCT1 siRNA-A and 39 ± 23% for OCT1 siRNA-B as shown in Figure 4.9. 20 nM of OCT1 siRNA-A and B caused significant decrease in the

percentage of protein expression by 61 ± 15% and 41 ± 13%, respectively (Figure 4.10).

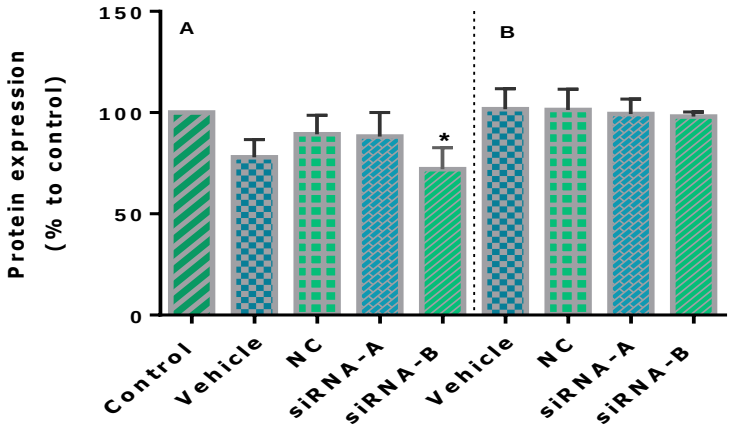

Figure 4.8: Percentage of OCT1 protein expression in A549 cells transfected with 1nM siRNA.

Cells were transfected with OCT1 siRNA-A and B at 1 nM as a final concentration.* indicates significant down regulation of OCT1 protein (One way ANOVA, followed by Tukey test, $p < 0.05$). Panel (A) represents % of OCT1 protein, whilst panel (B) represents % of *GAPDH* protein expression. Values represent means ± SED, (triplicate, n=3, n represent no of experiment).

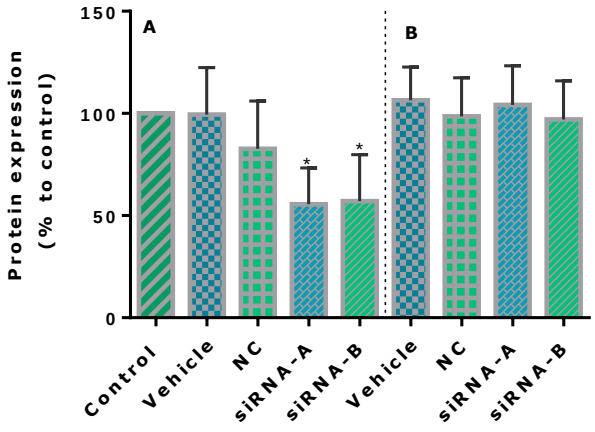

Figure 4.9: Percentage of OCT1 protein expression in A549 cells transfected with 5 nM siRNA.

Cells were transfected with OCT1 siRNA-A and B at 5 nM as a final concentration.* indicates a significant down regulation of OCT1 protein (One way ANOVA, followed by Tukey test, $p < 0.05$). Panel (A) represents % of OCT1 protein, whilst panel (B) represents % of *GAPDH* expression. The data represent means ± SEM, (triplicate, n=3, n represent no of experiment).

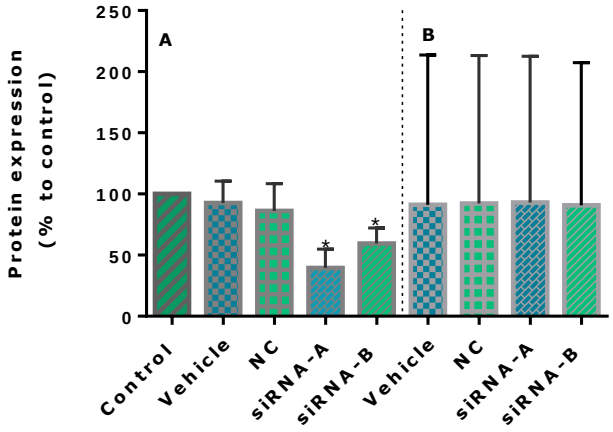

Figure 4.10: Percentage of OCT1 protein expression in A549 cells transfected with 2 0nM siRNA.

Cells were transfected with OCT1 siRNA-A and B at 20 nM as a final concentration.* indicates a significant down regulation of OCT1 protein (One way ANOVA, followed by Tukey test, $p < 0.05$). Panel (A) represents % of OCT1 protein, whilst panel (B) represents % of *GAPDH* expression. The data represent mean ± SEM (triplicate, n=3, n represent no of experiment).

4.4.5 Cell proliferation assay CyQUANT® NF

Cell proliferation assay CyQUANT® NF was conducted on transfected A549 cells 48 hrs post transfection, after confirming down regulation in OCT1 protein. According to obtained results, no significant change was reported between treated groups and control as shown in Figure 4.11.

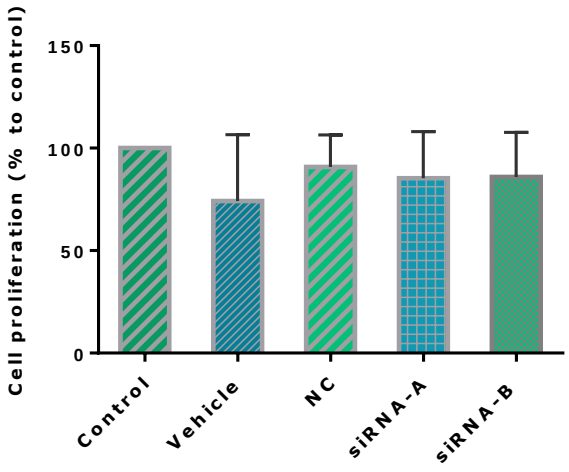

Figure 4.11: Cell proliferation assay on A549 cells transfected with *SLC22A1* siRNA at 20 nM.

Cell proliferation assay was conducted on the A549 cells transfected with *SLC22A1* siRNA- A and B 48 hrs post transfection. No differences were observed between groups (One way ANOVA, followed by Tukey test, $p \geq 0.05$). The values represent means ± SEM, (triplicate, n=3, n represent no of experiment).

4.4.6 PrestoBlue cell proliferation assay

PrestoBlue assay is also a cell proliferation assay which depends on measuring metabolic activity enzymes of mitochondria. No significant differences were observed between different groups.

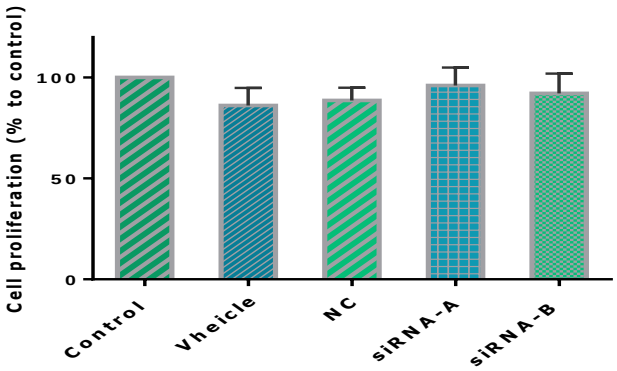

Figure 4.12: Cell proliferation assay on A549 cells transfected with *SLC22A1* siRNA-A and B at 20 nM.

The PB assay was conducted 48 hrs post transfection with OCT1 siRNA A and B. No differences were observed between groups (One way ANOVA, followed by Tukey test, $p \geq 0.05$). The data represents mean± SD, (triplicate, n=1, n represent no of experiment).

4.5 Discussion

A transient knockdown of the two genes *SLC22A1* and *5* was attempted via siRNAs using a peptide based vehicle, NTER. No impact on *SLC22A1* and *A5* mRNA level was observed in the A549 cells 24 hrs post transfection at 20 nM siRNAs. In contrast, Dr Manali Mukherjee obtained knock down of these genes in her work using the same materials except the qPCR primers. Several factors can affect the efficiency of knock down of genes since siRNA is a sensitive process. For example, cell density and their distribution in the well at the time of transfection, percentage of confluency, the use of nuclease free water and tubes for preparing siRNA/vehicle nano-particles, incubation time for allowing formation of the complexes, using either serum or serum free protocol all may affect the outcome of transfection.

An alternative vehicle, FuGENE® HD transfection reagent was tested as well for its knock down efficiency of the *SLC22A1* gene. Similarly to NTER, FuGENE® HD transfection reagent did not exhibit down regulation of the gene.

INTERFERin® transfection reagent exhibited down regulation of *SLC22A1* gene with 1 nM siRNA-B at 24 hrs, whereas siRNA-A caused decreased in the gene level at 48 hrs. Both sequences were shown to cause down regulation of the gene in comparison with control group, however, fluctuating effect was observed for siRNA-A and B. This may be because of difference in the rate of releasing the siRNA and incorporation with RISC complex to degrade the matching mRNA. Both targets of siRNA-A and B were also found to cause down regulation of the gene at 48 hrs, suggesting slow release of siRNA. Intracellular factors may also influence down regulation of the gene, for example, number of copies of the target mRNA available in the cytosol. Surprisingly, both siRNA at 20 nM did not show decrease in the gene level at the two points of measuring. This could be because of contamination of siRNA/vehicle complexes during the preparing steps for transfection or issues during collection of the cells, extraction of total RNA, transcription of RNA to cDNA or with the qPCR machine.

When treated groups were compared to vehicle as a main control, siRNA-B at 5 nM 48 hrs post transfection was found to be the only

dose to cause significant down regulation of *SLC22A1* gene (data not shown).

Measurement of post transcriptional level of genes at the protein level is the frequently applied step to measure the effectiveness of knock down after confirming down regulation of the mRNA of the gene of interest. As the mRNA of *SLC22A1* was reduced, it was predicted that the protein level would be diminished as well. The OCT1 siRNA- B at 1 nM caused significant decrease in the protein levels 48 hrs post transfection, whereas both siRNA A and B at 5 and 20 nM led to significant down regulation in the protein level at 48 hrs post transfection. When knocked down groups compared to vehicle as a main control, again 5 nM siRNA A and B were found to cause significant down regulation of OCT1 protein but not with 20 nM. This may explain that vehicle resulted decrease in OCT1 protein expression or caused death of cells which decreased protein expression since more volume of INTERFERin® was used with 20 nM compared to 5 nM.

Regarding pre and post transcriptional level of a particular gene, there is evidence that the relation between mRNA and protein levels is poor [446, 447]. Indeed, the number of mRNA copies will not all be translated into protein since intracellular factors may affect processing of the mRNAs before the translation process is completed. For example, the structure of mRNA and its length,

ribosomal abundancy as well as availability of tRNA, are all factors involved in the translation of the mRNA into the corresponding protein.

To screen whether OCT1 protein reduction is involved in or has an effect on cell proliferation, the CyQUANT® NF cell proliferation assay and PrestoBlue assays were conducted. The experiments have shown no influence in A549 cells transfected with siRNA-A and B at 20 nM 48 hrs post transfection although this dose for both targets of siRNAs has produced significant down regulation in OCT1 protein when compared to control group.

It was suggested that acetylcholine synthesize by airway epithelium may play a role in stimulation of cell proliferation [11, 161]. The alveolar adenocarcinoma, A549 cells were confirmed to be able to produce Ach which was measured by LC-MS/MS technique, and reported to be approximately 0.22 ng/ml [273]. It was previously shown that muscarinic receptor, particularly M3-subtypes were implicated in bovine tracheal smooth muscle cell proliferation in conjunction with platelet-derived growth factor [115]. By applying quantitative PCR, Buhling *et al*., have demonstrated that mRNA of five subtypes of muscarinic receptors, M1-M5 was expressed in A549 cells as well as other lung tissue, and higher level of M3 mRNA was identified [179]. The expression level of mRNA of M1-3 subtypes was further confirmed in A549

cells by qPCR [273]. In respect to nAchR, particularly α7 subunit was shown to stimulate proliferative effect in hippocampal cells in adult mice [448], in non-small cell lung cancer (NSCLC) [449], and in proliferation of basal cells in human respiratory epithelium [450]. Plummer *et al*., have shown that α7 subunit nAChR was expressed at pre and post-transcriptional levels in A549 cells as well as normal primary culture from small airway. Grozio et al., have also shown that this alpha subunit is expressed in A549 cells [451]. Alama et al., have also shown that α7 subunit nAChR was expressed in A549 applying qPCR. Adding α-Cobratoxin, which is alpha 7 subunit antagonist into A549 cells led to decrease in cell proliferation, indicating involvement of this subunit in regulation of cellular division in these cells [452].

According to the studies presented above, most of cholinergic components, Ach and its synthesizing enzyme, cholinergic receptors (muscarinic and nicotinic receptors) have been identified in A549 cells. Although we obtained down regulation of the OCT1 protein, the protein was still expressed at 40% of control and could exert its normal function to some extent. There could also be other factors in the medium that participate in triggering cellular signalling pathways controlling proliferation.

In addition to showing that A549 cells synthesize acetylcholine, a few investigations examined the effect of adding exogenous

acetylcholine to these cells and other cell lines including 16HBE14o-. It was found that acetylcholine is involved in inducing the release of pro inflammatory mediator leukotriene B4 (LTB4) as well as IL-8 and TGF-beta 1 [179, 273, 453, 454]. It seems that acetylcholine produced by airway epithelium plays an important role in inflammatory responses and maintenance of cell functions rather than in the stimulation of cell proliferation [162].

4.6 Conclusion

Down regulation of the OCT1 protein did not exert an inhibitory effect on A549 proliferation probably either because the protein was still expressed to some extent or the Ach molecules synthesized by these cells are not involved in their proliferation. A549 cell proliferation is presumably modulated via substances that are present in the medium.

Chapter 5 General discussion

5.1 Introduction and proposed hypothesis

It is well established that respiratory epithelium is considered a physical barrier and first defence mechanism against various inhaled insults. It also represents a source of a variety of substances and molecules including growth factors, cytokines, and metabolising enzymes. Synthesis of some molecules by airway epithelial cells is triggered as a result of respiratory inflammatory diseases such as asthma, COPD. The respiratory epithelium in these pathological conditions is fragile and can be easily removed or denuded. As a result, damage of underlying tissue can be easily induced via inhaled substances and biological molecules. Therefore, restoration of the epithelium is essential for the maintenance of normal respiratory function. Healing/restoration of the lost area of the epithelium involve a few primary processes. Firstly, the cells detach and migrate from the edge of the wound to compensate the lost region. This step is followed by proliferation and differentiation of cells. In order for the cells to move or migrate there have to be provisional extracellular substances. All these essential events are controlled via molecules produced originally by the affected/stimulated epithelial cells including epidermal growth factor (EGF), transforming growth factor alpha (TFGF-α), hepatocyte growth factor (HGF) and keratinocyte growth factor

(KGF) [298, 299, 301, 302]. Recently, bone marrow stem cells have been shown to have the capacity to differentiate into airway epithelial cells [326].

In addition to all described molecules, airway epithelial cells were shown to synthesize and release non-neuronal acetylcholine [11, 455]. The acetylcholine from these cells has been shown to be released via organic cation transporters, and particularly the OCT1 protein [163, 164]. It has also been suggested that this type of acetylcholine acts as an auto/paracrine molecules that may exhibit essential functions to maintain cells survival such as migration, attachment, cytoskeleton, differentiation and proliferation. Depending on these observations and suggestions the hypothesis of the current study was drawn. The main aim was investigating whether OCTs, and notably the OCT1 protein is implicated in non-neuronal Ach translocation from the cytosol to the extracellular spaces in airway epithelial cells and whether this type of Ach is involved in their proliferation.

5.2 Synopsis of findings

A549 cells cultured in submerged conditions were shown to express four members of the solute link carrier family: OCT1, OCT3, OCTN1 and OCTN2 using traditional PCR. In addition, the levels of OCT1 and OCTN2 were quantified using qPCR. The post transcriptional levels of these two genes were relatively quantified

as well applying ICW. A variety of well-known OCT/Ns inhibitors were examined on scratched and unscratched A549 monolayers. Most of these drugs were shown to cause inhibitor effect/decrease in fluorescence intensity on unscratched monolayers but ipratropium led to a decrease in cell proliferation on scratched layers. PrestoBlue® and Cyquant™ assays showed transient knockdown of OCT1 in A549 cells had no effect on their proliferation 48 hrs post transfection.

5.3 Selection of the cell culture model

Selection of A549 cell line to be used as a main model in the current study was based on expression profile of both OCT/Ns as well as cholinergic elements including acetylcholine, muscarinic and nicotinic receptors as previously explained in 2.1.2 and 2.1.3. The expression of OCT/Ns, especially OCT1 and cholinergic components was essential to examine the proposed hypothesis. The expression profile of OCT/Ns has been previously studied in A549 cells. A couple of studies have shown expression of transcripts of four genes, OCT1, OCT3, OCTN1 and OCTN2 applying PCR and qPCR [229, 267]. We further confirmed their expression using traditional PCR. In addition, the pre and post transcriptional levels of OCT1 and OCTN2 were measured using qPCR and ICW, respectively.

Selection of this cell line was also based on the high transfection efficiency previously obtained by Dr Manali Mukherjee compared to

the lower knock down efficiency for the Calu-3 cell line [445]. This may be partially because of adhesion molecules expressed in these cells as well as the secretion of mucin which act as a barrier and hinders influx/permeation of transfection nanoparticles. The transfection efficiency in differentiated Calu-3 was also shown to be lower compared to the undifferentiated cells COS-1 (green monkey fibroblasts) for the reasons described above [456]. Although Calu-3 cells exhibited higher expression of OCT1 mRNA compared to A549 cells which has been suggested to translocate non-neuronal Ach from the intracellular to the extracellular spaces no study regarding cholinergic components expression including Ach, muscarinic and nicotinic receptors has been found in Calu-3 cells. In addition, as a pilot study, the effect of OCT/Ns inhibitors was first examined on scratched differentiated Calu-3 monolayers. However, making scratches on these monolayers resulted in removal of a wide area of adherent cells and inconsistent wounds because of the difficulty in moving a sterile pipette tip on membrane supports. Using alternative culture condition to ALI for Calu-3 cells such as submerged condition was not envisaged because a recent study has shown that ALI/differentiated monolayers are closer to native epithelial cells in terms of function and gene expression [457]. Although ALI condition shows similarity with *in vivo,* still some considerations and limitations should be taken into account in term of gene expression profile. By using

microarray, it has been found that expression profile of human primary culture grown at ALI condition was similar to those of native airway epithelia but still differences can be observed in certain genes such as inflammatory and cilia related genes [458]. The previous investigation has also found variable profile expression between Calu-3 and primary culture grown in submerged condition. Dvorak et al., have also demonstrated that ALI condition reflected resemblance to native airway epithelium but difference should be considered for some genes [459]. Variable expression of miRNAs for many of genes was found to be dissimilar between differentiated airway epithelium and native respiratory epithelial cells (in vivo) [460]. A more recent study has also showed that ALI culture condition exhibited different expression profile of immune and wound repair genes in comparison with in vivo epithelia in response to flagellin [461]. The observations of comparative expression profile reflect presence of limitations of using ALI in spite of being the more suitable condition for study the biology of airway epithelium.

5.4 Efficiency of using PrestoBlue reagent to determine cell viability and proliferation/wound healing

In the current study, the PrestoBlue® (PB) cell proliferation assay was mainly used to examine the effect of the inhibitors tested on cell proliferation and their viability. The PB assay is based on

measuring the metabolic activity of mitochondrial enzymes. This assay was shown to be more efficient in determining minimum inhibitory concentration and half inhibitory concentration for a variety of tested compounds on different microorganisms compared to AmarBlue and p-iodonitrotetrazolium violet (INT) [370]. Again, the use of the fluorometric PrestoBlue assay was advised for determination of cytotoxicity towards human umbilical vein endothelial cells (HUVECs) in comparison to MTT [462]. In a more recent investigation the PB was found to be more reliable than MTT and AlmarBlue® in determining the viability of human corneal epithelial cells [371]. All these investigations strongly confirmed that PB assay can be applied and obtained more consistent/reliable data than other assays. Apart from comparing between cell viability assays, PB was used to examine the viability of glioblastoma cells and astroglia cells treated with aspirin for seven consecutive days [463]. Fraser et al., have also used the PrestoBlue® reagent to assess cell viability of different cell lines including breast cancer MCF7, MDA-MB-231 (triple negative breast cancer), and PC3 (prostate cancer) treated with different compounds at different concentrations [464].

The PB reagent is not only restricted to assessing cell viability but is also widely used as a cell proliferation reagent. The PB reagent has been used to assess cell viability of NIH 3T3 (Swiss mouse

embryonic fibroblast cell line) delivered at different volumes after 6 and 24 hrs [465]. The study also measured NIH 3T3 cell proliferation after days of delivery up to 120 hrs. The reagent was also used to monitor cell proliferation of human primary fibroblast printed in 3D dermis models at different time points up to 21 days of printing [466]. Again, the assay was used to monitor proliferation of human periosteal-derived cells (hPDCs) cultured in different patterns including static 2D, static 3D and perfused 3D [467]. Proliferation of human corneal stromal cells added onto different materials was monitored at different time points up to two weeks using PrestoBlue® reagent as well [468]. All these recent investigations suggest reliability and robustness of the results when using this reagent for probing cell viability and proliferation.

The assay was also used on A549 cells transfected with OCT1 siRNA A and B at 20 nM (Chapter 4). In addition to using this reagent, we used the alternative cell proliferation assay CyQUANT® which depends on measuring DNA quantity by a fluorescent dye. The fluorescence of this reagent directly reflects the cell number rather than the metabolic activity of mitochondrial enzymes like the PB reagent. The metabolic activity does not reflect cell number like the DNA assay does. This was noticed during monitoring cancer cell growth for four days. The metabolic assay AlmarBlue

over-estimated cell growth compared to the CyQUANT® assay [469].

There are alternative assays that can be used to monitor both cell viability and proliferation based either on measuring metabolic activity or DNA quantification. MTT and AlmarBlue assays are examples for measuring mitochondrial enzyme activity, whereas bromodeoxyuridine (BrdU) a DNA synthesis–based cell proliferation assay and a [^{3}H] thymidine incorporation assay are examples of DNA quantification which are based on incorporation of the thymidine/thymidine analogue BrdU within newly synthesized DNA. Cell proliferation can be measured with the former assay by using a BrdU antibody and with the latter assay by a scintillation beta-counter that detects the radioactivity of [^{3}H] thymidine. Both assays are widely used to assess cell proliferation in a variety of cell types including airway smooth muscle cells [470].

5.5 Influence of transient knock down of OCT1 gene on cell proliferation

It was difficult to determine whether OCT/Ns are involved in cell proliferation using a variety of inhibitors. Therefore, OCT1 was transiently knocked down using interfering RNA technique, small interfering RNA (siRNA). The OCT1 protein level was down regulated to about 60% of control group at 20 nM siRNA A and B using INTERFERin® reagent. However, no significant difference in

cell proliferation was observed between transfected and control groups.

A variety of vehicles can be used for gene silencing via siRNA . In our study, we have used three of them, and only INTERFERin® reagent resulted in significant down regulation compared to control. However, the OCT1 protein was still expressed to about 40%, and the remaining protein could exhibit its transportation function. Therefore, it is difficult to state whether OCT1 protein is involved in cell proliferation. To make it clear or to draw conceivable conclusion, it is suggested alternative vehicles are used, preferably lipid based such as Lipofectamine® [442]. If using the latter reagent is inefficient and fail to produce high or remarkable down regulation of the protein. shRNA could be used to obtain gene knock down instead of siRNA. In siRNA vs shRNA comparison studies, the latter was shown to be more robust and efficient in inducing gene silencing both *vivo* and *in vitro* empirical studies [471-473]. In addition of being more robust, shRNA effects last longer than those of siRNA since it is transcribed from the cellular genome. It also causes less off-target effects than siRNA as low number of copies can induce knock down [426].

5.6 Future work

It is well established that the respiratory epithelium plays a crucial role in protecting underlying tissues against multiple inhaled insults. It can be summarized that the airway epithelium functions include mucociliary clearance, secretion of a variety of mediators, and participation in immune responses.

These epithelial cells have been shown to express at least four members of organic cation transporters OCT/Ns which exhibit important role in absorption of inhaled drugs. Alteration in protein expression levels of these proteins has been shown in differentiated Calu-3 cells exposed to various inflammatory irritants such as LPS or human dust mite [474], suggesting their implication in respiratory inflammatory diseases.

OCTs, particularly OCT1 was shown to transport intracellular non-neuronal acetylcholine into the extracellular environment where it can bind to its cholinergic receptors and exert different function. We were unable to determine its involvement in cell proliferation but work could be focused on other suggested functions such as migration, attachment, and differentiation [11]. A recent review has suggested that non-neuronal Ach may implicate/interact with inflammatory cells, notably macrophages available on respiratory airway epithelial cell surface. This could be a task to screen the mechanism/effect of this type of Ach on macrophages [162]. It has

been found that inflammatory cells, in particular macrophage cells express nicotinic receptors, alpha 7 nAChR subunit. The latter has been found to exhibit anti-inflammatory effect through preventing release of TNF and other inflammatory cytokines [475]. It would worthy to investigate acetylcholine role and alpha 7 nAChR subunit role in modulation inflammatory conditions, and the possibility of using this to treat inflammatory disease. The alpha 7 nAChR subunit has been found to be expressed in endothelial cells and is responsible for angiogenesis via Ach and/nicotine [476, 477]. It is valuable to and suggested to carry out more investigation in this field.

A particular attention should be focussed on the OCTN2 transporter since an up-regulation in this gene has been reported in asthmatic patients [240].

5.7 Conclusion

The selection of the A549 cell line to examine the proposed hypothesis was based on the expression profile of OCT/Ns as well as cholinergic components. It was difficult to draw conclusions regarding the involvement of OCT/Ns in cell proliferation using a variety of transporter inhibitors. Therefore, we knocked down OCT1 gene to obtain clear understanding whether it is involved in respiratory cell proliferation via transporting non-neuronal acetylcholine. The knock down efficiency was not high enough to judge gene implication in cell proliferation since the protein was still expressed in A549 cells.

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