NOTTINGHAM TRENT UNIVERSITY

β_2-ADRENOCEPTOR INVERSE AGONISTS SIGNALLING IN HUMAN AIRWAY CELLS

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A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

December 2019

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Declaration

I Hafsatu G. Bawa-Sani, hereby declare that the research presented in this thesis was conducted by myself unless otherwise stated.

Dedication

This work is dedicated to the life and Memory of father, Garba Muhammad Bawa(1948-2019). May Allah reward you with al-jannatul Firdaus.

Acknowledgements

Special thanks and gratitude to Allah for the strength and courage and blessings that helped me throughout my thesis.

To my supervisor, Dr. Carl Nelson, I am grateful for the support and guidance I received throughout my thesis which has brightened my professional career. Thank you for being friendly and open minded.

To my second supervisor, Dr. John Dickenson thank you for your guidance and constant advice throughout my research.

Special thanks to my friends and colleagues most especially, Drs .Grace and Biola.Thank you for the friendship throughout my research at Nottingham Trent university.

I am also grateful to Elisa Tonoli, Maria Pia, Arif Surani, Merrell, Charlie, Belal Istec 139 and late Jordan Callear for making my research journey worthwhile.

To my husband, Auwalu and our sons, Sadiq, Umar and Ibrahim for their love and patience and the sacrifices you made throughout my research. To my parents, sisters and brothers thank you for being there for me when I needed you the most. I appreciate all the prayers and many more.

This would not be complete without mentioning my gratitude to TETFUND Nigeria and Umaru Musa Yaradua University Nigeria, for funding this research.

Special thanks to Dr .Grace and Dr .Tanze, Mr and Mrs Lucky-Eweh, Rita, friends, colleagues and family relations in Nigeria .

Conference communications and abstracts

- **2019 Thesis challenge**

  Nottingham Trent University research festival

  Topic presented: Asthma, New treatments.

  BAWA-SANI Hafsatu G

- **2019 Life sciences symposia**. University of Nottingham

  Poster (number 47) and Abstract Title: The effect of β_2-adrenoceptor ligands on wound healing in BEAS2B-R1 cells.

  BAWA-SANI Hafsatu G, DICKENSON John, NELSON Carl, The effect of β_2-adrenoceptor ligands on wound healing in BEAS2B-R1 cells.

- **2019 Women in Chemistry**

  Poster (number 24) Title: Impedance measurements in BEAS2BR-1 cells.

  BAWA-SANI Hafsatu G,DICKENSON John, NELSON Carl, Impedance measurements in BEAS2BR-1 cells.

- **2018 British Pharmacological Society meeting**

  University of Nottingham ,United Kingdom

- **2017 Science and Technology Conference**

  Nottingham Trent University.

  Oral Presentation, Abstract and Poster (number 7) title: β_2-adrenoceptor inverse agonist signalling in Human airway cells.

  BAWA-SANI Hafsatu G, DICKENSON John, NELSON Carl. β_2-adrenoceptor inverse agonist signalling in Human airway cells. School of Science and Technology, Nottingham Trent University.

Abstract

Bronchial asthma is characterised by airway inflammation, obstruction and reversible airway-hyper responsiveness (Lötvall *et al.,* 2011). One common treatment is the use of bronchodilator (particularly β agonists).Several studies have reported that the bronchodilators were less effective in chronic use, with worsening symptoms of poor asthma control (Bond *et al.,* 2007). In the quest for new modalities of treatment, antagonists (or inverse agonists) were found to be beneficial to patients on chronic administration. Previously, the agonists were shown to activate the receptor stimulating cyclic AMP production while inverse agonists do not, but act as "biased" agonists in promoting β-arrestin-dependent signalling through the β–adrenoceptor (Nguyen 2008, van der Westhuizen 2014). Because these cell signalling pathways are associated with modulation of intracellular β_2-adrenoceptor activity, it is plausible to hypothesize that the β_2-adrenoceptor agonists and inverse agonists directly affect β_2-adrenoceptor activity. In view of the this ,the thesis main aims were to investigate whether the β_2-adrenoceptor population are sensitive to inhibition by β_2-adrenoceptor inverse agonists and whether they are biased agonist which stimulate G-protein independent signalling pathways and stimulate the functional effects of the β_2-adrenoceptors on airway cell biology.

In the first part of the investigation, the production of cyclic AMP in Calu-3 and BEAS2B-R1 cells and real-time analysis of cellular impedance was investigated in BEAS2B-R1, Calu-3 and CHO-β_2 cells. Phosphorylation of ERK1/2 in Calu-3 and CHO-β_2 cells was assessed using western blotting techniques. The second part of investigation assessed the functional effect β_2-adrenoceptor stimulation using MTT reduction , MTT real time glo, Neutral red, incuCyte real time assays and cell counting. The third part assessed the effect of β_2-adrenoceptor stimulation in wound healing using mechanical,chemical and automated methods of wounding. The fourth part assessed the effect of TEER in Calu-3 cells and was investigated under air liquid interface experiments (ALI).

The β_2-adrenergic stimulation with isoprenaline and formoterol increased the cyclic AMP in Calu-3 cells and cellular impedance in BEAS2BR-1cells. Cellular impedance assay using the xCELLigence system decreased in BEAS2BR-1 and increased in Calu-3 cells and CHO-β_2 cells with formoterol and propranolol. The MEK 1 inhibitor, PD98059 did not decrease cellular impedance with both formoterol and propranolol. Formoterol and carvedilol increased ERK 1/2 phosphorylation in CHO-β_2 cells. The assessment of the functional effects of β_2-adrenoceptor stimulation showed increased cell viability in response to propranolol and nadolol in Calu-3 cells using MTT reduction assay. Responses to propranolol in neutral red assay increased cell viability in BEAS2BR-1 cells. Wound healing and repair assessed with incuCyte S3 system was inhibited by carvedilol and salmeterol and forskolin. The presence of tight junctions in Calu-3 cells was confirmed by sequential changes TEER measurements. The development of tight junctions was found to increase TEER in response to formoterol and propranolol. Overall, the findings highlight the β_2-adrenoceptor activity in the cellular function of β_2-adrenoceptor inverse agonist and shows the molecular mechanism involved via cyclic AMP and ERK1/2 dependent pathways.

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List of Abbreviations

7TM	7 transmembrane
AC	Adenylate Cyclase
AIDA	Advanced image data analysis software
ALI	Air liquid interface experiments
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BHR	Bronchial hyperresponsiveness
BSA	Bovine serum albumin
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCF	Congestive cardiac failure
CHO	Chinese hamster ovary cells
CI	Cell index
DAPI	Diamidino-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPR	opiod receptor
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	Extracellular signal-regulated kinase 1 and 2
F12	Ham's F-12
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FZD	Frizzled proteins

$GABA_B$ metabotropic glutamate receptors

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GIP Glucose independent insulinotropic polypeptide

GPCRs G-protein coupled receptors

GPCRs G-Protein-Coupled Receptors

GRK G-protein coupled receptor kinase

GTP Guanosine Triphosphate

GTPase

HH Hedgehog

HRP Horseradish Peroxidase

IBMX 3-isobutyl-1-methylxanthine

ICS inhalational corticosteroids

IL-6 Interleukin-6

JNK c-Jun N-terminal kinase

JNK3 c-Jun amino -terminal kinase 3

LABA long acting β_2-adrenoceptor agonists

MAPK Mitogen activated Kinase

MAPK Mitogen activated protein kinase

MAPK Mitogen-activated protein kinase

MAPKK MAP Kinase Kinase

MEK Mitogen Extracellular Signal-regulated Kinase

MENA Non-Essential Amino Acids

mRNA Messenger RNA

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUC5AC Mucin 5AC

MUC5B Mucin5B

NaOH Sodium Hydroxide

NHBE Normal human bronchial epithelial cells

P38 P38 mitogen-activated protein kinase

PACAP Secretin, pituitary adenylate cyclase-activating polypeptide

PBS Phosphate Buffered Saline

p ERK phosphorylated ERK

PKA Protein Kinase A

PKC Protein Kinase C

PMA Phorbol 12-Myristate-13-Acetate

PTCH transmembrane protein patched

RIPA Radioimmunoprecipitation assay buffer

RTCA DP Real time cell analysis dual purpose

RT-CES Real-time cell electronic sensing system

RWD Relative wound density

SDS Sodium Dodecyl Sulphate

SDS-PAGE Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SMO Smoothened

TBS Tris-buffered saline

TBST Tris Buffered Saline - Tween 20

TEER Transepithelial electrical resistance

TEMED N,N,N,N′-Tetramethyl ethylenediamine

VIP vasoactive intestinal polypeptide

WHO world health organisation

ZO-1 Zonula occludens-1

Chapter 1
Introduction

Introduction

1.1 General overview of the Human Respiratory System

1.1.1 Anatomy This is a complex structure which is involved in exchange of Oxygen and Carbon dioxide between air blood and body tissues. All animals breathe in oxygen and breathe out carbon dioxide. The lungs of animals developed complex internal lungs which divide into numerous tiny air sacs lined by thin moist epithelium, that permits the exchange of gases between the external environment and capillaries. The epithelium of the lungs is delicate, and as such it is protected by the condition of air as it passes through the air passages (Carlson 2019). The respiratory and circulatory system work together to transport oxygen from lungs to cells in exchange for carbon dioxide which is exhaled (Shier *et al.*, 2015).

The respiratory system consists of a conducting zone and a respiratory zone. The conducting zone consists of the trachea, bronchi and terminal bronchioles where air passes without any gaseous exchange (Germann and Stanfield 2004). Gaseous exchange occurs in the respiratory zone which consists of respiratory bronchioles, alveolar ducts and sacs. The airways branch forming up to 23 generations from trachea to alveolar sacs (Germann and Stanfield 2004). Inhaled air passes through these branches to reach the alveolar sacs where the gaseous exchange occurs. The respiratory tract at each level changes to adapt to the function of the region. Also, this condition the air and humidifies it as it flows along the respiratory zone and aids the removal of pathogens, production and transport of mucus. The mucus is important in removing trapped particles and pathogens in the inhaled air (Carlson 2017).

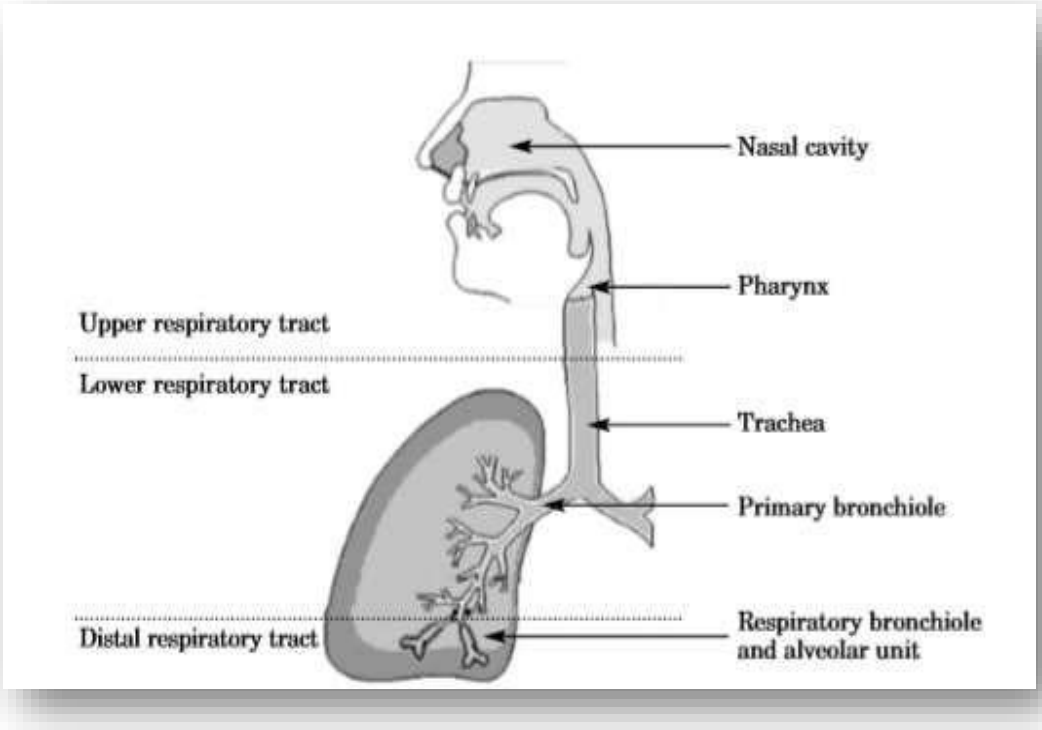


Figure 1.1 Diagram of the human respiratory system.

Diagram illustrating an adult respiratory system showing respiratory passages in relation to the 3 major divisions of the respiratory system; upper, lower and distal respiratory tracts (Berube et al., 2009).

1.1.2 Human Airway

The human airway is lined by a continuous layer of pseudostratified epithelial cells involved in maintaining important regulatory functions. These pseudostratified epithelial cells are basal, ciliated, secretory and subepithelial glands (Coraux *et al.,* 2005). The functions of these cells include barrier functions, mucociliary clearance, secretory functions (mucins and surfactants and peptides), repair and regeneration and inflammatory response mechanisms (Proud, 2008).There are 49 different cell types that were identified (Breeze and Wheeldon 1977) and the cell types differ from conducting zones to respiratory zones based on functions and defence mechanisms in each zone (Breeze and Wheeldon, 1997). The lower respiratory tract epithelium is pseudostratified columnar epithelium which includes basal, goblet and Clara cells and their secretions involved in mucociliary clearance. Cuboidal epithelium

containing Clara cells lines the bronchioles. The distal respiratory tract is lined by alveolar epithelium which consist of alveolar type I and type II cells.

The alveolar cells form a gaseous exchange barrier with endothelial cells. The alveolar cells regulate lung surfactant function and contain macrophages which remove inhaled pathogens (Breeze and Wheeldon, 1997).

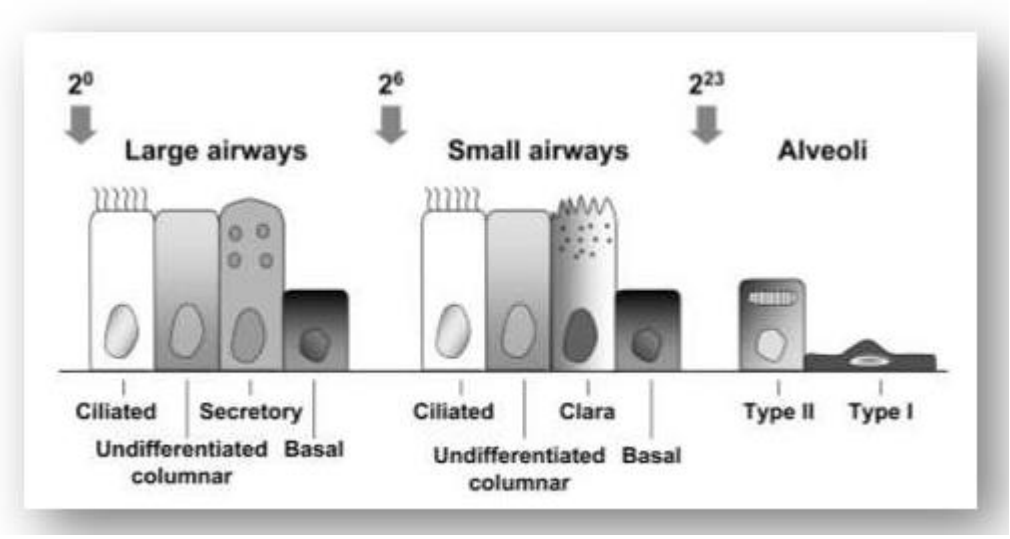


Figure 1.2 The cells of the respiratory epithelium.

The diagram illustrating the cells that line the respiratory airway, showing the cells of the large airways which are; ciliated columnar undifferentiated cells, basal and secretory cells. The cells line the respiratory epithelium along the tracheal partitions which are partitioned into 23 generations. These partitions form dichotomous branching extending from trachea termed generation 0(2^0) to the last order of terminal bronchioles generation 23(2^{23}) . In the smaller airway from $2^6$to 2^{23} the cells are also ciliated undifferentiated basal columnar cells but are more ciliated and the secretory cells have changed to Clara cell type. From 2^{23} the cells become alveoli type I and II. Adapted from Crystal et al., 2008.

The cell types along the airways in an adult human vary in function depending level they are located. The numbers of cartilage cells and submucosal glands are reduced and the Clara cells increase in number from larger to smaller airways (Crystal *et al.*, 2008).

1.1.3 Development and differentiation of respiratory cells

The development of the conducting airways and development terminal respiratory units. The bronchial structure develops at 16th week and is completed when the primordial segmental

airways have undergone repeated divisions and the number of airway generations have been formed (Hislop *et al.*,1974). After which the distal conducting airways are transformed into respiratory bronchioles. The tracheobronchial structures are completed at birth while acinar structures are completed after birth formed (Bucher *et al.*,1961). Alveolar structural development begins from 30-32nd week of gestation till term (Boyden *et al.*,1965).

The epithelium develops as primitive actively dividing foetal cells which appear as epithelial cells identified by the presence of tight junctions and desmosomes (Jeffrey et al.,1977). The epithelial develop from the trachea and further down the respiratory tract. The basal cells are developed at the 10th week. The basal cells develop into the ciliated and goblet cells in the central airways. In the lower part of the respiratory tract, a precursor of the Clara and ciliated cell appear (Jeffrey *et al.*,1977). The goblet cells appear in 13th week of the trachea and upper part of bronchi while submucosal glands appear at the 10th week in trachea and 13th week in bronchi. The cilia and mucus appear in the airways by the 16th week (Adamson 1991). The type I and II cells are developed by the 24th week (Aguayo *et al.*,1992).

The developing primary lung epithelium is important in lung department. For example, in foetal lamb lung fluid analysis showed increase level of k^+ and $cl^-$and low protein, HCO^-_3 and Ca^{2+}.This low protein content is lower than plasma or lymph protein. This suggests the epithelium is impermeable to macromolecules during foetal development (Adamson *et al.*,1969). Also in human foetal lung tissue investigated in submersion organ culture to determine whether bronchopulmonary epithelium secretes fluid during development. Transepithelial potential differences were measured after 3-8 days in culture. Isoproterenol increased Transepithelial potential differences which was inhibited by bumetanide. Cyclic AMP and 3-iosobutyl-1-methylxanthine increased transepithelial potential difference. This suggest the contribution of bronchopulmonary epithelium to lung fluid production and stimulation of Cl- secretion and fluid protection is agonist and cyclic AMP dependent (McCray *et al.*,1992). The production of cyclic AMP is necessary for normal lung function

which begins during intrauterine development as stated previously. The focus of this thesis is on the effect of β_2-adrenoceptor agonists and inverse agonist signalling in human airways which agonists and inverse agonists increase or decrease cyclic AMP production. A comprehensive description of these events would be advantageous.

1.1.4 Respiratory epithelial defence mechanism

The role of the epithelial lining of the airways is important in maintain the passage of air to and from the alveoli. It not only protects against infection from inhaled pathogens but it is also important in maintaining mucociliary clearance with combined effort of the secretory and ciliated cells (Knight *et al.,* 2003). The respiratory epithelial cells regulate defence mechanisms by secretion of mucous, regulating ciliary action of cells, ion secretions and regulation of anti-inflammatory mechanisms (Chang *et al.,* 2008). There exist intercellular and paracellular junctions between the epithelial cells which regulate the entry and exit of ions fluid in a regulated manner. As such the junctions form an effective barrier which prevents the passage of inhaled pathogens and noxious agents (Coyne *et al.,* 2003). This epithelial barrier is important in regulation of a stable ionic environment(homeostasis) and loss of which integrity results in leakage of ions and entry of pathogens causing impairment of homeostasis (Minuth *et al.,* 2006).

The epithelial barrier is prone to injuries on regular basis due to its interaction with the external environment which include; gases, fumes, droplets, biological matter, infectious agents and noxious physical agents. The injury to the epithelial barrier results in loss of permeability of the tight junctions which is a common feature of destruction of the epithelial surface. This could involve the partial or complete loss of the basal membrane (Coraux *et al.,* 2005). The loss of the epithelial function compromises pulmonary function such as loss of barrier function resulting physiological abnormalities, loss of secretory and ciliary functions leading to infective mucociliary clearance. It also affects the secretion from

pulmonary epithelial cells or from cells other than airway epithelial cells which are involved in specific challenges which affect the airway epithelium (Thomson *et al.*,1995)

The disruption of the epithelial layer of cells affect the structure and functions the epithelial barrier in the airway. This is important in understanding the pathogenesis of common respiratory diseases such as bronchial asthma (Knight and Holgate, 2003). The disruption of the epithelium exposes the damaged region to hyperreactivity to non-specific agents (Amishima *et al.*,1998). Also, the separation of epithelial cells from the basement membrane causes the upregulation of the Epidermal growth factor receptor. It is also an identifier of asthma severity in asthmatics. The bronchial epithelial repair processes showed Epidermal growth factor (EGF) enhanced repair of scrapped wounded monolayers in human bronchial epithelial cells. However, this was inhibited by its selective inhibitor tryphostin AG1478 and was accompanied by delay in wound closure (Puddicombe *et al.,* 2000). The wounding of confluent monolayers in the study of wound healing and repair is a widely used method in the study of migration of cells. The investigation of the effect of EGF on migration and proliferation of epithelial cells in human airway cells was assessed in this current study.

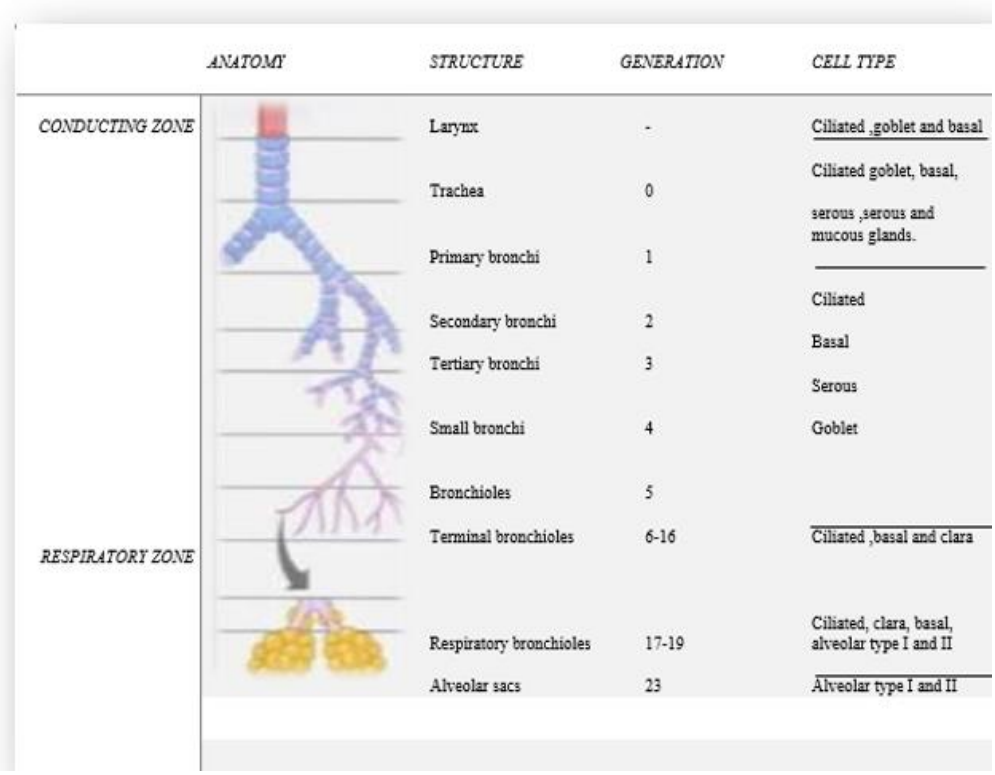


Figure 1.3 The structure of the respiratory airway

The diagram illustrating the structure of the respiratory airway showing the conducting and respiratory zones. The conducting zone is where there is no gaseous exchange. Air passes through the trachea, bronchi and terminal bronchioles. The respiratory zone is where the gaseous exchange between the lungs and pulmonary capillaries occurs. It consists of respiratory bronchioles, alveolar ducts and sacs. The airways divide from trachea to the alveolar sacs up to 23 generations. (Germann and Stanfield 2004).

1.2 Bronchial Asthma

According to the World Health Organisation there are 300 million people worldwide affected with asthma. There are more cases in children than adults and the prevalence of the disease is increasing in both developing and developed countries (Knight et al, 2004).The

cause of the increase in prevalence of the disease has been associate with change in lifestyle such as smoking and urbanization of less developed countries (Bousqet *et al.,* 2003).The cause due to urbanization is not clearly understood (WHO asthma facts 2017). There could be an additional 100 million people affected with asthma (Masoli *et al.,* 2004). Other risk factors for developing asthma include indoor allergens (house dust mites, pollution and pet fur), air pollution, moulds, pollens, smoke and chemical irritants from industries and factories (WHO asthma facts 2017).

Asthma is a disease of the respiratory system which causes narrowing of the airways, cough, wheeze and difficulty in breathing i.e. airway hyperresponsiveness. The airway hyperresponsiveness is associated with increased sensory irritability of the airways with increased secretion of mucous (Holgate *et al.,* 2008). The presence of clinical features of asthma might depend on different environmental factors which cause acute and chronic inflammation, contraction of airway smooth muscles, oedema and remodelling of airways. It has also been reported that Bronchial hyperresponsiveness (BHR) is a prominent feature of asthma. Although this was investigated in laboratory with bronchial provocation, environmental factors such as cold air, air pollutants, allergens and infections triggered BHR (Joos *et al.,* 2003). Asthma has also been identified as an inflammatory disorder associated with the presence of inflammatory cells cell such as mast cells, eosinophils ,basophils co-ordinate by anti-gen presenting cells via T and B lymphocytes. This inflammatory process is vital in allergic asthma which is supported by the effectiveness of leukotriene receptor antagonists and anti-human IgE monoclonal antibodies (Holgate *et al.,* 2004). Some gene-environmental interactions may influence some clinical features of asthma (Holgate *et al.,* 2008). For example, children and adults have different features of wheeze that is affected by age of onset, presence or absence of atopy and airway eosinophilia (Morgan *et al.,* 2005). Also, asthma has been characterised as a collection of different phenotypes. The phenotypes are different features of an organism from the interaction between its genetic composition

and the environment (Wenzel 2012). The understanding of phenotypes of asthma would provide insight into the link between genetics and environment (Wenzel 2006). In view of this, the difficulties in studying the disease and its phenotypes can be overcome by studying the genetics of asthma. Such as the use of improved genetic tools to investigate the heritable component of asthma, as well as the identification of alteration of genetic sequences in DNA base pairs. This would improve the understanding of the biology of asthma (Mutius 2008).

1.2.1 Pathophysiology of Bronchial asthma

The inflammatory response in allergic asthma results from the presence of eosinophils, neutrophils, $CD4^{+}$T lymphocyte and mast cells (Kay *et al.,* 2005).The complex interaction between the immune system and epithelial cells is responsible for the heterogenous presentation of the disease (Akdis *et al.,* 2019).In addition to this inflammatory cytokines such as interleukin are released in response to epithelial injury which play an important role in in providing rapid inflammatory response (Ziegler *et al.,* 2019).The inflammatory cytokines are a large group of proteins, peptides or glycoproteins (Brightling *et al.,* 2002). The inflammatory cytokines include lymphokines such as (Interleukin-2,3,4,5), proinflammatory cytokines (interleukin-1,6, tumor necrosis factor), anti-inflammatory cytokines (Interleukin-10,12,18) chemotactic cytokines (rantes, interleukin-8) and growth factors (platelet derived growth factor, epidermal growth factor). The contribution of cytokines is the pathogenesis of asthma is vast. This was investigated in this research. As part of the aims, the role of interleukin-6 in asthma was investigated with respect to its effect on airway cell biology.

The inflammatory response is localized in mild disease in conducting airways. In moderate and severe disease, the inflammation progresses into the smaller airways and alveoli (Kraft *et al.,* 1999). In non-allergic asthma the response is IgE mediated (Corrigan *et al.,* 2007). Some forms of late onset asthma are not IgE mediated and are related to occupational exposure to toxins (Slavin *et al.,* 2005). The presence of autoantibodies was discovered to

be directed towards epithelial components (Ye *et al.,* 2006). Previously in allergic asthma, circulating auto antibodies which targeted β_2-adrenoceptors in lung membranes prevented the binding of high affinity β_2-adrenergic ligand. The findings were presented in patients with β-adrenergic hypoactivity and airway hypo responsiveness (Venter 1980; Fraser 1981). These suggested an antagonist's role by the auto antibodies (Harrison *et al.,* 1982). Furthermore, studies have been conducted on the influence of the immune system on βadrenergic stimulation. During this response the CD4+ cells and T lymphocytes are activated to produce cytokines and antibodies (Kohm and Sanders 2001). Also, in norepinephrine depleted mice, The IgG_1 stimulation was abolished before adoptive transfer of antigen specific T helper 2 cells and B cells (Kohm and Sanders 1999). In addition, β_2-adrenoceptors increased IgG_1 production in (Kaprowicz *et al.,* 2000). This suggest the significance of the immune system and norepinephrine effect on antibody production.

1.2.2 Airway Epithelium in Asthma

The epithelial cells of the airway in asthma are involved in type 2 immune response as well as type 2 cytokine effect of airway epithelial cells. The airway epithelial cells form an effective barrier against infective agent in inspired as it passes the airways (Crystal *et al.,* 2008). The normal airway is lined by pseudostratified epithelium, the basal cells anchor the epithelium to the matrix (Rock *et al.,* 2010), while the ciliated cells transport mucous produced by secretory cells. In healthy airway the secretory cells produce MUC5B rich mucus gel which is easily transported by ciliary action of the ciliated cells. However, in asthma because of goblet cell hyperplasia, MUC5AC mucin is produced by secretory cells and it covers the epithelium and impairs mucociliary clearance (Bonser *et al.,* 2017). This is most likely the cause of mucous plugging airway obstruction. The presence of MUC5AC mucin in asthma has been studied and discovered to be a likely cause of airway obstruction in asthma (Bonser *et al.,* 2016). MUC5AC was also identified to be required in airway

obstruction in MUC5AC deficient mice (Evans *et al.,* 2015). Mucins are large glycoproteins which form the framework of mucus which form part of innate defence (Roy *et al.,* 2014).

1.3 The G-protein coupled receptors

These are one of the members of the G-protein coupled receptors, which represent the largest group membrane-bound receptor proteins in mammals which number approximately 1000 (Thomsen *et al.,* 2005, Lefkowitz 2004). They are the largest group of cell surface receptors and also the largest group of drug target proteins (Fredrickson and Schioth 2005). Thus, they are one of the most important drug targets in drug discovery and delivery. The G-protein coupled receptors (GPCRs), were previously thought to coupled only to G-proteins and activate intracellular effectors such as adenylyl cyclase or phospholipase C, which then initiates the activating of intracellular messengers such as cyclic AMP or Inositol triphosphate. However, the GPCRs have now been discovered to activate not only the G-protein dependent signalling pathways but in addition they activate G-protein independent signalling pathways (Pupo *et al.,* 2015). The G-protein independent signalling are the β-arrestin dependent- activation of ERK1/2 and other kinases such as c-Jun amino -terminal kinase 3 (JNK3), Mitogen activated Kinase (MAPK) (McDonald *et al.,* 2000, Shenoy and Lefkowitz 2011). This led to the discovery of the complexity of the multiple signalling pathways and how they are modulated by the GPCRs. Also, within this same period ligands were discovered which trigger a small fraction of responses produced by the effects of the GPCRs. This was also important in the discovery of a balanced agonist, which binds to a receptor and stabilises receptor confirmations to allow activation of downstream pathways (Pupo *et al.,* 2016). But it has now been discovered that alternate pathways can be preferentially activated over another by ligands called biased agonists. In support of biased agonism, Lefkowitz and colleagues, discovered that an angiotensin II analog did not activate G-protein coupled receptor (Angiotensin II) but stimulated β-arrestin recruitment and

activated ERK1/2 phosphorylation (Azzi *et al.,* 2003). In another study propranolol a β_2-adrenoceptor antagonist was identified as a biased agonist towards β-arrestin. This was also supported by another study in which propranolol stimulated the Mitogen activated (MAPK) kinase pathway and reduced cyclic AMP (Baker *et al.,* 2003). The above finding suggests more than one role for a β-adrenoceptor ligand. This could also be possible reason behind the outcome of drug treatment of patients with different β-adrenoceptor ligand for the same receptor (Kopecky 2006). For example, the β-adrenoceptor Carvedilol, used in myocardial infarction produced beneficial effects in heart failure (Kopecky 2006).

1.3.1 General activation of G-protein coupled receptors.

The G-protein coupled receptors exist in equilibrium between an active state (R*), which couples and activates G-proteins, and inactive (R) confirmation (Gether *et al.,* 1995). The inactive R does not couple or activate the receptor (Seifert and Wenzel-Seifert 2002). The R* active state is associated with a conformational change in Transmembrane domain 3,6,7 and helix 8 (Zacarias *et al.,* 2018). The binding of agonists(A) to the active receptor R* stabilise the active confirmation.

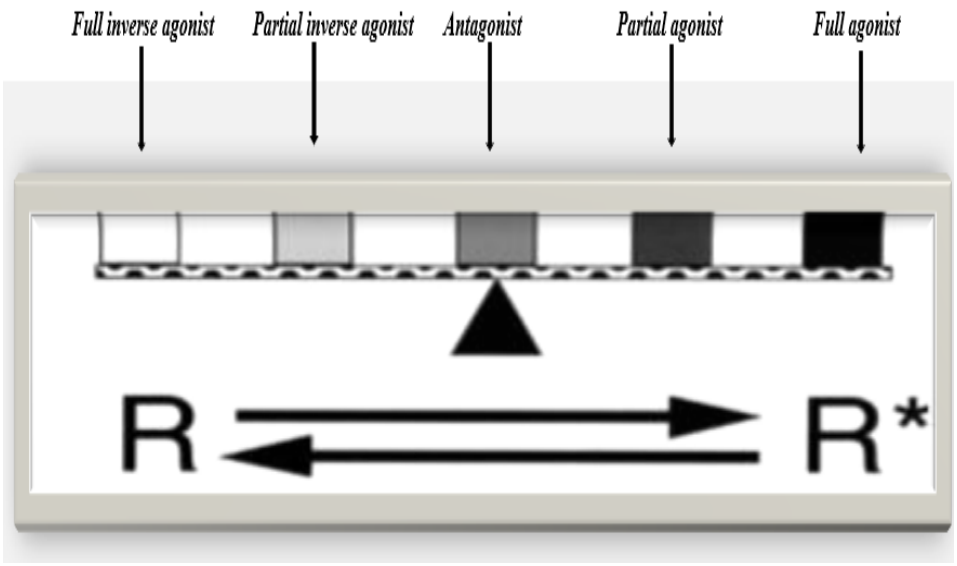


Figure 1.4 Constitutive activity of G-protein coupled receptors.

The diagram illustrating the 2-state model of receptor activation in which the GPCRs isomerise from an inactive state R to an active state R .The full agonist binds to the receptor and shift the equilibrium to the active confirmation R* of the receptor. The full inverse agonists favour the inactive state of the receptor, they shift the equilibrium state of the receptor towards the inactive R of the receptor (Seifert and Wenzel-Seifert 2002).*

1.3.2 Constitutive activity of β-adrenoceptors

This constitutive activity of a receptor is the activation of a receptor from the inactive state (R) to the active (R*) without the influence of a ligand. It is also referred to as the basal level of activity of the receptor (Bond and Ijzerman, 2006).Constitutively active receptors are capable of becoming active in the absence of an agonist (Seifert and Wenzel-Seifert 2001).This behaviour was first described in the opiod receptor (DOPR) in 1982 (Koski *et al.,* 1982) and in 1984 in the β_2-adrenoceptor (Cerione *et al.,* 1984) and the first naturally occurring constitutively active mutation in the GPCR was first described in 1991.This mutation was identified in rhodopsin and implicated in some diseases. Also, this has been identified in animal models. The identification of these mutations and how they occur lead to the discovery of the mechanisms of action of the mutations. Also, inverse agonists were discovered to be effective in treating diseases caused by constitutively active mutants than antagonists of the GPCRs (Tao 2008). The β_2-adrenoceptor was identified to be constitutively active in the absence of a hormone (Cerione *et al.,* 1984). This was also identified in a neuroblastoma cell line which expresses the opioid receptor in which some antagonists of the receptor with negative intrinsic activity, decreased the GTPase activity in the absence of agonist. This suggested the existence of a basal level of activity of the opioid receptor (Costa and Cotecchia 2005)

Several other mutations in GPCR were identified to show a reduction of constitutive activity. Such as β_2-adrenoceptors mutations which increased the constitutive activity which was studied by Lefkowitz (Cotecha *et al.,* 1990, Ren *et al.,* 1993, Semama *et al.,*1993). This led to the development of the extended ternary complex of receptor activation (Semama *et al.,*1993). In this model the wild type of receptor exists in an equilibrium between active and inactive confirmations. This was modified to the cubic ternary complex model (Weiss *et al.,* 1996) This cubic ternary complex of the receptor activation accommodated the existence of multiple receptor confirmations. The agonist stabilizes the receptor in active confirmation

while the inverse agonists stabilize the inactive receptor confirmation. The neutral antagonists have equal affinity for active and inactive confirmation (Weiss *et al.*,1996).

The mutations in GPCR do not always lead to constitutive receptor activation. There are many wild type receptors which have constitutive activity. Such as the opioid receptor, cannabinoid receptor, growth hormone secretagogues (Seifert and Wenzel-Seifert 2002). Also, receptors of the same subfamily of receptors different constitutive activity. This has been displayed by dopamine receptors in which dopamine 1 has more constitutive activity than the remaining members of the family (Tiberi and Caron 1994). Another example is in the β-adrenoceptor family in which the β_2-adrenoceptor has significantly higher constitutive activity than β_1-adrenoceptor (Chakir *et al.*, 2003). Although mutations have been identified in β_2-adrenoceptor, the mutation does not affect the β_2-adrenoceptor cell signalling (Tao *et al.*, 2000).

1.3.3 Molecular structure of G-protein coupled receptors.

The G-protein coupled receptors are single polypeptides with different numbers of amino acids in each class. G-protein coupled receptors exist as seven transmembrane hydrophobic α-helices, each of 25-35 amino acids. The α-helices are connected to an NH_2-terminus (extracellular) and COOH-terminus (intracellular) by 3 intracellular and 3 extracellular loops (Dickenson et al., 2012). An 8^{th} α-helix or intracellular loop 4, was identified at the beginning of the C-terminus parallel to the cell membrane (Conner *et al.*, 2008).

The first G-protein coupled receptor to identified using cryoelectronic microscopy, was Rhodopsin (Unger *et al.*, 1997). The rhodopsins , also represent the largest family of GPCR which are activated by light and dark. Mutations in the rhodopsin gene cause abnormalities in the retina (Dryja *et al.*, 1990).

1.3.4 The functions of G-protein coupled receptors

The GPCRS are widely distributed and expressed in the human body and as such perform a range of functions. They share a common ancestry, structure, sequence and sometimes similar pharmacology. The GPCRs are also able to activate many multiple signalling pathways. As a result of these many unwanted side effects of GPCR activation occurs.

The GPCRs are involved in basic cellular functions which include; cellular proliferation, regulation of ionic transport, neuronal function, cellular secretion and regulation of cellular metabolism. They are also involved detection of stimuli such as light, taste, ions, peptide hormones (insulin, endothelin , nonpeptide hormones (adrenaline) and neurotransmitters (acetylcholine). The G-protein coupled receptors respond to external stimuli, either environmental (such as light) or internal (such as hormones). These responses cause intracellular changes in cellular behaviour which occur based on the G-protein signalling pathway stimulated.

1.3.5 G-protein coupled receptors in disease

There are some mutations in which the role of GPCRs has been identified. The mutations include: Nephrogenic diabetes insipidus caused by vasopressin receptor mutations, Retinitis pigmentosa from mutation in the rhodopsin gene and female infertility caused by Follicle stimulating hormone receptor mutations. Also, the presence of autoantibodies such as, thyroid stimulating receptor hormones targeting GPCRs cause Graves' disease. The altered expression of coupling of GPCRs has also been studied in some diseases such as asthma (β_2-adrenoceptor), M_1 muscarinic acetylcholine receptor in Alzheimer's disease and angiotensin II receptor AT_1 receptor in hypertension. The GPCRs are very useful therapeutic targets and they are the most exploited drug targets. It has been estimated that 30-50% of drugs act

through GPCRs. The unexploited GPCRs are termed orphan receptors. These orphan receptors number about 400 out the more 800 GPCRs. Many of the orphan receptors have been de -orphanised (about 300).

However, the GPCRs are expressed in multiple tissues and they perform many different roles. Such as the antagonism of the M_3mACh receptor produces bronchodilation in asthma The GPCRs are also closely related with similar structure and pharmacology which makes it possible for a particular drug to act on closely related receptors. The GPCRs can also couple multiple signalling pathways which makes them promiscuous. For example, adenosine A3 (Palmer *et al.*, 1995) receptor, α_2-adrenergic receptor (Eason *et al.*, 1992) β_2-adrenoceptor (Kilts *et al.*, 2000). The GPCRs have also been identified to signal through G-protein independent signalling such as the arrestin dependent signalling.

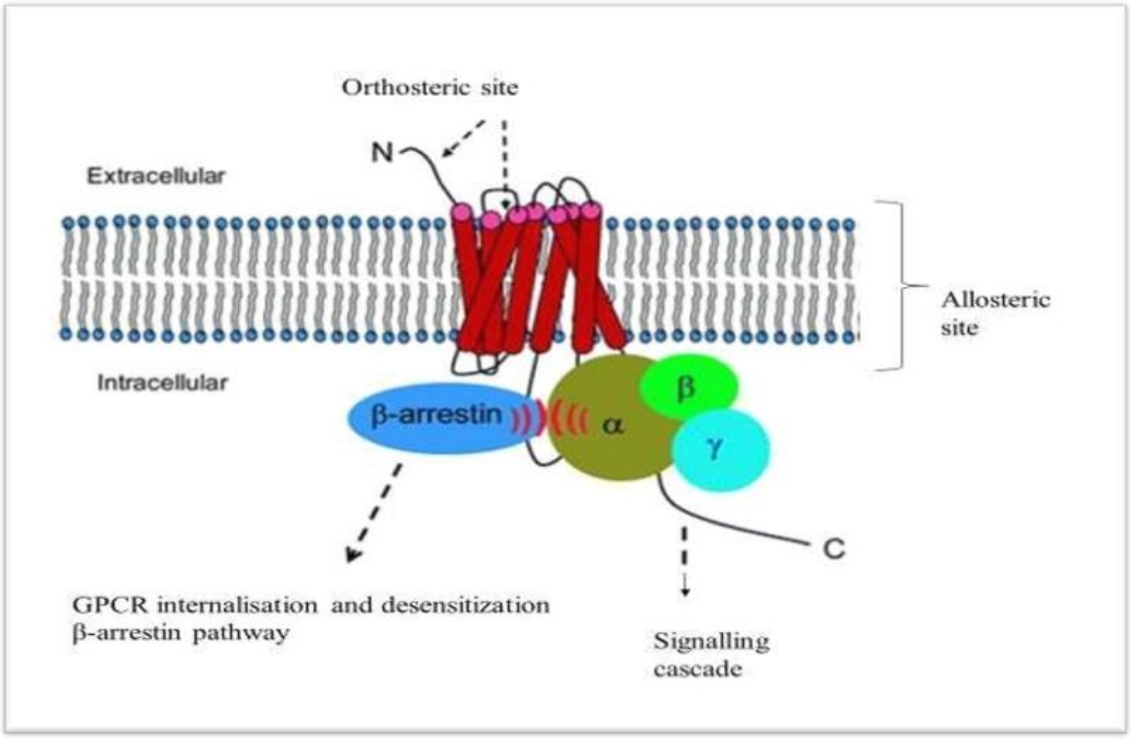


Figure 1.5 Molecular structure of G-protein coupled receptors.

Diagram illustrating the plasma membrane which is made up phospholipid bilayer which consist of the G-protein coupled receptor. The polypeptide chain of the GPCR passes 7 times through the plasma membrane. The diagram also shows the orthostatic site of the receptor, where many GPCR drugs bind to the receptor. The binding at allosteric site are subtype selective and have physiological relevance. (Sharma et al., 2017).

The ability of the GPCRs to perform a wide range of functions gives them the ability to activate many intracellular signals. These intracellular signals may also cause undesired effects of many novel GPCRs drugs.

Pharmacological terms

A receptor is a specialised molecule located on the exterior of a cell membrane within the plasma membrane of a cell. The receptor also receives chemical signals which bind to the receptor and elicit a response. The chemical signals are from the chemicals which bind to the receptors and are called agonists. The agonists activate the receptor to produce ma cellular response (Kanekin,2004). The antagonists are chemical substances which prevent the response of a receptor. They do not activate the receptor but they compete with the agonists (Range and Dale's pharmacology). The agonists /antagonists (ligand)actions are characterised based affinity and efficacy and intrinsic activity. Affinity is the occupation of a receptor by a chemical substance or ligand. It is also the strength with which a ligand binds to a receptor. The efficacy of a ligand is its ability to activate a receptor which it has bound to. It is also the maximal response elicited by a drug on a receptor. Intrinsic efficacy, however, is the property of the drug which determines the amount. Potency is the activity of a drug expressed in terms of the amount or dose of the drug required to produce an effect. The amount of the ligand required to elicit a response is the dose.

1.3.6 Classification of G-protein coupled receptors (GPCRs)

G-protein coupled receptors (GPCR) have phosphorylation sites are mostly located in the third intercellular loop and C-terminus, which are targets for second messenger-dependent protein kinase (PKA and PKC) and G-protein coupled receptor kinase (GRK) (Lefkowitz, 2004). There are 5 main classes of the G-protein coupled receptors;

1.3.6.1 Class A: Rhodopsin-like

This group comprises of receptors from small molecules, neurotransmitters, peptides, hormones, visual pigments, olfactory receptors, taste type 2 receptors and 5 pheromone receptors.

1.3.6.2 Class B: Secretin-like

The members of this group are encoded by 15 genes in humans. The ligands of this family include polypeptide hormones of27-141 amino acids and residues, glucagon, glucagon like receptors, glucose independent insulinotropic polypeptide (GIP), vasoactive intestinal polypeptide (VIP), Secretin, pituitary adenylate cyclase-activating polypeptide (PACAP).

1.3.6.3 Class B: Adhesion

The members of this group are similar to the class B receptors. They are derived from 7TM domains at GPCR proteolysis site. They have an extra extracellular N terminus.

1.3.6.4 Class C: Glutamate

The members of this group comprise of the metabotropic glutamate receptors, $GABA_B$ receptors, 3 taste type 1 receptors, family of pheromone receptors (V2 receptors).

1.3.6.5 Class F: Frizzled

This group consists of 10 Frizzled proteins FZD (1-10), and Smoothened (SMO). The FZD are activated by secreted lipopolysaccharides of the WNT family while, the SMO is activated by the Hedgehog (HH) family of proteins which act on transmembrane protein patched (PTCH) (Alexander *et al.*, 2015).

1.4 Discovery of β-adrenoceptors

Raymond Ahlquist in 1948 discovered the binding of adrenaline to β and α -adrenoceptors. In 1958 Eli Lilly laboratories invested the first β blocker. In 1988, Sir James W. Black pioneered the use of Propranolol in the management of angina pectoris for which he received a Nobel prize (Stapelton *et al.,* 1997).

1.4.1 The β-adrenoceptors

These are located on gene 5 of the human chromosomes which code for 1,200 base pairs. They have been characterized in detail. They exist as β_1 and β_2- adrenoceptor which are pharmacologically and physiologically distinct subtypes of the receptor (Koblika *et al.,* 1987). The 3rd subtype, β_3-adrenoceptor was identified later by cloning which was later investigated in adipose tissue of laboratory animals. The β_3-adrenoceptor has also been identified mRNA in human fat cells of living donors (Kreif *et al.,*1993). These receptors belong to Class A: Rhodopsin-like receptors

1.4.1.1 β_1-adrenoceptors

These are in the heart, kidney and adipocytes. (McGraw *et al.,* 2000, Abraham *et al.,* 2004, Dorn 2010).

1.4.1.2 β_2-adrenoceptors

They are expressed in high density in the lungs, bronchial smooth muscles, broncho epithelial cells, immune cells, kidneys, urinary system, eyes, adipocytes, liver, skeletal muscles,uterus,peripheralblood vessels and heart (McGraw *et al.,* 2000, Abraham *et al.,* 2004, Dorn 2010).

1.4.1.3 β_3-adrenoceptors

These are widely distributed in the subcutaneous adipose tissue (Enocksson *et al.,* 1995).

1.4.2 Pharmacology of β-adrenoceptors and function

Catecholamines have been identified as a natural endogenous ligand which act through β_1, β_2 and β_3-adrenoceptors. Catecholamines such as adrenaline and noradrenaline are required

for the activation of β-adrenoceptors (Ahles *et al.,* 2014). The β-adrenoceptors bind to stimulatory G proteins (Gs) and increase the formation of cyclic AMP (Ahles *et al.,* 2014). They regulate important physiological functions of the sympathetic nervous system.

1.4.3 Structure of β-adrenoceptors

1.4.3.1 β_1-adrenoceptor

The crystal structure of the β_1-adrenoceptor was investigated in turkey β_1-adrenoceptor bound to cyan pindolol, an antagonist (Warne *et al.,* 2008). The structural analysis of mutated turkey β_1-adrenoceptor showed the amino acid sequence was 82% identical to human β_1-adrenoceptor. The structural features of extracellular loops 1-3 in β_1-adrenoceptor were similar to β_2-adrenoceptor, this suggests the amino acid sequences in all β-adrenoceptors are highly conserved. The ligand binding pockets consists of 15 amino acids side chains comprising 4 from helix 3, 3 from helix 5, 4 from helix 6, 2 from helix 7 and from 2 extracellular loop2. Mutated β_1-adrenoceptor bound to full agonists isoproterenol and camerterol and partial agonists salbutamol and dobutamine, were displayed that full agonists to with serine 212(5.42) and Ser 215(5.46) in helix 5 and partial agonists interact specifically to serine (212). The interaction with agonists produced small contraction of ligand-binding pocket (Warne *et al.,* 2011).

1.4.3.2 β_2-adrenoceptor

The structure of the β_2-adrenoceptor bound to inverse agonist was used to investigate and establish the human G-protein coupled receptor (Bang *et al.,* 2015). The structure of the β_2-adrenoceptor was crystallized and bound to an inverse agonist (Rasmussen *et al.,* 2007). The receptor was identified to have long flexible intercellular loop 3. This was stabilized using an inverse agonist, carazolol and the flexible intercellular loop 3(IL3) was reduced using by introducing IL3-specific Fab fragment or by replacing it with T4 lysosome(T4L). Carazolol interaction with Asp113(33.2), Asn312(739), and Tyr316(743) of β_2-adrenoceptor was

revealed through crystallization studies. Nuclear magnetic resonance (NMR) technology has also been used to study the characteristics of activated β_2-adrenoceptor (Nygaard *et al.*, 2013).

1.4.3.3 β_3-adrenoceptor

The 3rd subtype was recently discovered and not extensively investigated as the previous 2 classes. The crystal structure of the β_3-adrenoceptor has not been established. It was identified in 1984 and was investigated by the stimulation of lipolysis with Isoprenaline in brown adipocytes (Arch *et al.*, 1984). The stimulation of the β_3-adrenoceptor caused increase in thermogenesis, anti-obesity and anti-diabetic activities. This also led to the molecular characterization of the β_3-adrenoceptor (Emorine *at al.*, 1989).

The amino acid sequence of the β_3-adrenoceptor has 51% and 46% similarity with β_1-adrenoceptor and β_2-adrenoceptor respectively (Skeberdis *et al.*, 2000). In the C-terminal domain of the β_3-adrenoceptor, there are no phosphorylation sites for Protein kinase a (PKA) and G-protein coupled receptor kinases. In β_3-adrenoceptor the intracellular loops 3,4,5 and 6 are important in ligand binding while , 2 and 7 are important in G protein binding and activation of the receptor (Cannavo *et al.*, 2017).

1.4.4 β-adrenoceptor activation and cell signalling.

1.4.4.1 Cyclic AMP dependent signalling

The activation of β-adrenoceptor is initiated by agonist binding which produces a conformational change in the receptor. The GPCRs have 3 subunits (αβγ) bound to GDP in an inactive state (Figure 1.6). The agonist bound receptor undergoes a conformational change that releases α subunit from GDP replacing it with GTP. The α-GTP and βγ dissociate from the activated receptor. The α-GTP and βγ bind to effector proteins such as adenylyl cyclase, phospholipase C or ion channels (Oldham and Hamm 2008). The G-α subunit has 4 subunits; G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$ which are selective for different GPCR subtypes and effector proteins. Adenylyl cyclase is associated with $G\alpha_s$ stimulation. $G\alpha_s$ is widely

distributed in the body and stimulates the synthesis of second messenger cyclic AMP from ATP, which activate Protein Kinase A. Protein Kinase A phosphorylates β-adrenoceptor and causes a conformational change that uncouples the receptor to produce a biological response (Billington *et al.*, 2003, Evans *et al.*, 2010). The active α-GTP is inactivated by hydrolysis of GTP back to GDP and recombines with βγ subunit.

Cyclic AMP is hydrolysed into AMP by phosphodiesterase. The phosphodiesterase regulates intracellular levels of cyclic AMP (Boswell-Smith *et al.*, 2006). The cyclic AMP dependent signalling pathway has been further identified to be responsible for the stimulation of additional signalling pathways. This is because the binding of cyclic AMP to protein kinase A results in release of subunits which phosphorylate a number of cellular targets that cause relaxation of the bronchioles in asthma (Billington and Hall 2011).

1.4.4.2 β-arrestin dependent signalling

The $G\beta_{\gamma}$ subunit recruits G-protein coupled receptor kinase-2 which phosphorylates the β_2-adrenoceptor leading to binding of β-arrestins and desensitization of the receptor (Gupta *et al.*, 2015). The β-arrestins and G protein-coupled Receptor Kinase are other proteins that interact with activated β_2-adrenoceptor. They participate in receptor silencing by uncoupling of the receptor by desensitization, receptor trafficking by internalization, resensitization and degradation. They are also involved in facilitating G Protein independent pathway (Reiter and Lefkowitz, 2006). They regulate the G protein signalling by inhibiting the G protein pathway and stimulating the β-arrestin pathway. The β-arrestins also scaffold the c-Jun N-terminal kinase (JNK), extracellular regulating kinase (ERK) and p38 mitogen activated kinase (MAPK). The activation of ERK by β-arrestins is slow and the mechanism why it happens is not well understood (Reiter and Lefkowitz 2006).

The kinases are enzymes that phosphorylate proteins, lipids and sugar which result in enzyme activation, cellular translocation or interaction with other proteins and signal

transduction (Manning *et al.*, 2002). There are many subtypes of the kinases; MAPK are the largest group which are involved in signal transduction. The major MAPK pathways are the c-Jun N-terminal kinase (JNK), extracellular regulating kinase (ERK) and p38Mitogen activated kinase (MAPK). The inhibition p38 MAPK is of special interest because some molecule where discovered to be inhibitor of the pathway.

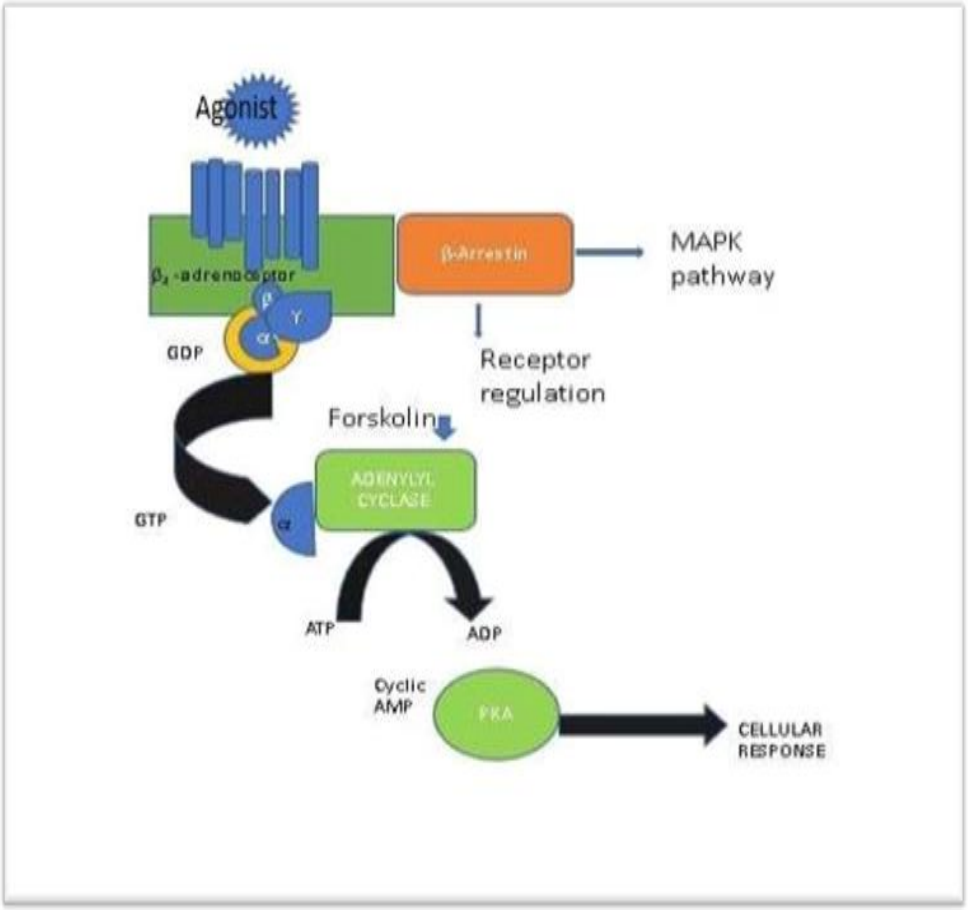


Figure 1.6 The β2-adrenoceptor cell signalling pathway.

Diagram illustrating agonist binding to the β_2-adrenoceptor which, alters the receptor confirmation of G-protein releasing the Gα segment of the G-protein from the βγ segment and activating adenylyl cyclase initiating a cascade of cellular events. The Gα subunit is categorised into Gαs, i/o ,q and $\alpha_{12/13}$ .The Gαs activates adenylate cyclase which triggers the production of cyclic AMP from ATP. The second messenger cyclic AMP activates protein kinase A which causes phosphorylation of downstream targets involved in producing cellular response(Benovic 2002).

1.4.4.3 Desensitisation of the β-adrenoceptor

The activation of the β-adrenoceptor for long duration worsens airway inflammation in asthma. Because of this, the use β_2-adrenoceptor agonist which act via the cyclic AMP pathway have deleterious effects which limit its use in severe asthma. This has also been a cause of increase in asthma related deaths because the effectiveness of the β_2-adrenoceptor agonists has reduced (Salpeter *et al.,* 2006). The reduction of the effectiveness of the drug has been related to failure of the β_2-adrenoceptor agonist to reduce cytokine release from inflammatory and airway epithelial cells. This also increases the concentration of cytokines, reduces mucociliary clearance in the airway and increases the responsiveness of the airway to allergens (Penn *et al.,* 1999).

The repeated stimulation the β_2-adrenoceptor causes rapid desensitization because of the uncoupling of the receptor from the Gs protein and adenylate cyclase. The receptor is internalised into the interior of the cell into the intracellular endosome that removes the receptor from the cell surface (Johnson 2001). This causes degradation of the and downregulation of the receptor which the reduction in number of the β_2-adrenoceptor and cyclic AMP accumulation (Barnes 1999). Furthermore, in asthmatics who are unresponsive the β_2-adrenoceptor agonist at risk of becoming more responsive to allergens (Penn *et al.,* 1999).

The regulation and control of the β-adrenoceptor activation is essential in maintaining normal body functions. The β-adrenoceptor is an important pharmaceutical target and understanding these regulatory mechanisms is necessary in drug discovery and development (Inglese *et al.,* 1993). Long-term use of β_2-adrenoceptor agonists significantly reduces the effectiveness in the treatment of asthma (Penn *et al.,* 1993). The repeated or constant stimulation of the receptor reduces the activation of the receptor. Also, in asthmatics, reduced responses to β-adrenoceptors has been related to desensitisation (Heijink *et al.,*

2005). This indicates that the inflammatory mediators and cytokines have can influence reduction of β-adrenoceptor responsiveness and desensitisation related to β-adrenoceptor agonist usage.

Desensitisation is therefore the absence or loss of a response of a receptor due to prolonged or frequent stimulation of a receptor by an agonist (Shenoy *et al.,* 2018). This is accompanied by a reduction of receptor expression at plasma membrane a process called downregulation. Downregulation is further divided into (1) Receptor internalisation and destruction in lysosomes (2) Reduction of mRNA effect through mechanisms which are not clearly understood (Tsao *et al.,* 2001). However, receptor responsiveness can be restored if agonist stimulation can be reduced by removal of an agonist or by addition of an antagonist. This then causes the receptor to become responsive which then leads to resensitization of the receptor (Kelly *et al.,* 2008). Phosphorylation of the receptor by kinases has been identified as the mechanism behind desensitisation (Stadel *et al.,* 1983). However, the return of the responsiveness depends on the duration of drug treatment during sensitization (Kelly *et al.,* 2008). Lefkowitz studied desensitization using β_2-adrenoceptor, in which catecholamine induced desensitization of adenylyl cyclase was associated with phosphorylation of theβ_2-adrenoceptor (Stadel *et al.,* 1983).

The major mechanism behind desensitization was phosphorylation (Stadel *et al.,* 1983). The second messenger-dependent protein kinases such as protein kinase A(PKA) and protein kinase C (PKC) were behind the phosphorylation of β-adrenoceptors (Benovicet *et al.,* 1985). However, this was discovered that cells which lacked PKA were responsible for the phosphorylation and desensitization of β_2-adrenoceptors (Strassel *et al.,* 1986). Also the G-protein coupled receptor kinase (GRK) was discovered to mediate agonist-specific phosphorylation and other forms of phosphorylation (Premont and Gainetdinov 2007). However, the possibility of the GRKs participating in extensive desensitization was doubted. This led to the discovery arrestins which bind to agonist – occupied GRK-phosphorylated

GPCR. The arrestins uncouple the G-protein activation and induce desensitization (Lohse *et al.,* 1990). This led to the development of the classical model of agonist induced sensitization which is applicable to all GPCRs. Desensitization is subdivided into homologous or heterologous desensitization (Kelly *et al.,* 2008).

1.4.4.3.1 Agonist-specific (homologous) desensitisation

In this desensitisation, phosphorylation of specific serine and threonine residues located in carboxyl-terminal tail of agonists-activated receptor by G-protein coupled receptor kinase (GRKs). It is the loss or absence of response only to agonists which act on a specific GPCR subtype. It usually occurs at the level of the receptor (Kelly *et al.,* 2008).

1.4.4.3.2. Agonist non-specific (heterologous) desensitisation

Phosphorylation of specific serine and threonine residues located in 3rd intracellular loop of the receptor by second-messenger dependent kinases such as protein kinase A (PKA) or protein kinase C (PKC). The phosphorylated receptor is bound to G-protein coupled receptor kinas which uncouple the receptor from the G-protein and initiate GPCR endocytosis via clathrin coated pit-dependent pathway. This is also called internalisation or sequestration. The internalised GPCRs are either dephosphorylated and returned to the plasma membrane or targeted for degradation and loss of the receptor (Bohm *et al.,* 1997). This is also called down regulation and does not always occur with desensitization (Kelly *et al.,* 2008). Heterologous desensitisation is widespread and involves the loss of agonist response at several GPCR subtypes and in the absence of agonists occupation at other receptors. It might also involve cell signalling components downstream of the receptor (Kelly *et al.,* 2008).

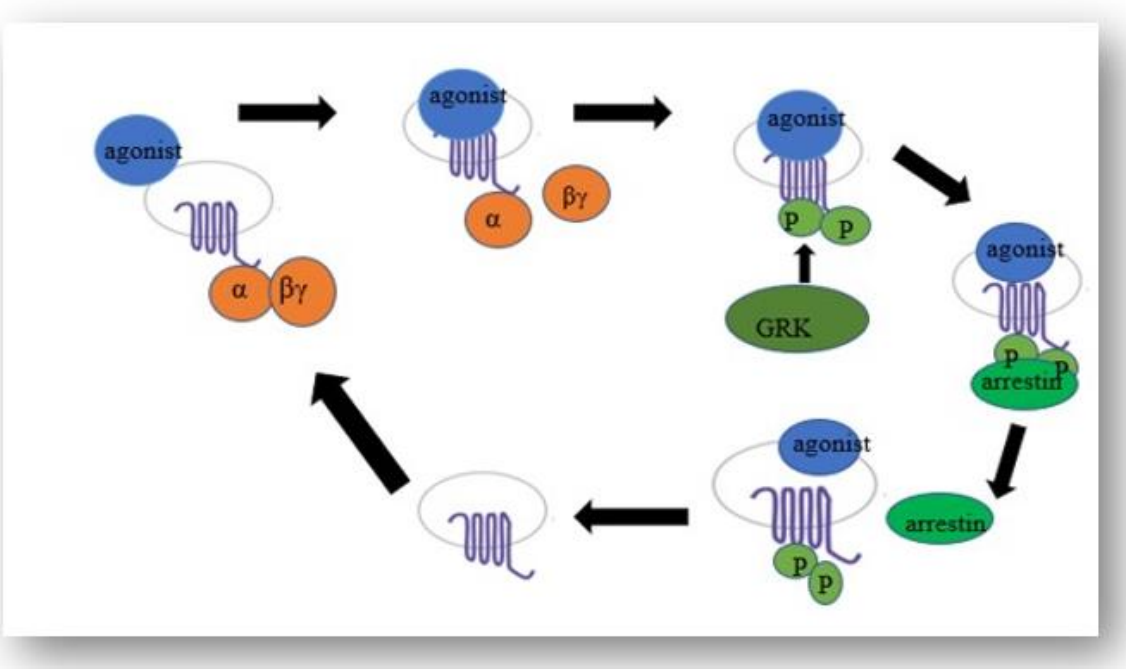


Figure 1.7 Schematic representation of desensitisation showing activation of the GPCR by agonist binding.

Diagram illustrating the desensitisation the GPCR by G-protein coupled receptor kinase (GRK) and arrestin which leads to uncoupling of the receptor. The agonist bound receptor is phosphorylated by G-protein coupled receptor kinase (GRK). This is then bound to arrestin , which causes the desensitisation, internalisation, dephosphorylation and recycling of the receptor(Kelly et al.,2008).

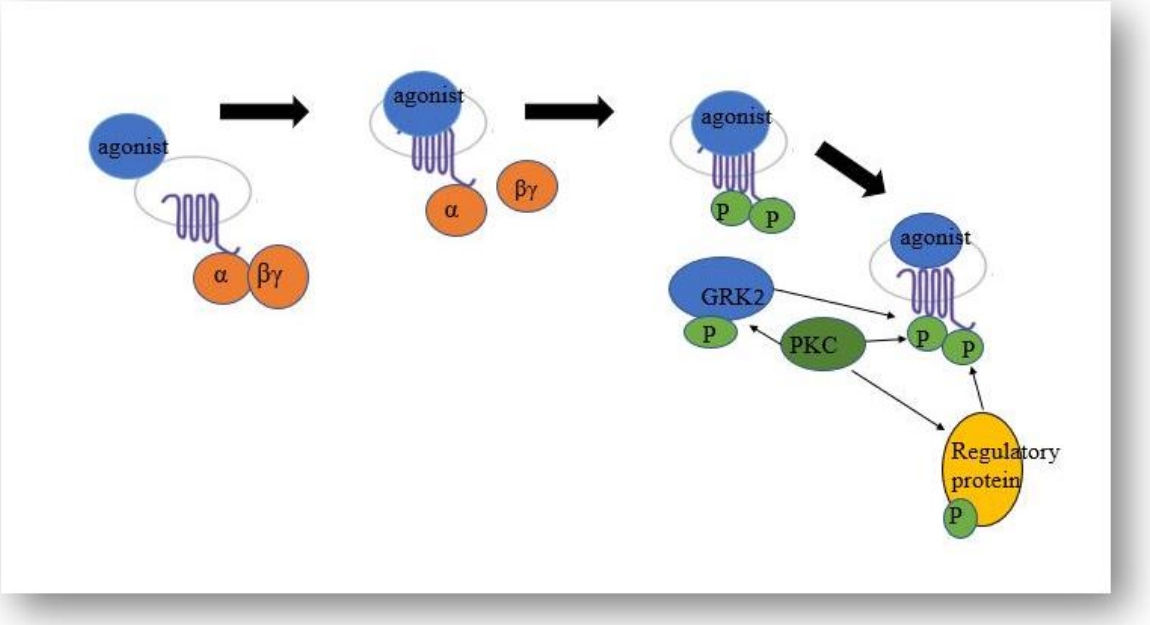


Figure 1.8 Schematic representation of desensitisation showing GPCR desensitisation and receptor regulation by protein kinase C.

Diagram illustrating phosphorylation and desensitisation of the receptor by protein kinase C. This could also occur by phosphorylation of the receptor by PKC and activation of GPCR receptor kinase 2. PKC can also phosphorylate a regulatory protein which causes desensitisation(Kelley et al.,2008).

1.5. The role of β-adrenoceptors in human bronchial epithelial cells

The bronchial epithelial cells have been studied as targets of β-adrenoceptor agonists and as such are very important in the pathophysiology of asthma (Barnes 1995). They inhibit secretion of mediators of bronchoconstriction from mast cells (Barnes 1997). Also, as initiators of inflammatory response they are capable of stimulating ant-inflammatory mediators while deactivating pro-inflammatory mediators to influence the of activity of both inflammatory and parenchymal cells. They are therefore the determinants the severity of asthma (Abraham *et al.,* 2004). For example, the bronchial epithelial cells mediate second messengers such as cyclic AMP which is activated by adenylate cyclase system (Nijkamp *et al.,* 1992). The β-adrenoceptor agonists and catecholamines are the stimulators of the β_2-adrenergic receptor adenylate system. The β-adrenoceptor agonists also increase cyclic AMP

levels via the β-adrenoceptor and could mediate inflammatory response (Aksoy *et al.,* 2002). Also, the activation the β_2-adrenergic receptor by β_2-adrenergic agonists and the catecholamines are important in initiating mucociliary clearance, regulating water and electrolyte transport across epithelial cells (Sanderson *et al.,* 1989). The β_2-adrenergic receptor activation is also important in the release of bronchodilators such as nitric oxide (Smith *et al.,* 1982). Furthermore, the β-adrenoceptor agonists and catecholamines also decrease pro-inflammatory cytokines and chemokines (Izeboud *et al.,* 2000). The β_2-adrenoceptor agonists besides increasing cyclic AMP, they also reduce the production of pro-inflammatory cytokines and chemokines (Izeboud *et al.,* 2000). This was investigated in Phosphodiesterase inhibitor drugs, which inhibited the release of pro-inflammatory cytokines by increasing cyclic AMP (Kambayashi *et al.,* 1995).

The physiological and pathophysiological responses which occur following stimulation of β_2-adrenoceptor have not been clearly assigned to one cell type (Turki *et al.,* 1995). This is because the β_2-adrenoceptor has been identified in high density in more than one cell type (Kelson *et al.,* 1997). In view of this, the BEAS-2B cell line has been investigated and its β_2-adrenoceptor expression and physiology has been characterized (Kelsen *et al.,* 1997). In the BEAS-2B cell line, the β_2-adrenoceptor functional expression has been studied as well as the mechanisms underlying the β_2-adrenoceptorl activation response (Amstad *et al.,* 1988). Other cell lines investigated for the effect of β_2-adrenoceptor agonists effects include Calu-3 cells. (Mizuno *et al.,* 2000, Zhang *et al.,* 2001).

The β_2-adrenoceptor population has been studied to be highly expressed in Calu-3 and 16HBE14o$^-$. The production of cyclic AMP in these cells was stimulated by Isoprenaline and blocked by ICI118551(Abraham et al 2004). This suggest the coupling of the β_2-adrenoceptor to adenylate cyclase has occurred. Also, the β_2-adrenoceptor was localised on the cell membrane using immunohistochemical staining (Abraham *et al.,* 2004). These findings make Calu-3 cells suitable cells model for β_2-adrenoceptor research.

1.5.1.Modulators β_2-adrenoceptors

The ligands of the β_2-adrenoceptor are classified based on their effect on the cyclic AMP pathway, as inverse agonists, antagonists or agonists. Ligands are balanced when they equally activate G protein and arrestin signalling.

1.5.2 .Inverse agonists

The modulators of the β-adrenoceptor which bind with the receptor to stabilise inactive state of the receptor are defined as inverse agonists. The inverse agonists are a subset of β adrenoceptor drugs, which inactivate spontaneously/constitutively active receptors and turn off empty active receptors and reduce the basal level of activity of a receptor which inhibit receptor-dependent signalling (Nguyen *et al.,* 2008). This is responsible for reducing efficacy values to zero (Bond *et al.,* 1995). Results of clinical studies conducted in myocytes of failing heart and murine models of asthma, indicated that inverse agonists on chronic administration improved symptoms in murine models and congestive cardiac failure (Callaerts-Vegh *et al.,* 2004). The administration of inverse agonists over a long duration enhanced haemodynamics in congestive cardiac failure and also reduced mortality (Packer *et al.,* 1996). In the study some patient groups were administered carvedilol while other groups were on placebo. The carvedilol administered group had less adverse reactions of the treatment during the study than the placebo (Packer *et al.,* 1996). This suggest a protective role of carvedilol in congestive cardiac failure patients.

Despite being clinically and pathological different, both bronchial asthma and congestive cardiac failure are affected by similar β-adrenergic modulators which interact with β-adrenergic receptors (Callaerts-Vegh *et al.,* 2004). The administration of β-adrenergic agonists such as salmeterol, was removed from a study because the increase in asthma related deaths (Nelson 1995). As a result of the above findings, it was suggested that similar administration of β-adrenergic agonists might cause either beneficial of detrimental effects of the drugs in congestive cardiac failure. Also, the β-adrenergic antagonists had detrimental

effects on asthma because of the effects of the drugs which worsen airway narrowing (*Isreal* et al.1996) Furthermore, these β-adrenergic inverse agonists had beneficial effects on congestive cardiac failure (Callaerts-Vegh *et al.,* 2004). In view of this similar drugs were administered with β-adrenergic antagonists in asthma patients. Nadolol and carvedilol in murine model asthma improved asthma symptoms when administered over 28days. The reduction of symptoms with chronic duration of nadolol was similar to the effect produced with administration of a single dose of salbutamol. This suggest reciprocal effect of β-adrenergic agonists and inverse agonist on cell signalling based on duration of administration of the drugs (Callaerts-Vegh *et al.*, 2004).

The inverse agonists reduce symptoms of asthma by reducing constitutive activity of β_2-adrenoceptor or preferentially stimulating the non-G protein pathway to stimulate multiple signalling pathways (biased agonism) (Wisler *et al.*, 2007). The β_2-adrenoceptors have some constitutive activity and the beneficial effect of inverse is due biased agonism. In a study to determine the cell signalling pathway necessary for expression of the full asthma phenotype, β-arrestin2 was deleted in murine models of asthma. These mice lacking β_2-arrestin displayed increased attenuation of cough, wheeze and difficulty in breathing. This suggests the involvement of the non-canonical or non-G protein pathway in the development of asthma phenotype. (Walker *et al.*, 2003).

1.5.3 Agonists

The activation of a receptor by agonists show variation in magnitude of response produced by different agonists due to differences in efficacy. Efficacy is the degree of response produced by different agonists that are occupying the same number of receptors. Receptors with high efficacy show a maximal response while occupying smaller number of receptors. Low efficacy agonists show minimal response despite occupying all available receptor sites (Perez and Karnik, 2005). Agonists promote the active confirmation of receptor (R*). Based

on the differences in eliciting response agonists are classified as full, partial agonists (Wenzel-Seifert *et al.,* 2002).

1.5.4 Antagonists

These drugs bind to receptors but, do not alter R/R* equilibrium. They block agonist from binding to the receptor. As result they prevent agonist induced activation of receptor (Dickey *et al.,* 2010).

1.5.5. Ligand Bias

This is the ability of a ligand to differentially influence the behaviour of a receptor (van der Westhuizen et al., 2014). Ligand bias also determines how effective a ligand is when it is stimulating a receptor. Ligand biased was introduced because of the functional selectivity of some ligands stimulating the same G-protein coupled receptor with specific physiological and pharmacological response (Ingram *et al.,* 2009). The response could be a desired or a detrimental response (Russo *et al.,* 2009). The first studied example of ligand bias was in β_2-adrenoceptor inverse agonists, such as ICI118551 and propranolol with inverse agonists properties which inhibit the cyclic AMP pathway and also stimulated the MAPK activation (Baker *et al,* 2003, Azzi *et al.,* 2003).

1.5.6 Biased agonists

Biased agonists are ligands which can preferentially activate a receptor to stimulate an alternate pathway (Walker *et al.,* 2010) or differentially influence receptor behaviour. For example, biased agonists can stimulate either the G protein or beta arrestin-dependent signalling (Callaerts-Vegh *et al.,* 2004). These are agonists that can preferentially activate a receptor over another signalling pathways (Strachan *et al.,* 2014).

Carvedilol stimulates β_2-adrenoceptor beta arrestin–dependent signalling and serves as an inverse agonist when it inhibits the cyclic AMP pathway. Propranolol is an inverse agonist at β_2-adrenoceptors and weakly stimulates beta arrestin-dependent signalling (Wisler *et al.,* 2007).

1.6. The relationship between cardiac failure and bronchial asthma

The use antagonists in the management of heart failure was contraindicated until a clinical trial involving its use showed it was beneficial (Parker *et al.,* 1996). Congestive cardiac failure (CCF) is a decrease which reduces cardiac output. β_2-adrenoceptor agonists increase contractility of the heart while antagonists and inverse agonists do not. In clinical trials results indicated prolonged administration of β_2-adrenoceptor agonists did not improve the contractility of the heart but worsened the condition and increased patient deaths of patients when administered chronically (Parker *et al.,* 1996). This reversal of the effects based on duration of treatment lead to an investigation to determine the reason for this change in congestive heart failure. It is also the reason for using a similar approach with β-adrenoceptor antagonists in bronchial asthma (Dickey *et al.,* 2010). In the management of bronchial asthma, the β_2-adrenoceptor agonists are the mainstay of treatment. These drugs are beneficial when administered acutely. However, the chronic use of the drugs is responsible for worsening the symptoms of cough, wheeze and difficulty of breathing (Nelson *et al.,* 2006). Due to the detrimental effects, it was suggested that the chronic use of β_2-adrenoceptor agonists was associated with asthma related deaths (Salpeter *et al.,* 2006). Furthermore, in a randomised control study, patients on chronic treatment with the β_2-adrenoceptor agonist, salmeterol presented with increased incidence in respiratory related deaths (Nelson *et al.,* 2006).

The similar responses obtained in congestive cardiac failure and bronchial asthma made comparisons possible. They both have similar receptors and the use of bronchodilators acutely improved symptoms while their chronic use resulted in mortality. The β_2-adrenoceptor antagonists were previously contraindicated in the management of both CCF and bronchial asthma but recently some β_2-adrenoceptors are now effective in CCF (Maack *et al.,* 2000).

In the management of bronchial asthma, β_{2-} adrenoceptor antagonist on chronic administration in murine model of asthma significantly reduced airway hyperresponsiveness (Callaerts-Vegh *et al.,* 2004). Airway inflammation occurs from interactions between immune cells and parenchymal cells such as epithelial and smooth muscle cells. The reduction of airway inflammation by reduction of cytokines and mucin in chronic administration of ICI118551 an inverse agonist (a selective β-antagonist) was recorded in antigen challenged mice. This was also seen in Nadolol an inverse agonist (a non -selective β-antagonists) significantly reduced Bronchial alveolar lavage eosinophil counts (Ngyuyen *et al.,* 2007). Furthermore, recent data have shown chronic treatment with inverse agonist produced widespread effects on airway epithelium and mucous metaplasia in murine model of asthma (Lin *et al.,* 2008). Previously an inverse agonist administered in an antigen challenged of β_2-AR mice showed reduction in mucous metaplasia, airway hyperresponsiveness (Nguyen et al.,2009). This suggest β_2-adrenoceptor signalling was required for full development of the cardinal features of asthma.

1.7. Bronchodilator drugs/β adrenoceptor drug use in the management of bronchial asthma

Table 1.1. Showing the properties of β_2-adrenoreceptor ligands as previously published for human β-adrenoceptor subtype.

Ligand	**β_1-adrenoceptor**	**β_2- adrenoceptor**	**β_3- adrenoceptor**
Endogenous agonists	Noradrenaline, Adrenaline (Freille *et al.*,1988)	Adrenaline, Noradrenaline	Noradrenaline, Adrenaline (Freille *et al.*,1988)
Nonselective agonists	Pindolol (Kurshinski *et al.*, 2007), Isoprenaline (Sato *et al.*, 1996), Dobutamine (Isoqaya *et al.*, 1999)	Isoprenaline, (Sato et al.,1996), Dobutamine (Liapakis *et al.*, 2004) Ephedrine	Carazolol (Mejean *et al.*, 1995)
Selective agonists	Ro363 (Molenaar *et al.*, 1997), Xamoterol (Isoqaya *et al.*, 1999), Denopamine (Isoqaya *et al.*, 1999)	Formoterol (Baker 2010), Salmeterol (Baker 2010)	Mirabegron (Takasu *et al.*, 2007) CGP 121777(Blin *et al.*, 1993)
Antagonists	Carvedilol (Candelore *et al.*, 1999), Bupranolol (Candelore *et al.*, 1999), Nadolol (Candelore *et al.*, 1999), Metoprolol (Candelore *et al.*,1999)	Carvedilol, (Candelore *et al.*,1999) Propranolol, Alprenolol (Baker 2005), Nadolol (Candelore *et al.*, 1999)	L-748337 (Candelore *et al.*, 1999) L748328 (Candelore *et al.*, 1999)
Inverse agonists		ICI118551(Hopkinson et al.,2000)	
		Nadolol, Carvedilol(Dickey et al .,2010)	

In the management of bronchial asthma, β_2-adrenoceptor agonists such as Salbutamol were and still are the mainstay of treatment. Terbutaline has also been used in the management of asthma due to its ability to cause bronchodilation in the airways (Rang *et al.,* 2003). Isoproterenol is also a β-adrenoceptor agonists, which is used the management of heart related conditions (Mizuno *et al.,* 2000). It causes decrease in peripheral resistance and reduces blood pressure but, can also cause receptor desensitisation and increased heart rate. The desensitization of the receptor commonly occurs when high dose of isoprenaline is administered. It has been replaced by the antagonist metoprolol (Lanzara *et al.,* 2005).

In the management of glaucoma, the antagonist timolol has been used in the reduction of intraocular pressure, which is a common cause of blindness (Fieldman *et al.,* 2004). In the management of essential tremors, propranolol, alprenolol and oxprenolol have also been investigated (Costain *et al.,* 1978). Timolol and oxprenolol have been used in the management of cardiac rhythm abnormalities from hypertension and angina pectoris. Furthermore, it has been suggested that these drugs might be useful in the management of cancer, malaria and osteoporosis (Baker *et al.,* 2010).

1.7.1 Long acting β_2-adrenoceptor agonists

The β_2-adrenoceptor agonists Formoterol and Salmeterol are both long acting β_2-adrenoceptor agonists (LABA) but Formoterol has a rapid onset of action than Salmeterol (Lotvall 2002). Formoterol also has a rapid onset of bronchodilation after inhalation (Palmqvist et al.,1997). The long acting agonists have a long duration of action that could last up to 12-24hours after drug administration (Rabe *et al.,*1993). However, both Formoterol and Salmeterol have different chemical structures and pharmacological properties which account for the more rapid onset of action of Formoterol compared to salmeterol (Lotvall *et*

al., 2002). Formoterol is more hydrophilic and less lipophilic than salmeterol, which is more lipophilic and less hydrophilic, this enables Formoterol to diffuse through the bilipid layer of cell membranes (Lotvall *et al.,* 2002). Despite both Formoterol and Salmeterol effect on improvement of lung function ,the management guideline for management guidelines of asthma recommend the use of inhalational corticosteroids(ICS) with the long acting β_2-agonists,which have been shown to be effective in the management of asthma symptoms.(Pauwels *et al.,* 1997). The use of ICS in the management is the mainstay of anti-inflammatory therapy in the treatment of mild asthma. Also, in clinical trials the ICS have been shown to effective in alleviating asthma symptoms when used in combination with long acting β_2-adrenoceptor agonists than the ICS administered alone (Vermetten *et al.,* 2009).

1.7.2 β_2-adrenoceptor agonists and inverse agonists in wound healing

Bronchial asthma has been associated with injury to airway epithelial cell from exposure to toxins and allergens(Knight *et al.,* 2003).The damage to the epithelium with the release of pro -inflammatory cytokines and inflammatory cells in to the lung tissue show histologically characteristic wide spread epithelial damage(Ware *et al.,* 2000).The airway epithelial cells have been shown to express β_2-adrenoceptor and regulate mucociliary clearance in the airways .The β_2-adrenoceptors also regulate transepithelial anion transport and ciliary beat frequency in the airway epithelial cell (Peitzman *et al.,* 2015).The activation of the β-adrenoceptors increases the production of cyclic AMP via the adenylate cyclase system. The β_2-adrenoceptor agonists administered to patients affected by pulmonary diseases such asthma (Bassford *et al.,* 2012) promote anti-inflammation and epithelial repair (Perkins *et al.,* 2004). This makes the role of β_2-adrenoceptors and the downstream signalling pathways in wound healing associated with their activation important. Previously it was shown that, Isoprenaline stimulated wound closure in airway epithelial injury via the cyclic AMP

pathway (Spurzem *et al.,* 2002). Also, in another study, the β_2-adrenoceptor agonists salbutamol, delayed wound closure in normal human bronchial epithelial cells and Calu-3 cells. However, the treatment with carvedilol delayed wound closure and did not produce any significant change in ERK phosphorylation in treated cells. The inhibition of protein kinase activity delayed wound healing stimulated by Isoprenaline. This suggest PKA dependent phosphorylation was necessary to increase the rate of wound healing. Both β_2-adrenoceptor agonists and inverse agonists do have some effects in the closure of wounds. Isoproterenol improved wound healing in bovine bronchial epithelial cells (Spurzem *et al.*, 2002). Salbutamol was reported to have improved wound closure, but this was reduced when was used salbutamol in combination with propranolol (Perkins *et al.,* 2008). The difference in wound closure seen in lung epithelium was different to that observed in corneal epithelial cells, keratinocytes from oral and dermal origins. In keratinocytes, Isoprenaline reduced wound closure by the inhibition of ERK phosphorylation The β_2-adrenoceptor antagonists by increasing ERK phosphorylation and delayed wound closure (Chen *et al.,* 2002). This was also displayed by antagonists which enhanced wound closure in corneal epithelial cells (Ghoghawala *et al.,* 2008, Pullar *et al.,* 2007). In a study conducted in adult human corneal cells, the activation of protein phosphatase A(PPA) by β-adrenoceptor agonists reduced ERK phosphorylation which reduced wound closure. Also, the inhibition of PPA in adult human keratinocytes reversed delay in wound closure and enhanced wound closure. The suggests the β-adrenoceptor agonists mediate migration of cells through PPA (Pullar *et al.,* 2002).

1.8. β_2-adrenoceptor inverse agonist use in bronchial asthma

The discovery of the β_2-adrenoceptor in the airway epithelial cells with the use of radiolabelled β_2 -adrenoceptor agonists and inverse agonist, showed surface epithelium expressing the β -adrenoceptor and β_1 and β_2-adrenoceptor which co-exist in mucosal glands (Carstairs *et al.,* 1985, Mak *et al.,* 1994). Other methods were hybridization techniques to

detect β-adrenoceptor mRNA in lung tissues and immunodetection of cyclic AMP which showed that the β-adrenoceptor was functional in ciliated cells (Hamid *et al.,* 1991).

The identification of the β-adrenoceptor in the human airways led to investigations of the receptor. For example, the β-adrenoceptor agonist such as isoprenaline, feneterol, albuterol and salmeterol increased ciliary beat frequency in mammalian airway cells (Yanuara *et al.*, 1981). The mechanism underlying the response is cyclic AMP mediated that is accompanied by increase in cyclic AMP levels. However, the increase cyclic AMP level via cyclic AMP independent mechanisms have also been identified in human, bovine and lupine cilliary beat frequency and was blocked by a protein kinase inhibitor (Wyatt *et al.*, 1998; Salathe *et al.*, 2000).

Previously congestive cardiac failure (CCF) patients were treated with the β-adrenoceptor agonist, dobutamine which was however associated with increased mortality in clinical trial in CCF and asthma (Lin *et al.,* 2008). The chronic use of β-adrenoceptor agonist with harmful effects such as asthma related deaths, was suggested by studies conducted on detrimental effect of the drugs. Recent data suggest β_2-adrenergic inverse agonists may be useful in the chronic treatment of asthma (Dickey *et al.,* 2010). Previous data showed Carvedilol, a β_2-adrenergic antagonist which was a contraindicated drug, improved symptoms of congestive cardiac failure on chronic administration (Bristow *et al.,* 1996). This led the research into possible use of similar drug in bronchial asthma.

Furthermore, the modulation of the β_2-adrenoceptor inverse agonists was investigated whether it was due to inhibition of constitutive activity of the β_2-adrenoceptor or due to biased agonism (Wisler *et al.,* 2007). Also, in CCF, biased agonism was suggested to be reason for the beneficial effects of Carvedilol. This led to the study of genetic deletion in knock out mice after chronic administration of a β_2-adrenergic inverse agonist, which produced reduction of airway hyperresponsiveness airway inflammation and reduction of mucin production in asthma (Nguyen *et al.*, 2009).

In view of this further studies on the action of inverse agonists on the β_2-adrenoceptor based on duration of treatment in asthma would be worthwhile. The effects of long duration of treatment was investigated with carvedilol and nadolol in murine model of asthma, which acutely increased airway responsiveness but chronically reduced airway hyperresponsiveness (Callerts-veigh *et al.,* 2004). Alprenolol ,however did not reduce airway hyperresponsiveness despite being a β_2-adrenoceptor antagonist with weak agonist properties (Callerts -veigh *et al.,* 2004).

Clinical trials with the inverse agonist nadolol was conducted in which the safety and efficacy of nadolol was determined in 10 patients with mild asthma (Hanania *et al.,* 2008). The chronic administration of nadolol was tolerated after 9weeks of investigations. A second study was conducted with similar results (Dickey *et al.,* 2010). To determine the beneficial effects, murine models of asthma were investigated for chronic beneficial effects of inverse agonists. The acute administration of the agonist albuterol reduced asthma symptoms while nadolol worsened it. However, on chronic administration of both drugs, albuterol worsened asthma symptoms while nadolol improved symptoms by providing protective effects against airway hyperresponsiveness (Callaerts-Vegh *et al.,* 2004). ICI118551 was investigated for its effect on the reduction of airway hyperresponsiveness in murine models of asthma, which provided a bronchoprotective effect (Lin *et al.,* 2007). The findings suggest that the chronic administrator of the inverse agonists were responsible for the bronchodilator effect which reduced airway hyperresponsiveness.

Two of the main clinical signs of asthma, airway inflammation and airway mucin production, were investigated to determine the mechanism behind the beneficial effects of chronic administration with inverse agonists. Nadolol was administered for 28 days which reduced the eosinophil count in bronchoalveolar lavage fluid. The inverse agonist also reduced the amount of mucin produced. Similar results were obtained with an alternative inverse agonist, ICI118551. Both inverse agonists were ineffective in reducing airway inflammation or

mucin production when administered for only 7 days (Nguyen *et al.*, 2008). This suggest the chronic administration of inverse agonists is effective in reducing airway inflammation and mucin production reduction.

1.9 Project aims

The activation of the β_2-adrenoceptor by produces a wide range of responses in the human airway epithelium (Graham *et al.*, 2004). These responses could be as result of the constitutive activity of the β_2-adrenoceptors. The β_2-adrenoceptor has been studied to be expressed in high density in multiple cell types such as epithelial cells, immune cells and bronchial smooth muscle cells (Turki *et al.*, 1995, McGraw *et al.*, 2000). As such any observed response from the receptor activation cannot be assigned to a single one cell type. The β_2-adrenoceptor has been shown to be sensitive to inhibition by inverse agonists in clinical trials which have reduced the symptoms of asthma and the progression of the disease (Callaerts-Veigh *et al.*, 2004). The inverse agonists have also been shown to activate a G-protein independent signalling via the β-arrestin-dependent signalling. However, not much is known in the general population about the effects of the inverse agonists on human airway cells.

Therefore the aims of this study were to:

1. Investigate whether the β_2-adrenoceptor populations present in human airway cells show any form of constitutive activity, that is sensitive to inhibition by β_2-adrenoceptor inverse agonists.

2. Investigate whether the inverse agonists found to be effective in clinical trials exhibit biased agonism, stimulating G protein-independent signalling via β-arrestin-dependent signalling.

3. Investigate the functional effects of inverse agonists on airway cell biology e.g. cytokine release, mucous secretion and cell proliferation and migration in wound healing and repair.

Chapter 2
Materials and Methods

2.1 Materials

Materials used for this project were mostly purchased from Fisher Scientific UK, Promega UK, ABCAM, Bio-Rad, ACEA Biosciences and NTU Stores department, unless stated otherwise.

2.1.1 General laboratory reagents

The general laboratory reagents used in the research were; Bovine serum albumin (BSA),

phosphate buffered saline (PBS), dimethyl sulphoxide (DMSO), 1M sulphuric acid,

ethanol, propanol, methanol, triton-x, tween20, paraformaldehyde, hank's balanced salt solution and trypsin EDTA.

2.1.2. Cell culture reagents

Table 2.1 Table showing the constituents of cell culture media.

Cell culture reagent	Catalogue number	Company
Gibco™ DMEM/F-12, no glutamine	11540566	Fisher
DMEM, high glucose	11584486	Fisher
Gibco™ Ham's F-12 Nutrient Mix	11500586	Fisher
Gibco™ Penicillin-Streptomycin 10,000 units/ml penicillin and 10mg/ml of streptomycin)	11548876	Fisher
Foetal Bovine Serum	11573397	Fisher
MEM non-essential amino acids solutions(100x)		Fisher
Phosphate buffered saline	11593377	Fisher
Trypsin, 0.05% EDTA	11590626	Fisher

2.1.3 General laboratory reagents

Table 2.2 List of general laboratory reagents

Chemical	Catalogue number	Company
Gibco™ HBSS with Calcium and Magnesium	15266355	Fisher
BCA protein assay kit	10678484	Fisher
Phosphate buffered saline (tablets)	P4417-100TAB	Sigma
Poly-L-lysine solution	P4707-50ML	Sigma
Protease Inhibitor Cocktail	P8340 -1ML	Sigma
RIPA Buffer	R0278-50ML	Sigma
Thiazolyl Blue Tetrazolium Bromide	M2128	Sigma
Epidermal growth factor(Recombinant Human EGF Protein, CF	236-EG-200	R and D systems
Page ruler plus pre-stained protein ladder	11832124	Fisher
Thiazolyl Blue Tetrazolium Bromide	M2128-1G	Sigma
3-Isobutyl-1-methylxanthine	I5879-250MG	Sigma
Laemmli Sample Buffer	161-0747	Bio-Rad
cyclic AMP Select ELISA kit	CAY501040-480	Cambridge bioscience
Anti-mouse IgG, HRP-linked Antibody	7076S	New England Biolabs
Anti-rabbit IgG, HRP-linked Antibody	7074S	New England Biolabs
p44/42 MAPK (Erk1/2) antibody	9107S	New England Biolabs
Phosphor-p44/42 MAPK (Erk1/2) antibody	9101S	New England Biolabs
GAPDH (14C10) Rabbit mAb	2118S	New England Biolabs
IL-6 Duo set ELISA	DY206-05	Bio Techne
cAMP-Glo Max Assay	V1682	Promega
Real-time-Glo MT Cell Viability Assay	G9712	Promega
Neutral red	57993	Sigma-Aldrich
Triton-X100	X100	Sigma
β-Mercapto ethanol	M3148	Sigma
Sodium Dodecyl Sulphate	B2008	Melford
Enhance chemiluminescence solution	RPN2109	GE,Healthcare
Sodium Phosphate Dibasic Heptahydrate (Na2HPO4)	S2317	Melford
Acrylamide/Bisacrylamide solution	A2-0072	Geneflow
Ammonium per sulphate	A0502	Sigma
Tween 20	P1379	Sigma
Tris base	B2005	Melford
Glycine		
N,N,N,N′Tetramethylethylenediamine (TEMED)	T9281	Sigma
Nitrocellulose membrane	1620115	Biorad
Sulphuric acid (H2SO4)	59160PB	Fisher

2.1.4.1 Antibodies

Table 2.3 Primary antibodies used in this study.

Primary antibody	**Dilution in western blotting**	**Catalogue number**	**Company**
GAPDH	1:1000	2118S	Cell signalling Technology
p44/42 MAPK (Erk1/2) antibody	1:1000	9107S	Cell signalling Technology
Phosphor-p44/42 MAPK (Erk1/2) antibody	1:1000	9101S	Cell signalling Technology
	Dilution in immunocytochemistry		
MUC5AC	1:250	ab3649	Abcam
ZO-1	1:125	orb153344	Biorbyt

Table 2.4 Secondary antibodies used in this study.

Secondary antibody	**Dilution in western blotting/immunochemistry**	**Catalogue number**	**Company**
Anti-mouse IgG, HRP-linked Antibody	1:1000	7076S	New England Biolabs
Anti-rabbit IgG, HRP-linked Antibody	1:1000	7074S	New England Biolabs
Goat anti-Mouse FITC	1:100	F0257	Sigma -Aldrich
Rabbit anti Goat IgG H and L Rhodamine	1:100	E.AB-1066	Elabscience

2.1.4.2 Kinase inhibitors

Table 2.5 Kinase Inhibitors used in this study

Inhibitor	Catalogue number	Molecular weight	Company	Concertation
PI3-kinase inhibitor (LY 294002)	ab120243	307.35	Abcam	10µM (Brunn et al.,1996).
MEK1 inhibitor (PD98059)	ab 120234	267.28	Abcam	10µM(Newton et al.,2010).

2.1.4.3. Modulators of β_2-adrenoceptor

Table 2.6 Modulators of β_2-adrenoceptor.

Modulators of β_2-adrenoceptor	**Catalogue number**	**Company**
Carvedilol	CAY15418-100mg	Cambridge Bioscience
Forskolin	SM18-2	Cell guidance systems
Formoterol	CAY15584-10mg	Cambridge Bioscience
Isoprenaline hydrochloride	I5627-5G	Sigma-Aldrich
Propranolol	ab120757	Abcam
Nadolol	N1892-1G	Sigma-Aldrich
ICI118551	ab120808	Abcam
Salmeterol	ab120771	Abcam
Alprenolol	A0360000	Sigma-Aldrich

2.1.4. Technology systems

Table 2.6. Technology systems used in this study.

System	Company	Experiments	Plates
Epithelial Volt/Ohm meter	World Precision instruments	Transepithelial electrical measurement	12 well plates and trans well inserts
Incucyte S3 system	Essen Bioscience	Proliferation, Scratch wound assay and cell imaging.	96 well plates Image lock plates
XCELLigence RTCA	ACEA Bioscience	Cellular impedance	E-16 plates

2.1.5 Western blotting reagents and equipment.

The list of western blotting reagents and equipment are;

sodium dodecyl sulphate polyacrylamide gel Electrophoresis set up ,5% non- fat milk, Tris buffered saline, Enhanced chemiluminescence solution, RIPA buffer, protease inhibitor, Running buffer, Transfer buffer, nitrocellulose membrane, filter paper, Turbo blot transfer buffer and Turbo blot transfer machine.

2.2. Cell lines

2.2.1. Cell lines with culture conditions.

2.2.1.1.Relevance of cell lines.

The cell lines used in this study have been previously investigated and used for research. There are many in vitro cell models derived from the bronchial epithelial cells that have been used in asthma studies. They are also a target of β_2-agonist and are involved in the pathophysiology of asthma*(Salathe 2002;Barnes1999).*The cell lines used for this study were selected based on the level of expression of the β_2-adrenoceptor which are listed below.

2.2.1.2 Calu-3 cells (ATCC HTB-55).

Calu-3 cells are human adenocarcinoma cells of epithelial origin. Calu-3 cells were derived by Germain Trempe and Jorgen Fogh of the memorial Sloan Kettering Cancer centre. The Calu-3 cells were derived from the bronchial epithelial carcinoma of 25-year-old Caucasian male(*Fogh and Trempe 1975).*

2.2.1.3. BEAS2BR-1

This is an immortalised cell line, derived from Human Bronchial Epithelial cells. Provided by Dr Ray Penn, university of Maryland Baltimore, USA.

2.2.1.4 CHO-β_2. (T26J-1/09 (CHO-Beta-2 (ADRB2)).

This is a cell line of epithelial origin is clonally derived from the CHO-K1 cell line which has been transfected with a human β2-adrenergic receptor cassette to allow the expression of the human β2-adrenergic receptor. It was supplied by the European collection of authenticated cell cultures (*Nehlson et al., 2009)* .

2.3. Methods

2.3.1 Cell culture and culture growth conditions .

Cells were sub-cultured in appropriate media and incubated in a humidified environment at 37^0C and 5% carbon dioxide and 95% air until 80-90% confluent. Cells were washed with 5 ml PBS then trypsinised with 2-3 ml trypsin EDTA for 5 minutes. The trypsin-EDTA was deactivated with 7-8 ml medium. The suspension was centrifuged at 300g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 10 ml of medium. A split ratio of 1:8 or 1:10 was done for BEAS2BR-1 and CHO-β2 and 1:6 for Calu-3 cells.

BEAS2BR-1 and CHO-β2 achieved 80-90% confluency in 3-4 days. The Calu-3 cells achieved 80-90% confluency in 10-14 days. For Calu-3 cells, 70% of culture medium was removed and replaced with fresh medium every 3 days.

Table 2.7 Table showing the constituents of cell culture media**.**

Cell line	**Serum supplemented media**	**Serum free media**
Calu-3	Gibco™ DMEM/F-12 no glutamine (11540566, fisher) 10% (v/v/) Foetal bovine serum (11573397, fisher), Gibco™Penicillinstreptomycin 10,000 units/ml penicillin and 10mg/ml of streptomycin (11548876,fisher) MEM Non-Essential amino acids	DMEM/F-12 Media penicillin-streptomycin
BEAS2B- R1	DMEM Media High glucose(11584486, fisher) 10% (v/v) Foetal Bovine serum Penicillin-streptomycin	DMEM Media High Glucose penicillin-streptomycin
CHO-β_2	F-12 nutrient mix ham 10% (v/v) Foetal Bovine serum. penicillin-streptomycin	F-12 nutrient mix ham penicillin-streptomycin

2.3.2 Proliferation assays

2.3.2.1 Cell counting

BEAS2BR-1 cells were seeded at 13000 or 100000 cells/well in serum free media before treatments were added. The treatments were added and the cells were incubated for 48 and 72hrs. The cells were trypsinised at 48 and 72h and centrifuged. The pellets were resuspended in 1 ml serum free media. The cell suspension was stained with Trypan blue and cell counts were determined with an automated cell counter by BIORAD cell counter, Countess ^{TM} Automated cell counter by Thermo Fisher or haemocytometer.

2.3.2.2 MTT assay

Cell proliferation was investigated using the MTT (3,4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide).The MTT dye is converted to formazan crystals determines mitochondrial activity(van Merloo et al.,2011). This assay was investigated to assess cell proliferation of cells following treatment. Cells were obtained from the cell culture flask when 100% confluent. The culture medium was removed, and the cells were washed with PBS before trypsinised using 0.05% trypsin. The cell suspension was centrifuged at 300g for 5minutes, and the resultant pellets were resuspended in 1ml of media. The cells were counted, and 9000 cells/well (75-200µl well size) were plated in 96 well plates. The plates were incubated in a humidified environment at 37^0C and 5% carbon dioxide and 95% humidified air until 80-90% confluent. The cells were treated with DMSO (0.1 and 1%), IBMX (500 µM), forskolin (FSK, 10 µM), isoprenaline (ISO, 1µM), formoterol (FM, 100 nM), forskolin and IBMX (FSK (10 µM) + IBMX (500 µM)), formoterol and IBMX (FM (100 nM) + IBMX (500 µM)) and inverse agonists ICI118551(1µM), propranolol (1µM), nadolol (1µM). The treatments were made in the presence and absence of 10% (v/v) foetal bovine serum-containing medium and incubated for 72 hours at 37^0C and 5% carbon dioxide and 95% humidified air .The treatments were replaced with MTT reagent (0.5 mg/ml

dissolved in warmed serum free medium) and incubated for 4 hours at 37^0C and 5% carbon dioxide and 95% humidified air. The MTT reagent was removed and the formazan crystals were dissolved in DMSO (200µl) and the luminescence levels across the cells were recorded using a FLUOstar optima luminometer (BMG Labtech Ltd,UK).

2.3.2.3 MTT real time glo assay

The MTT real-time glo assay was used to assess cell proliferation in Calu-3 and BEAS2B-R1 cells. The assay determines the number of viable cells in culture by measuring the reducing potential of cells and metabolism which is a marker of cell viability(Duellman et al. 2015). The treatments were made in the presence and absence of 10% (v/v) serum containing media and incubated for 72 hours at 37^0C and 5% carbon dioxide and 95% humidified air. Medium from all wells of the 96-well plate was removed and replaced with 80µL/well of luminescent reagent containing a 1X concentration of luminescent substrate and luciferase enzyme diluted in serum-free media according to manufacturer's instructions. After a 10-minute incubation at 37ºC, the luminescence levels across the cells were recorded using a FLUOstar optima luminometer (BMG Labtech Ltd,UK)..

2.3.2.4 Neutral red assay

Calu-3 cells and BEAS2B-R1 were seeded at 10000 cells/well in a 96 well plate (75-200µl size) and they were incubated in a humidified environment at 37^0C and 5% carbon dioxide and 95% air for 24 hours. Treatments were added for 72hours.There after the treatments were remove and the Neutral red solution was added to all wells and incubated for 1.30minutes. The Neutral red solution was extracted using NaH_2PO_4 in 50% ethanol. The absorbance was measured with a plate reader.

2.2.3 Incucyte proliferation assay

This was investigated in all the 3 cell lines. BEAS2BR-1 and CHO-β2 were seeded at 5000 cells/well and Calu-3 cells at 10,000 cells/well in a 96 well plate (75-200µl size) , in serum free media. They were incubated in a humidified environment at 37^0C and 5% carbon dioxide and 95% air for 24 hours. Treatments were added, and the plates were kept in the IncucyteS3 system. The scans were recorded every 3 hours were for 72 hours.

2.2.4 Scratch wound assay

Calu-3, CHO-β_2 and BEAS2B-R1 cells, were investigated in this assay. In the initial part of the investigation using BEAS2B-R1 cells(mechanical method only),the wells of 24 well plates were demarcated with vertical lines . The BEAS2B-R1 cells were seeded at a density of 100000 cells per well, in poly-L-lysine coated 24 well plates(15.6mm). The plates were incubated in a humidified environment at 37^0C and 5% carbon until 100% confluent. The cells were then wounded using the mechanical or chemical method. The wounded cells were briefly rinsed with 200µl PBS/serum free medium before the addition of appropriate β-adrenergic agonist or antagonist. The images were captured at time 0,24 and 48 hours after addition of treatments using an inverted microscope .The distance between the wound edges was measured using the image J .

2.2.4.1 Cell wounding

In this investigation,3 types of cell wounding were used to access wound healing. These were applied in BEAS2BR-1(chemical lysis ,mechanical and automated methods) and one method(automated) in CHO-β_2 and Calu-3 cells. The 3 methods of wounding were used to compare the duration of wound closure. All plates were coated with 96µl of poly-lysine for 1 minute prior to seeding with cells.

Chemical lysis method

In the chemical lysis method ,2 lines at right angles were drawn of the bottom of the 12 and 24 plates, prior to seeding of the cells. 1M NaOH was used to create to a wound on confluent monolayers of cells and a circular wound was created. The medium in the wells was replaced by serum free media for 24 hours.1µl of 1M sodium hydroxide was deposited at the centre of the confluent monolayers where the horizontal and vertical lines crossed. The wounds were washed with PBS for less than a minute. The PBS was removed and replaced with treatments in serum free medium in all wells except the control wells. The images were captured using an inverted microscope at time 0, 24 and 48 hours.

Mechanical method

Using the mechanical method of wounding, the wounds were created using 10µl pipette on confluent monolayers that formed rectangular in shaped wounds. A horizontal line was drawn of the bottom of the 12 well plates, prior to seeding of the cells. The medium in the wells was replaced by serum free media for 24 hours. A sterile 10 µl pipette was used to create vertical scratches at right angles to the horizontal lines. The medium was removed, and the plates were washed with PBS. The treatments added were made in serum free media except the control wells. The images were captured with an inverted microscope at time 0, 6, 24 and 48hours.

Automated method

In the automated method, a wound maker (Essence bioscience) was used to create equal sized wounds in a 96 well image lock plates pre-coated with 96 µl poly-L-lysine for 1 minute before seeding the cells. The BEAS2B-R1 ,Calu-3and CHO-β_2 cells were seeded at cell densities ranging from 5-7x10^4 cells per well until 100% confluent. Equal sized wounds were created using a wound maker from Essence Biosciences. The

wounds were washed with serum free medium before adding treatments. Images were captured 3 hourly using Incucyte S3 system.

2.2.5. Cyclic AMP assay

2.2.5.1 Cyclic Amp Glomax™ kit (Promega)

The cyclic AMP assay was investigated using the cyclic AMP Glomax™ (Promega). In this assay, Calu-3 and BEAS2B-R1 cells were seeded at 9000 cells per well in serum free medium, in 96-well microtitre plates with clear bottom well(Corning ,Fisher Scientific, Loughborough UK). These cells were incubated in a humidified environment at 37^0C and 5% carbon dioxide and 95% air until 80-90% confluent. The medium was then removed and the cell monolayers were treated with Forskolin (10 µM), β-adrenoceptor agonists

Isoprenaline (1 µM), Formoterol (100 nM) or inverse agonists ICI1185511 (1 µM), Propranolol (1 µM), Nadolol (1 µM).The treatments and control were made up in the in serum free medium presence of 20mM $MgCl_2$ and phosphodiesterase inhibitor IBMX (500µM) and added to cells for 20 minutes. Following stimulation ,cyclic AMP levels within cells were determined using cyclic AMP Glomax™ assay kit (Promega; Southampton ,UK). Then, briefly the cells following treatment ,10µl of cAMP detection was added to all wells and incubated for 20 minutes at room temperature. Then after the incubation ,Kinase Glo reagent (50µl)was added to all wells and incubated for 10 minutes at room temperature. After the incubation, the luminescence levels across the cells were recorded using a FLUOstar optima luminometer (BMG Labtech Ltd,UK). The control wells treated with (500µM) IBMX were used as positive control and the luminescence values were converted to cyclic AMP levels using a cAMP standard curve (0-100nM) according to manufacturer's protocol.

2.2.5.2 cyclic AMP ELISA assay from Cayman

Calu-3 cells were seeded at a concentration of $1x10^5$ cells/ml were plated in 12 well micro plates(22.1mm) ,(Corning, Fisher Scientific, Loughborough, UK) in serum free

medium until 100% confluent in a humidified environment. The medium was removed from the wells and the cells were treated with Forskolin (10µM), agonists Formoterol (100nM), Isoprenaline (1µM) and inverse agonist Propranolol (1µM), each made up in IBMX

(500µM) and control wells were made in the presence and absence of IBMX (500µM). The Cells were incubated for 20 mins in a humidified environment at 37^0C and 5% carbon dioxide and 95% air. The culture medium was removed and 0.1M Hydrochloric acid was added to wells. The cells were then incubated at room temperature for 20 minutes and scraped with a scraper. The suspension was centrifuged at 1000 g for 10 minutes, and the supernatant was used to determine the cyclic AMP levels by ELISA using the cAMP select kit (Cayman Chemicals). The samples were diluted at 1:3 with 0.1M HCl 1M HCl. The assay performed comprised of three independent experiments. These were plated in 96 well plates together with standards and assay reagents. The standards were made from serial dilution of bulk standard and ELISA buffer. The wells were made up as shown in the table. The plates were developed after the addition of the Elman's(which contains substrate to (cyclic AMP acetylcholinesterase conjugate) reagent to all wells. The plates were incubated for 18hours at 4^0C and then washed 5 times with wash buffer .After the washing, the Elman's reagent was added to all wells. Total activity well had 5µl tracer at the time of development. The plate was developed in the dark for 90-120minutes. The absorbance was detected at 405-420nm.The absorbance values were converted to cyclic AMP levels using a cyclic AMP standard curve.

Table 2.8. Pipetting summary

Well	ELISA Buffer	Standard/Sample	Tracer	Antiserum
Blank	-	-		-
Total activity	-	-	5 µl (at dev. Step)	-

Non-Specific Binding	100µl	-	50 µl	-
B_0(maximum binding)	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

2.2.6. Protein Estimation assay

The estimation of protein concentration in cell lysates was determined using the Bicinchoninic acid assay method ,first described by Smith et al in 1985 (Walker 2009). The Bicinchoninic assay kit(BCA),(Thermofisher, Loughborough, UK) was used to determine the protein concentration of unknown samples from cell lysates. The bovine serum albumin (BSA) standards were prepared by following the manufactures instructions as detailed in the table. All materials were contained within the BCA assay kit and the diluent used was PBS. Into a 96 well plate 25µl of each standard and 10µl of each unknown sample was pipetted in triplicates. To this 200µl of working reagent was added, prepared from 50 parts of BCA reagent A with 1-part BCA reagent B. The plate was then incubated at 37^0C for 30 minutes. After the incubation , the absorbance was read at 570nm on a Bio-Rad iMark Microplate reader. Using the standards' absorbance readings, a standard curve was plotted, and the unknown sample concentrations were obtained by measuring them against the BSA standard curve. The calculated concentrations were used to load equivalent protein levels of the samples in an SDS electrophoresis gel.

Table 2.9. Bicinchoninic assay reagent dilution

Vial	Volume of Diluent (PBS) (µl)	Volume of BSA (µl)	Final of BSA concentration (µg/ml)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	375	325 of stock	1000
D	175	175 of vial B	750

E	325	325 of vial C	500
F	325	352 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0

2.2.7 Sample generation for western blotting

Cells from all cell lines: Calu-3, BEAS2BR-1 and CHO-β2 cells were cultured in 6 well plates until 80% confluent. The medium was removed and treatments were added in fresh medium followed by incubation at 37°C for 30 minutes. The treatment was then removed and cells were washed with ice cold PBS. The PBS was removed and 100µl of radioimmunoprecipitation assay buffer (RIPA buffer) from Sigma, UK, was added with the addition of protease inhibitor at 1:100 (Sigma, Poole, UK). This was incubated on ice for 30 minutes. The cells were then scraped using a cell scrapper and the resultant suspension collected in pre-cooled 1.5ml Eppendorf tube. The lysed cells were placed on ice with mild agitation for 30 minutes then cell lysates were placed into centrifuge pre-cooled to 4^0C and spun at 15,600g for 20 minutes to remove any cell debris. The supernatant was removed and placed into a fresh Eppendorf and the pellet was discarded. The supernatant was used for SDS electrophoresis or frozen at -20^0C.

2.2.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After the determination of protein concerntration,30µg of each sample was diluted 3:1 ratio with 4x Laemmli buffer(8% w/v, SDS 40%(v/v),β-mercaptoethanol,0.01%(w/v) bromophenol blue 250Mm ,Tris-HCl ,pH 6.8) and boiled on a heat block at 95^0C for 5 minutes. The polyacrylamide gels were prepared using the Bio-Rad Mini-PROTEAN Tetra Hand cast systems. The gels were prepared according to manufacturer's instructions(Geneflow ltd, Staffordshire, UK). Each gel consisted of a running gel, topped with a stacking gel as described in tables(2.10 and 2.11) below.

Table 2.10 Resolving gel recipe (10ml)

Composition	Volume
Acrylamide percentage	10%
Water	3.8ml
Acrylamide /bis acrylamide (30% w/v)	3.4ml

1.5 tris pH8.8	2.6ml
10% SDS(w/v)	0.1ml
10% Ammonium persulphate	0.1ml
TEMED	0.01ml

Protein (kDa)	Gel percentage (%)
15-100	10

Table 2.11 Stacking gel recipe

Composition	**Volume**
Distilled H_20	2.975ml
Acrylamide/bisacrylamide(30%\w/v)	1.25ml
1.5M Tris (pH = 8.8)	0.05ml
10% SDS (w/v)	0.67ml
10% (w/v) ammonium persulphate	50µl
TEMED	5µl

Reagents used include: Ammonium per sulphate(APS) (Fisher, Loughborough UK), Acrylamide (Fisher, Loughborough) and TEMED (Sigma, Poole UK). A Bio-Rad-mini-Protean electrophoresis chamber was used to make the gels. The 10% acrylamide (w/v) resolving gel was prepared as shown in the table 2.9. The ammonium per sulphate and TEMED polymerised the acrylamide. The mixture of the resolving gel was made up in 50ml tube and added to the glass cast .The amount added was enough to allow some space for the stacking gel, which was added after the resolving gel had polymerised. Some distilled water was added to the top of the resolving gel layer to prevent the gel from shrinking .The resolving gel polymerised after 40 mins or when the remaining unused mixture in 50ml tube had polymerised. The distilled water was then removed by carefully tilting the get cast. The stacking gel made up at 4 % (w/v) was polymerised by 10% APS and TEMED as shown in the

table 2.9. The stacking gel permits the passage of proteins and accumulation of the protein at the interface with the resolving gel. Once the resolving gel had solidified the stacking gel was poured and a comb was inserted. This produced the wells for loading the samples. The gel was placed into the Invitrogen gel tank containing ice cold running buffer (SDS 0.01%,2.5mM Tris, 19.2mM glycine, pH 8.3), at this point the comb was removed which produced 12 wells. The protein samples were loaded into separate wells (30µg/well) along with 5µl of protein ladder. The whole apparatus was attached to a power pack for 30minutes at 120volts then increased to 160 volts for 1hour or until the dye front had just run off the gel.

2.2.9 Protein transfer

At the end of the SDS-PAG electrophoresis, the gel cassette was removed from the running buffer and chamber was opened to remove the gels. The gels were placed in the transfer buffer (200ml of 5X Bio-Rad Trans blot buffer,600ml distilled water ,and 200ml of ethanol) for 5 minutes to equilibrate, as were the sponges, nitrocellulose membrane (GE Healthcare, UK) and filter paper. A set up of a dry transfer was made using the Trans-Blot® Turbo™ transfer system . The turbo blot transfer cassette was opened and the transfer stack was made consisting of the following layers: Filter paper, membrane gel and filter paper. The whole transfer stack was inserted into the transfer cassette and inserted into the Turbo transfer blot. The transfer was then run for 7 minutes.

2.2.10 Immunoblotting

After the electro transfer of the proteins, the membranes were removed from the transfer stack and blocked with 5% non-fat milk in Tris buffered saline in 0.1% tween 20(v/v) (TBST) or 1% (w/v) Bovine serum albumin, for 1 hour to prevent non-specific

binding. The membrane was then probed with the relevant primary antibody diluted in 5% (w/v) skimmed milk powder in (TBST) for 24 hours at 4^0C on rocker at room temperature. The membrane was removed and the primary antibody made up milk or BSA was frozen for further use. After the incubation the membrane was washed in TBST 5 times for 5 minutes on a rocker at room temperature. After the incubation, the blots were washed again with TBST 5 times for 5 minutes on a rocker room temperature. After the washes the blots were probed with appropriate horse-radish peroxidase (HRP)-conjugated-secondary antibody for 1 hour at 4^0C.After the incubation the secondary antibody was removed and the wash with TBST was repeated 5 times for 5 minutes. Following the washes, the blots were developed using the enhanced chemiluminescence reagent (GE,Healthcare ,United Kingdom) (ECL).The ECL consisted of 1-part reagent A to reagent Bathed immunoblot was then exposed to a chemiluminescence viewer and images were taken for 60 minutes. Images of the blots were captured by LAS 4000 system and the bands were quantified by densitometry using Advanced image data analysis software (AIDA) (Fuij version 3.52).The band densities were measured and corrected for background and normalised to band densities of control. The data were expressed as percentage of the average value of peak area compared to its corresponding control ± SEM

2.2.11. Impedance Measurements using the xCELLigence DP system.

The xCELLigence DP system (ACEA Biosciences San Diego,CA,USA) is a real-time analyser (RTCA) based on the assessment cell impedance changes. The RTCA is made up of 4 main parts; The RTCA DP station with 3 independent E-16 well plate platforms for insertion of E-plates, placed inside a tissue culture incubator ;the RTCA computer (Control unit) which has an integrated software to acquire and show data in real-time and E-16 plates. The E-plate is a 16 well plate with glass bottom coated with gold plated

electrodes which cover about 75% of the well area(*Lebourgeois et al.*,2018).

The measured electrical impedance is displayed as a parameter ,Cell index (CI).The CI is zero when no cells have adhered to the electrodes or are absent. The absence of cells on the electrodes indicates background impedance measurements. The value of CI increased as the cells began to adhere the electrodes. The changes in CI are shown in real-time plot by the software. The data obtained from the software were exported to Microsoft excel and Graph pad prism was used to plot and analyse the data .In the beginning of the impedance measurements, 50µl of culture media was added to each well of an E-16 plate. This were incubated for 30 minutes at 37^0C and 5% CO_2 and a background impedance reading in the absence of cells was recorded. After the background measurements, BEAS2B-R1, CHOβ_2 and Calu-3 cells seeded, at $2.50X10^5$ cells per well cells per well for Calu-3, 5000 cells per wells for BEAS2B-R1 cells and $5.0X10^4$ cells per well for CHO-β_2 cells in E-16 well plates

.

The E-plate containing the cells was incubated for 30 minutes in an incubator, at 37^0C and 5% CO_2 before it was placed into the cradle of the RTCA DP station which was kept in the tissue culture incubator throughout the experiment. The cell attachment, adhesion and proliferation were monitored every 15 minutes for 24hours, until a plateau was reached to obtain an optimal assay window or a stable baseline for addition of agonists or inverse agonist. The background CI was then subtracted from subsequent CI as they are generated after cell attachment.

The first reading prior to addition of treatments was paused, which served as the basis for normalizing the cell index. The 100µl of media in the wells were removed and replaced 50 µl of treatments. The measurements were monitored every 15 seconds for 30 minutes, then every 15 minutes for 24-72h. The data was analysed by normalising the CI values by the dividing the CI index values with the CI prior to treatment addition time.

2.2.12. Culture of Calu-3 cells at Air liquid (ALI)

The Transepithelial electrical resistance measurement was measured in Calu-3 cells using an EVOM2 epithelial volt-ohm meter (over 14-24 days at Air liquid interface to investigate the development of tight junction.Calu-3 cells were cultured as using air liquid culture to differentiate the cells. The Calu-3 cells ,seeded at 250000 cells per well, were grown on 12 well plate trans well inserts(1.12cm^2,12mm,0.4µm diameter, Corning Costar ,Fisher Scientific).The cells were maintained in 10% serum containing medium. In basolateral compartment of the transwells,1.5ml of medium was added to the basolateral compartment and the media was changed after every 3 days until cells were 100% confluent. The medium in the apical compartment was removed when the cells were 100% confluent, to raise the cells to air liquid interface after 72hours.

2.2.12.1 Transepithelial electrical resistance (TEER) measurements

The transepithelial electrical resistance was measured in differentiating Calu-3 cells using EVOM2 epithelial volt-ohm meter(World Precision Instruments UK, Stevenage) over 21 to 28days to ALI to investigate development of tight junctions. Briefly, the medium was aspirated and replaced with 1.5 ml in basolateral compartment and 0.5ml of Hank's balanced salt solution was used to wash the cells in the apical compartment before adding 0.5 ml to the apical compartment. The differentiating cells were equilibrated 30 minutes in incubator. The EVOM2 chopsticks were equilibrated will 10 ml of media in a biosafety hood for 20 minutes before measurement of TEER. The TEER of the blank (insert and media only) was subtracted from the measured TEER and the Ωcm^{-2} was calculated by multiplying the insert area. The media in the apical compartment was removed after measurements were taken, to resume ALI conditions.

Treatments were added to the wells on days 10, 14 or 24days for 30 min -7 days. Measurements were taken to assess a change in Transepithelial electrical resistance in Calu-3 cells.

2.2.12.2 Immunocytochemistry

Cell morphology was investigated in Calu-3 cells seeded at $2.5x10^5$ cells per well grown on trans well inserts grown under ALI conditions. Immunohistochemistry was performed using indirect immunofluorescence staining followed by confocal laser imaging microscopy. The inserts were released with a scalpel and transferred to 12 well plates containing PBS/1% (v/v) FBS. The inserts were rinsed in PBS and fixed with 4% paraformaldehyde for 10 minutes at 37^0C or with 95% ice cold methanol which was incubated for 10mins on ice. 0.1% (v/v) Triton-X in PBS was used to permeabilize the inserts at room temperature for 15 minutes. The inserts were blocked with 2% (w/v) BSA in PBS at room temperature for 60 minutes. The preparation steps were performed on a shaker at room temperature. Primary antibody was added to the inserts and incubated at 4^0C overnight. The inserts were then washed with PBS ,5 times for 5minutes.After the washes ,the secondary antibody was added with a counter stain, DAPI for 45 minutes . The inserts were mounted on glass slides using antifade reagent with DAPI and covered with a cover slip. The glass slides were viewed with a confocal microscope.

2.2.13 Cytokine ELISA assay (Duo set ELISA Development system)

The cytokine levels in Calu-3 cells was investigated by ELISA using matched monoclonal capture and biotinylated anti human(IL-6) detection antibody(Duoset ELISA Development system, R and D ,Europe Limited).The experiment was performed according to manufacturer's instructions. Briefly ,96 well ELISA plates were coated overnight at room with 100µl IL-6 capture antibody and added to each well and incubated on a rocker at room temperature. Each well was aspirated and washed with 300µl of wash buffer (PBS with 0.5% (v/v) Tween-20) 3 times. The plate was blocked by adding 300µl of block buffer(1%

(w/v) BSA in PBS) to each well and incubated 1hour.The aspiration and wash process were then repeated. After the washes,100µl of samples/standards in reagent diluent(1% (w/v) BSA,0.05% (v/v) Tween-20 in Tris -buffered saline) was added to each well. Standards were set up as shown in Table 2.13.

Table 2.7 Cytokine ELISA Standards dilution recipe

IL-8	Buffer Reagent diluent	Final Concentration
18.1µl	981.9 µl	2000 pg/ml
500 µl of 2000 pg/ml	500 µl	1000 pg/ml
500 µl of 1000 pg/ml	500 µl	500 pg/ml
500 µl of 500 pg/ml	500 µl	250 pg/ml
500 µl of 250 pg/ml	500 µl	125 pg/ml
500 µl of 125 pg/ml	500 µl	62.5 pg/ml
500 µl of 62.5 pg/ml	500 µl	31.25 pg/ml
0	100 µl/well	0 pg/ml

Material details

Reagent diluent for IL-8:0.1% (w/v) BSA,0.05% (v/v) Tween-20 in Tris -buffered saline

(20Mm Tris base, 150mM NaCl) pH 7.2-7.4. Block buffer consisting of 1% (w/v) BSA in PBS. Wash buffer used was PBS with 0.5% (v/v) Tween-20. 2N H_2S0_4 was used to stop the reaction. Substrate solution consist of 1:1 mixture of Colour Reagent 1 and Colour Reagent B (R&D). Standards were loaded in triplicate and samples were set up in duplicate. The plate was sealed with a plate-sealer and incubated for 2 hours at room temperature and the wash process was repeated. After the washes,100µl of detection antibody (20ng/ml) was added to each well. The plate was sealed and incubated for 2hours and the wash process was repeated. Then 100µl of streptavidin-HRP was added to each well, covered in foil and incubated for 20 minutes at room temperature which displayed development colour in the wells. The aspiration and washes were repeated and the substrate solution was added(Colour Reagent 1 and Colour Reagent B at 1:1) (R&D). The reaction was stopped by adding 50µl of stop

solution(2N H_2S0_4) to each well. The degree of colour generated was determined at 450nm with 570nm .The standard curve was determined using a four-parameter logistic equation. The concentrations of cytokines produced were read off from the standard curve.

2.2.14. Statistical analysis

Graphs were produced and statistics One-way ANOVA, followed by Dunnett's multiple comparison test) performed using GraphPad Prism software, version 8.1.2 (332) (GraphPad software, inc, USA). The results obtained were presented as mean ± SEM and '$p< 0.05$ was considered significant.

Chapter 3

Modulation of the β_2-adrenoceptor by inverse agonists

3.1 Introduction

The β-adrenoceptors are largely distributed in the lungs. The β_2-adrenoceptor sub-type has been isolated in airway smooth muscle, epithelial cells, mast cells and type II alveolar cells (*Johnson 1998*).

The activation of the receptor increases the production of cyclic AMP. The β_2-adrenoceptors exist in different structural states. A small population of the β_2-adrenoceptors is in an active state which increases the production of cyclic AMP. The activation of the β_2-adrenoceptors by agonists increases the population of receptors in the active confirmation (*Anderson 2006*). Antagonists block receptor activation by competing for the binding sites of agonists. The inverse agonists reduce the activation of the receptor by reducing its spontaneous activity (*Strange 2002*). This spontaneous activity of β-adrenoceptors is the ability of a receptor to undergo agonist independent signal transduction (*Milligan 2003*). The presence of these receptors in a spontaneously active form was previously identified (*Cotecchia et al.,* 1990; *Kjelsberg et al.,* 1992). From these studies the extended ternary complex model for receptor activation was developed, in which the receptor (R) exists in 2 states: inactive (R) and the R^* active state, in equilibrium with each other (*Samama et al.,* 1993). It was also stated that high efficacy agonists bind to R* with high affinity and shift the equilibrium towards R* (*Lefkowitz et al., 1993*; *Samama et al.,* 1993). In addition to this, if the available receptors are in R* state in an intact signalling system in the absence of an agonists, then the system would be spontaneously or constitutively active (*Hopkinson et al.,2000*). The inhibition of constitutive activity by inverse agonists such as ICI118551 has been demonstrated in constitutively active mutant receptors (*Hopkinson et al.;2000*). In support of this ICI118551 displayed similar effect in Calu-3 cells when it reduced the amount of cyclic AMP produced. The β_2-adrenoceptors have also displayed multiple signalling pathway interactions with both G-protein and non-G-protein effectors. These multiple signalling events arise from

interactions with G-protein couple receptors and the Mitogen activated protein kinase (MAPK), which convert extracellular stimuli into multiple signalling events (Cargnello *et al.,* 2011). For example, extracellular regulated kinase (ERK)1/2, c-Jun amino N-terminal kinase and 1/2/3(JNK1/2/3) and p38 isoforms α,β and γ (Kyriakis *et al.,* 2001). Also, the MAP Kinases have been identified as the triggers for the production of second messengers and activation of phosphorylation cascades (*Hermans 2003*). The β_2-adrenoceptor agonists and inverse agonists have also been shown to modulate different types of cell signalling by preferentially stimulating a different pathway over another. This is called ligand bias or functional selectivity. A form of ligand biased signalling has been displayed by ligands behaving as inverse agonists in cyclic AMP production and as agonists for extracellular regulated kinase (ERK)1/2 activation (*Galandrin et al.,* 2006). For instance, inverse agonists ICI118551 and propranolol have been known to reduce cyclic AMP signalling but they behaved as partial agonists towards ERK activity (*Azzi et a.,l* 2002). The activation of ERK1/2 was previously investigated in HEK293S cells, where the β_2-adrenoceptor agonist isoprenaline, β_2-adrenoceptor agonist inverse agonist propranolol and β_2-adrenoceptor antagonist, carvedilol, phosphorylated ERK1/2 (Galandrin and Bouvier 2006). Furthermore salbutamol, a β_2-adrenoceptor agonist was reported to have induced the expression of MAP kinase in human airway epithelia cells which was inhibited by propranolol and MEK1 inhibitor ,PD98059 *(Nishimura et al.,2002*). This suggests a potential role for the β_2-adrenoceptor agonists and inverse agonists in modulation of MAP kinase activation and cyclic AMP production in human airway epithelial cells.

Investigating the complete signalling profile or description of the behaviour of agonists or inverse agonists towards a receptor is time and resource consuming. This involved the use of many different assay formats and can lead to inadequate conclusions (*Sandroglio et al.,* 2010). This is because the knowledge about a complete signalling pathway of receptors has not been adequately studied. An integrative approach would provide broad signalling profile

in a single assay, that would enhance identification of individual agonists, inverse agonists and other drugs acting on the receptor. This would also aid their classification into distinct pharmacological groups (*Peters et al.,* 2009). In view of this, a label free cell-based technology such as the X-xCELLigence RTCA system, was developed recently to monitor real-time changes in cellular events such as cell morphology, viability,adhesion and cell distribution. Another assay that is also label free was recently developed that measures the cellular impedance, based on the principle that adhesion of cells in a local ionic environment at an electrode solution interface produces increase in electrode impedance has been used to investigate GPCR signalling. The assay uses the real-time cell electronic sensing system (RT-CES) which investigates cytotoxicity, cell adhesion, proliferation and cell mediated signalling (Xi et a.,2008).Furthermore, because GPCRs are widely studied in drug discovery, the impedance assay could be a valuable tool to explore and monitor GPCR activity. One such study was the investigation of the β_2-adrenoceptor signalling profile. This was studied using the impedance assay in HEK293S cells expressing β_2-adrenoceptor. Isoprenaline produced a concentration dependent response which was blocked by inverse agonists (e.g. ICI118551) and not by antagonists, such as CGP20712A (*Stalleart et al.,2012*). Furthermore ,the investigation of various other GPCRs showed the possibility of conducting similar studies using agonists and inverse agonists (Yu *et al.,* 2006). In view of this, the research was extended to use this technology to monitor the activity of the an endogenous β_2-adrenoceptor population in human airway epithelial cells for the first time.

3.1.1 Aims

- To investigate whether the β_2-adrenoceptor population shows any form of constitutive activity that is sensitive to inhibition by inverse agonists.
- To investigate whether any β_2-adrenoceptor inverse agonists are biased agonists.

3.2 Results

3.2.1 Assessment of cyclic AMP production coupled to β_2-adrenoceptors

The β_2-adrenoceptor was previously found to be expressed in the human bronchial epithelial cell lines Calu-3 cells and 16HBEI4o$^-$. The activation of the receptor by β_2-adrenoceptor agonists, increased cyclic AMP levels but was inhibited by β_2-adrenoceptor inverse agonists (Abraham *et al.,* 2004).

Modulation of the β_2-adrenoceptor by β_2-adrenoceptor agonists and inverse agonists was investigated in human airway epithelial cells in this chapter. The initial assessment was investigated in Calu-3 cells and using the cyclic AMP assay using the cyclic AMP promega kit and cyclic AMP ELISA assay using the Cayman kit. The cyclic AMP was also investigated in BEAS2-R1 using cyclic AMP assay Promega kit. The real-time analysis of human airway epithelial cells was studied Cau-3, BEAS2-R1 and CHO-β_2 cells, using the X-xCELLigence RTCA system. Western Blotting experiments were carried out to investigate for activation of MAP kinase in Calu-3 and CHO-β_2 cells.

β_2-adrenoceptors are Gs protein coupled receptors which stimulate the production cyclic AMP via the adenylate cyclase system (Nishimura *et al.,* 2002). The activation of the receptor cause acute and long term down regulation responses which affect the signalling of the receptor (Anderson 2006). Furthermore, the activity of airway epithelial cells is regulated by the β_2-adrenoceptors, therefore epithelial damage in airway diseases and altered β_2-adrenoceptor function lead to airway hyperresponsiveness (Nijkamp *et al.,* 1992). In view of this the cyclic AMP production was investigated in Calu-3 and BEAS2B-R1 cells.

The stimulation of the β_2-adrenoceptor in Calu-3 cells, significantly increased cyclic AMP in formoterol and isoprenaline treated cells relative to control (Fig3. 1) $p<0.05$. This was not

significantly increased in forskolin treated cells and was not significantly reduced by ICI118551, propranolol and nadolol.

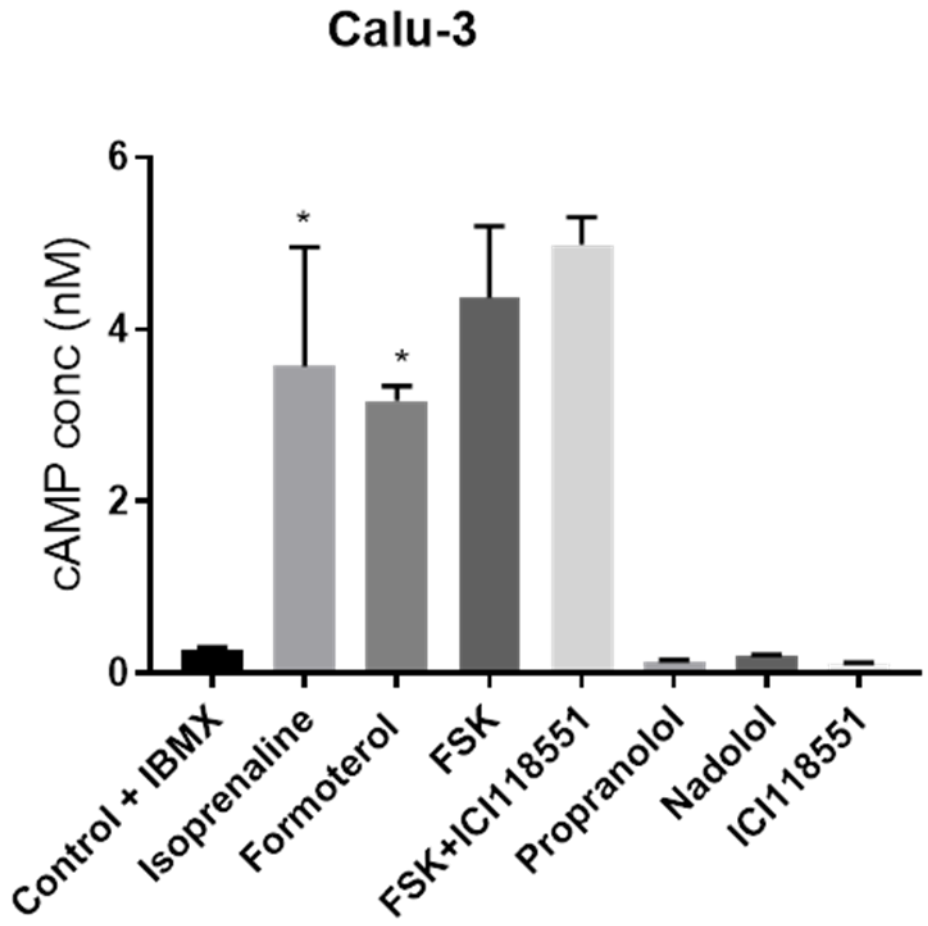


Figure 3.1 β2- adrenoceptor agonist stimulation of cyclic AMP production.

Calu-3 cells were treated with forskolin (10 µM), isoprenaline (1 µM), formoterol (100 nM), propranolol (1 µM) and ICI118551 (1 µM) for 20 minutes in the presence of IBMX (500 µM), before performing the assay. Cyclic AMP was investigated as described in section (2.2.5). Data are presented as mean ± SEM, n=3 for control, isoprenaline, formoterol, n=2 for forskolin, fsk+ICI118551,propranolol, nadolol and ICI118551. Cyclic AMP assay showed a significant increase in cyclic AMP with isoprenaline and formoterol ($p \leq 0.05$) ($p<0.05$, **$p<0.01$, analysed using Dunnett's post –hoc test following one-way ANOVA).*

The cyclic AMP assay in BEAS2B-R1 cells showed a non-significant increase in cyclic AMP in isoprenaline, forskolin and formoterol relative to control. The β-adrenoceptor inverse agonists propranolol and ICI118551 did not significantly inhibit cyclic AMP production in BEAS2B-R1 cells (Figure 3.2).

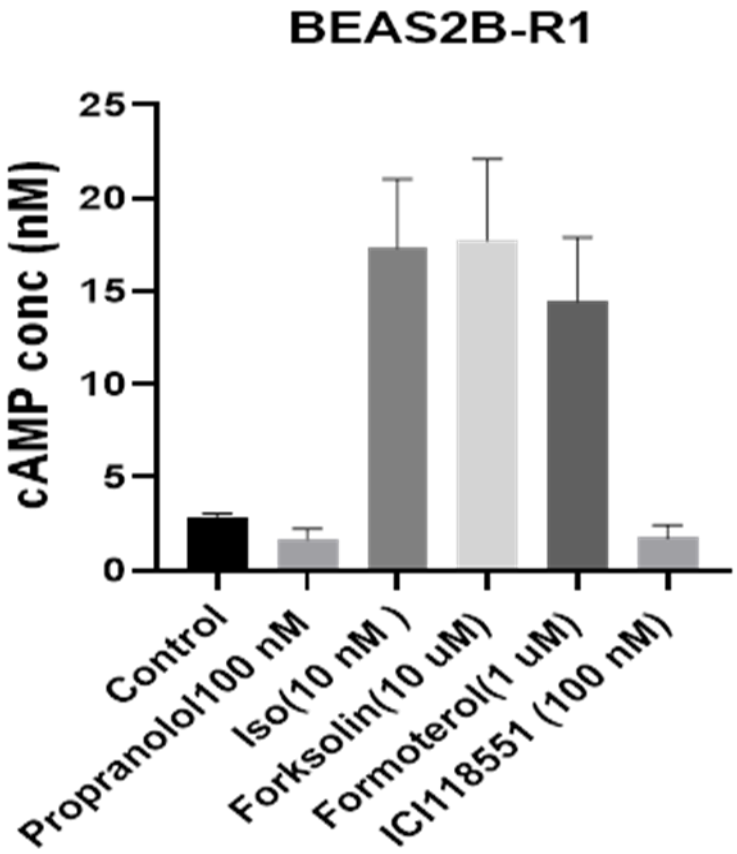


Figure 3.2 β2- adrenoceptor agonist stimulation of cyclic AMP production in BEAS2B-R1.

BEAS2B-R1 cells were treated with forskolin (10 µM), isoprenaline (1 µM), formoterol (100 nM), propranolol (1 µM), and ICI118551 (1 µM) for 20 minutes in the presence of IBMX (500 µM), before performing the assay. The cyclic AMP was investigated as described in section (2.2.5). Data are presented as mean ± SEM, n=2.

3.2.2 Measurement of cyclic AMP using an ELISA assay

The cyclic AMP ELISA assay displayed a non-significant increase in cyclic AMP with forskolin and the β_2-adrenoceptor agonists formoterol and isoprenaline increased cyclic AMP relative to control. Propranolol however did not significantly reduce cyclic AMP production (Fig 3.3).

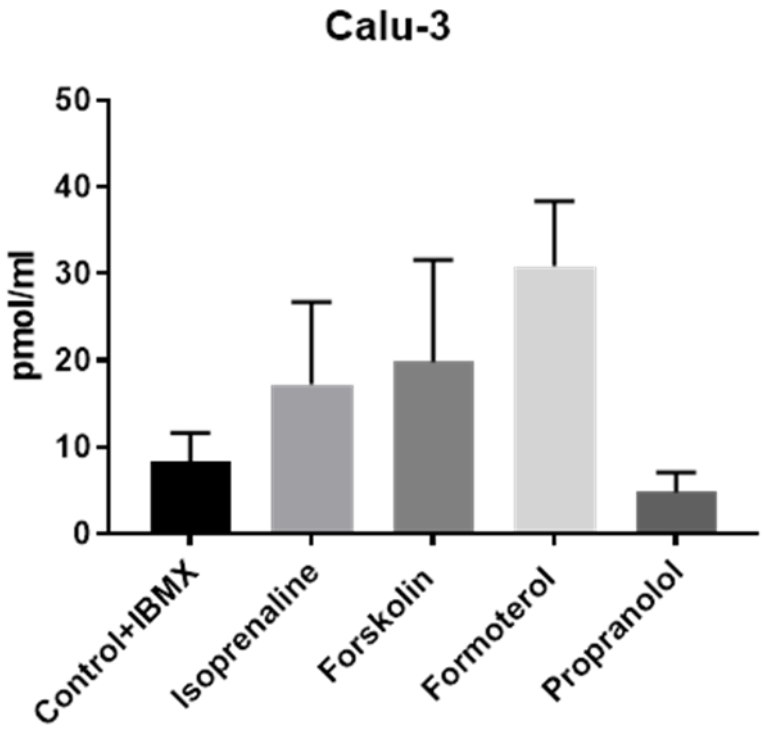


Figure 3.3 cyclic AMP measurements by ELISA.

Calu-3 cells were treated with forskolin (10 µM) isoprenaline (1 µM), formoterol (100 nM), propranolol (1 µM) for 20 minutes. The treatments were made with IBMX (500 µM). The experiment was investigated as described in section 2.2.5. Data are presented as mean ± SEM, n=3 for Control+IBMX, isoprenaline, formoterol and propranolol, forskolin n=4.

3.2.3 Modulation of $\beta_2$adrenoceptors investigated using impedance measurements in human airway epithelial cells.

Impedance measurements in human airway cells

Calu-3 cells were seeded at a concentration of 250000 cells/well, BEAS2B-R1 cells were seeded at 5000 cells/well and CHO-β_2-cells at 5000 cells/well. Cells were serum starved for 24 hours prior to stimulation with β_2-adrenoceptor agonist, β_2-adrenoceptor inverse agonist and PD98059(MEK 1 inhibitor).

In the first part of the cellular impedance assay, BEAS2BR-1 were assessed for cellular impedance changes to determine if impedance changes could represent signalling events. In the first part the cell index measurements were measured for 26:26 hours to allow for cell attachment and to assess proliferation of cells (Figure 3.4). The cells were stimulated with β_2-adrenoceptor agonists and inverse agonists after 26:26hours which displayed a change in cellular impedance prior to agonist stimulation. The results were normalised at 26:27 hours i.e. 1 minute prior to addition of treatments.

The impedance changes shortly following treatment displayed a down ward spike with a negative impedance response initially, which then reverted to a positive slope (Figure 3.4b).Shortly after addition of treatments, each ligand displayed its own impedance responses as indicated in the tracing(Figure 3.4b).The impedance changes produced after stimulation showed a highly significant increase in impedance in response to forskolin, isoprenaline and ICI118551 relative to control (Figure 3 .4 a). Formoterol, propranolol and formoterol did not produce significant increase in impedance. (Figure 3.4a).

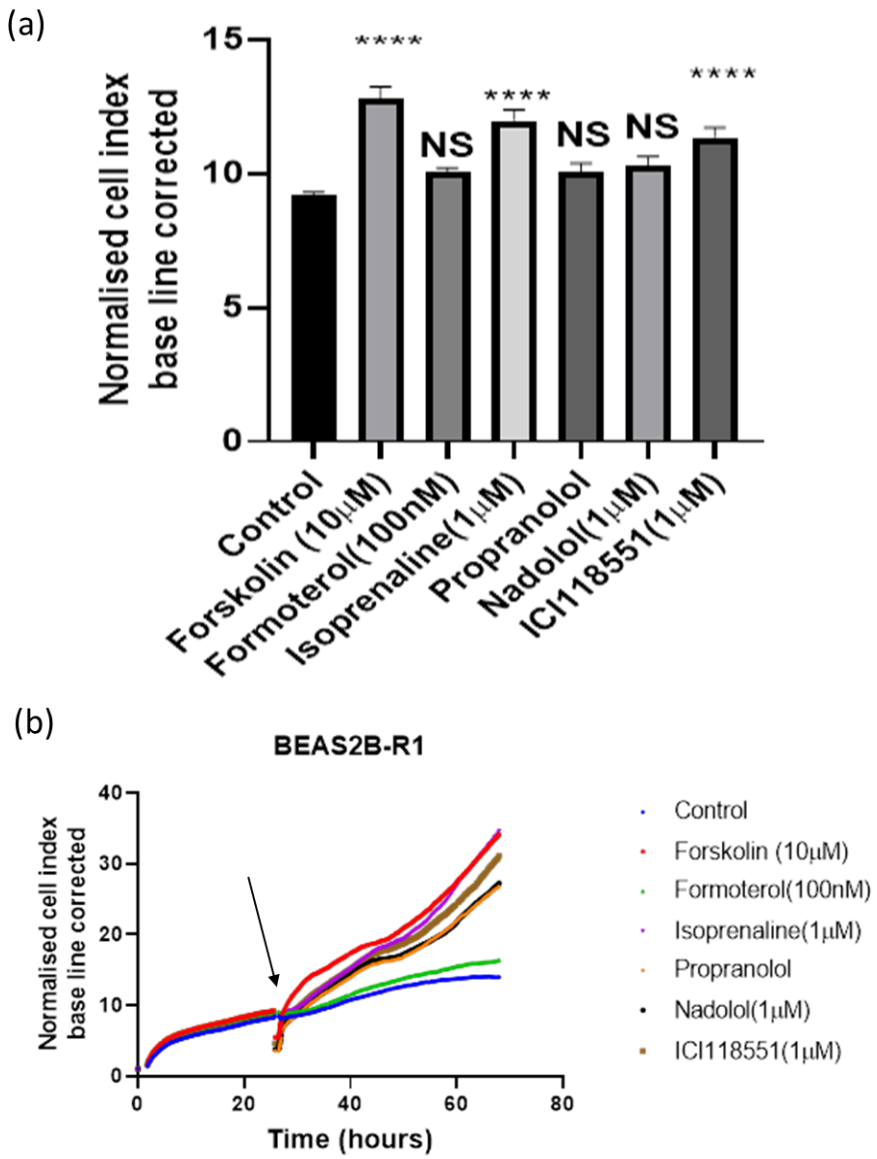


Figure 3.4 Impedance measurements in BEAS2B-R1 cells (a.b).

*The cells were seeded at 5000 cells/well and treated with forskolin (10 μM); inverse agonists propranolol (1 μM), ICI118551 (1 μM) and nadolol (1 μM); agonists isoprenaline (1 μM) and formoterol (100 nM) in serum free media. Impedance tracing showing impedance measurements in BEAS2B-R1 cells. The rapid ascending phase of the impedance measurements was achieved in 26h:26 mins. The black arrows indicate addition of treatments. The downward spike produced after addition of treatment produced a reduction of impedance measurements which reverted to a positive slope media .Cells were stimulated after 26:26: of cell growth from time point 0. forskolin. isoprenaline and IC118551 significantly increased cellular impedance within 20mins of addition of treatments The cell indices in response to treatment were normalized at time 26:27:01, to control. Data are presented as mean ± SEM (****p<0.0001, analysed using Dunnett's post–hoc test following ANOVA.*

3.2.4 The role of ERK1/2 activation in β_2-adrenoceptor induced changes in cellular impedance

Impedance measurements investigated in BEAS2BR-1 cells

BEAS2BR-1 cells were serum starved for 24 hours. This produced a rapidly ascending slope which reached a plateau after 24 hours (Figure 3.5b). The MEK 1 inhibitor PD98059 ,was added to the cells for 30 mins prior to addition of formoterol, propranolol or ICI118551.The impedance response when paused prior to the addition of treatments produced a downward spike which gradually produced an increase in impedance. Thereafter different impedance response profiles were observed for different treatments. The data were normalised to 1 minute prior to addition of treatment and for a duration of 20 minutes after receptor stimulation and recorded for this period. The impedance measurements were recorded every 15 seconds for 20 minutes after addition of treatments. The formoterol, propranolol, ICI118551 and PD98059 treated cells significantly decreased cellular impedance relative to control ,$p \leq 0.0001$(figure 3.5a). The propranolol+PD98059 treated cells significantly increased cellular impedance relative to PD98059,$p \leq 0.001$ relative to PD98059. However,formoterol+PD98059 and ICI118551+PD98059 did not produce any significant effect on cellular impedance relative to PD98059(figure 3.5a).

The impedance changes were assessed for the 24-48hours and all treatment groups significantly reduced cellular impedance relative to control(figure3.6a).

Furthermore,formoterol+PD98059 and propranolol+PD98059 significantly increased cellular impedance relative to PD98059 but, was significantly reduced in ICI118551+PD98059 relative to PD98059 (figure 3.6a).

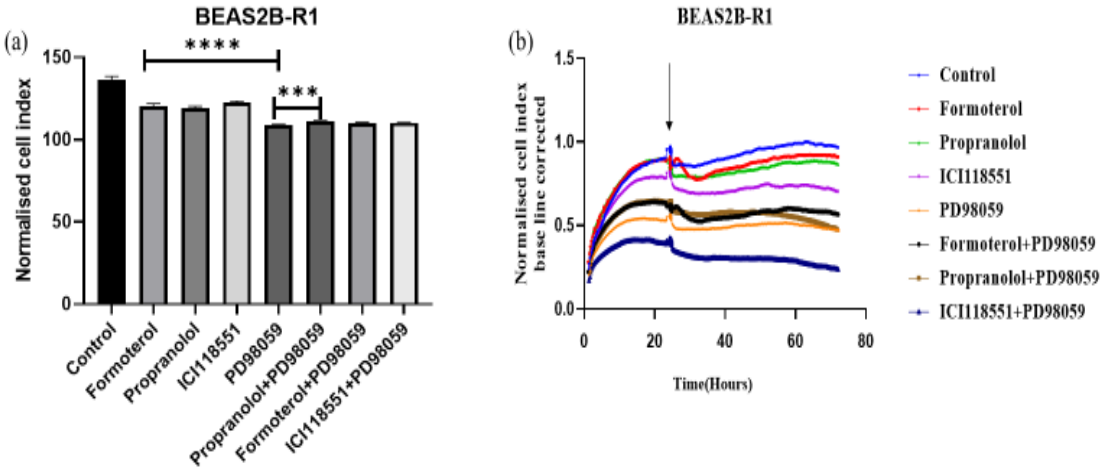


Figure 3.5 Impedance changes in BEAS2B-R1 cells in response to PD98059 a MEK 1 inhibitor, β2-adrenoceptor agonist and inverse agonists.

(a) Impedance measurements obtained in BEAS2B-R1 cells treated with β_2-adrenoceptor agonist formoterol (100 nM) and inverse agonists propranolol (1 µM), ICI118551 (1 µM) and PD98059. The effects of PD98059 after 30 mins pre-treatment prior to addition of formoterol, ICI118551 or propranolol were investigated. The impedance assay was performed as described in section 2.2.7. The data were normalised to 1 minute prior to addition of treatment and for a duration of 20 minutes after receptor stimulation and recorded for this period. The impedance measurements were recorded every 15 seconds for 20 minutes after addition of treatments. The measurements were then recorded every 15 minutes for 72 hours.Formoterol,propranolol,ICI118551 and PD98059 significantly reduced cellular impedance relative to control,$p \leq 0.00001$.The effect of PD98059 on cellular impedance showed significant increase in impedance in propranolol+PD98059 relative to PD98059,$p \leq 0.001$ with no significant effect in formoterol+PD98059 and ICI118551+PD98059.(b) Impedance tracings for BEAS2BR-1 cells showing a rapid ascending phase of impedance measurements for 72 hours. The black arrow indicates the point of addition of treatments. The addition of treatments reduced the impedance measurements which later reversed to a positive slope of the impedance Data are presented as mean ± SEM and represent 3 experiments performed in duplicate ($p<0.05$, $**p<0.01$, analysed using Dunnett's post–hoc test following ANOVA).

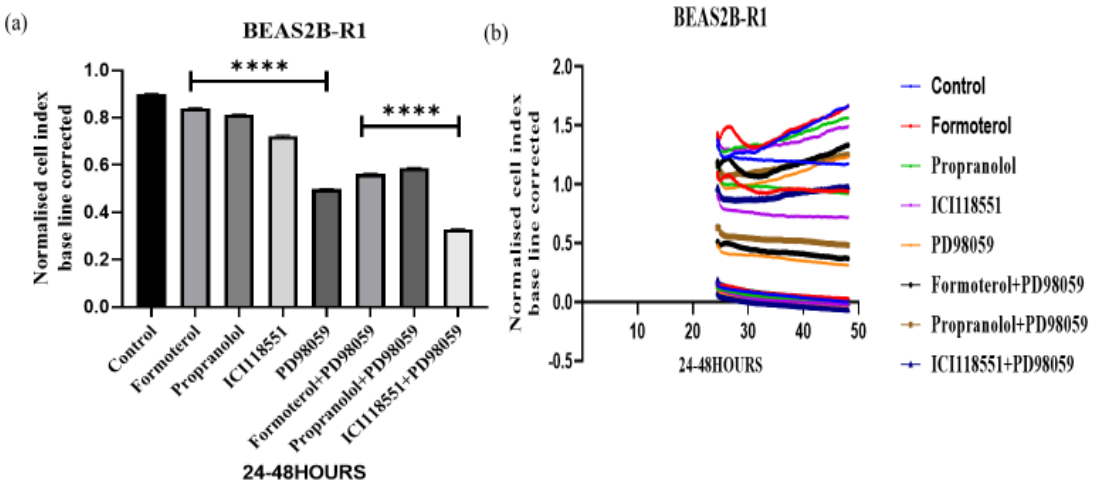


Figure 3.6 Impedance changes in BEAS2B-R1 cells in response to PD98059 a MEK 1 inhibitor, β2-adrenoceptor agonist and inverse agonists.

(a)Impedance tracings for BEAS2BR-1 cells in response to PD98059. The effect of the PD98059 on cellular impedance within 24-48hours of addition of treatment. All treatment groups significantly reduced cellular impedance.Formoterol,propranolol,ICI118551 and PD98059 significantly reduced cellular impedance relative to control,p≤0.0001.The effect of PD98059 on cellular impedance showed significant increase in cellular impedance in formoterol+PD98059,propranolol+PD98059, and a significant decrease in ICI118551+PD98059 ,p≤0.0001.(b)Impedance tracings showing the impedance measurements within 24-48hours.

3.2.4 The impedance measurements in CHO-β_2 cells

As displayed in figure 3.7a and 3.7b, serum starved CHO-β_2 cells induced an increase in cellular impedance for 24 hours indicating cellular proliferation. The impedance measurements changed after the addition of the treatments (β-adrenergic agonist and inverse agonists). The 20-minute monitoring of the impedance/cell index showed an upward spike of the graph as shown in Figure 3.7a. To investigate the effect of MAPK activation in these responses, the MEK1 inhibitor PD98059 was applied to cells for 30 mins prior to addition of the treatments; formoterol, propranolol and ICI118551. The cells treated with formoterol and propranolol displayed significantly increased cellular impedance($p \leq 0.0001$) as shown in the data Figure 3.7a, as normalised cell indices relative to control. The cells treated with ICI118551 and PD98059 did not produce any significant effect in cellular impedance relative to control. However, formoterol+ PD98059,propranolol+PD98059 and ICI118551 +PD98059 significantly increased cellular impedance relative to PD98059 ,$p \leq 0.0001$(figure 3.7a).In the 24-48hours assessment of impedance changes after addition of treatment a significant effect in cellular impedance in formoterol and propranolol ($p \leq 0.0001$) relative to control. Furthermore,ICI118551 significantly increased cellular impedance ($p \leq 0.001$)but ,PD98059 did not significantly decrease cellular impedance relative to control(figure 3.8a). The effect of formoterol+PD98059 and ICI118551+PD98059 significantly increased cellular impedance relative to PD98059 ,$p \leq 0.0001$ and was significantly increased by propranolol+PD98059,$p \leq 0.001$ relative to PD98059 (figure 3.8a).

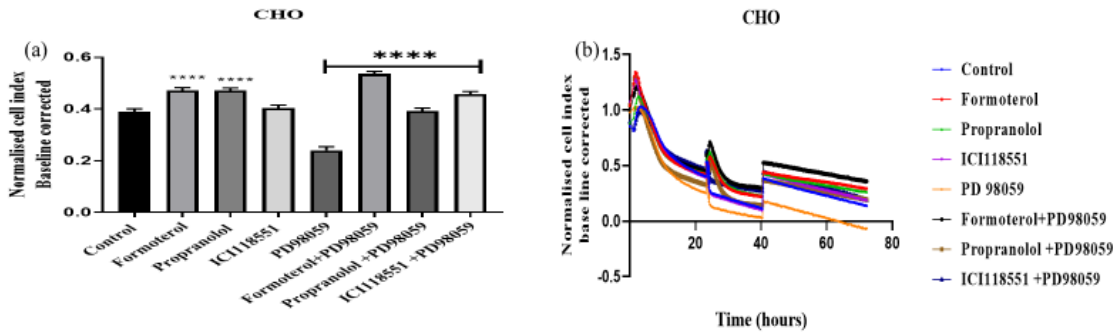


Figure 3.7 Impedance changes in CHO-β2 cells in response to PD98059 a MEK inhibitor, β2-adrenoceptor agonist and inverse agonists.

(a) CHO- β_2 cells were seeded at 50,000 cells/well and treated with β_2-adrenoceptor agonists Formoterol (100 nM) and inverse agonists Propranolol (1 µM) and ICI118551 (1 µM) in the absence and presence of PD98059. The effects of PD after 30 mins pre-treatment on the cells prior to addition of formoterol, ICI118551 and propranolol The impedance assay was investigated as described in section 2.2.7. The data were normalised to 1 minute prior to addition of treatment and for a duration of 20 minutes after receptor stimulation and recorded for this period. The impedance measurements were recorded every 15 seconds for 20 minutes after addition of treatments. The measurements were then recorded every 15 minutes for 72 hours. Formoterol ,propranolol significantly increased cellular impedance,$p \leq 0.0001$ relative to control while,ICI118551 and PD98059 did not produce any significant effect relative to PD98059 . Data are presented as mean ± SEM and represent 3 experiments performed in duplicate. $p<0.05$, $**p<0.01$, analysed using Dunnett's post–hoc test following ANOVA.(b)Impedance tracings in CHO-β_2 showing rapid ascending phase of impedance measurements for 72 hours. The black arrow indicates the point of addition of treatments. The addition of treatments reduced the impedance measurements which later reversed to a positive slope of the impedance.

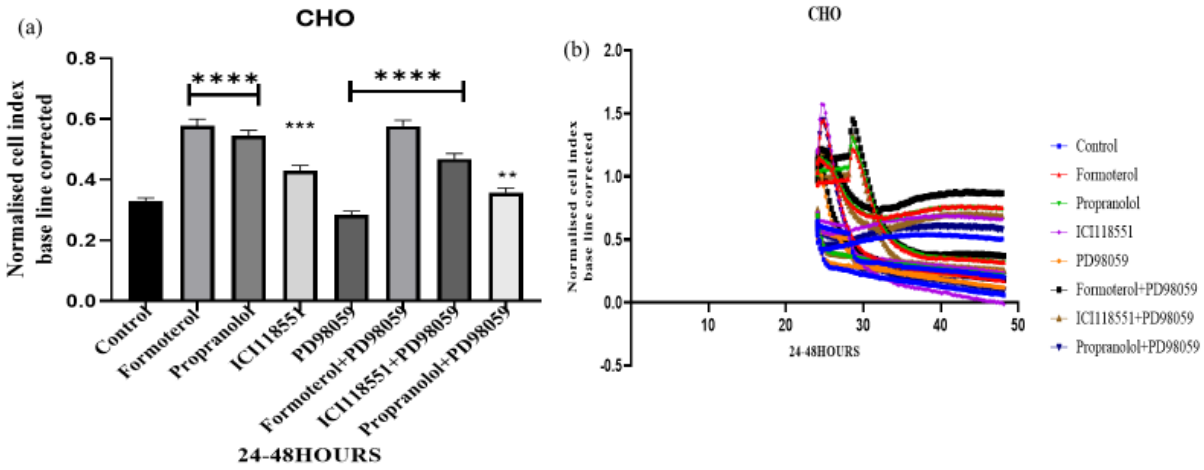


Figure 3.8 Impedance changes in CHO-β2 cells in response to PD98059 (24-48hours).

(a). *The data was normalised to 24 -48hours after addition of treatments. The cellular impedance was significantly increased in formoterol, propranolol p≤0.0001 and ICI118551 p≤0.001,with no significant effect on PD98059 relative to control. The cellular impedance was increased in formoterol+PD98059,ICI118551+PD98059,p≤0.0001and propranolol+PD98059.p≤0.01 relative to PD98059. (b)Impedance tracings for* CHO-β2 *cells measurements for 24-48 hours*

3.2.5 The impedance measurements in Calu-3 cells

Calu-3 cells seeded at 250000 cells per well (Figure 3.9) which produced increase in cellular impedance for 24hours indicating cellular proliferation. The impedance measurements changed after the addition of the treatments; β-adrenergic agonist and inverse agonists and PD98059 MEK 1 inhibitor. The treatments produced cellular responses which were measured as a transient changes in cellular impedance (Fig3.9a). The treatments were monitored for 20mins after an interval of 15 secs. The results were normalised to 1 minute prior to stimulation (Fig 3.9b). The impedance changes were investigated to determine if the measurements could provide distinct representation of more than one signalling event, using the PD98059, MEK 1 inhibitor. The impedance measurement (Fig 3.9b) displayed significant increase in normalized cell indices with formoterol and propranolol,$p \leq 0.0001$ relative to control .However ,the cellular impedance was decreased with the MEK 1 inhibitor PD98059,$p \leq 0.0001$ relative to control. The formoterol+PD98059 and propranolol+PD98059 significantly increased cellular impedance ,$p \leq 0.0001$ but was not significantly decreased with ICI118551+PD98059 relative to PD98059.

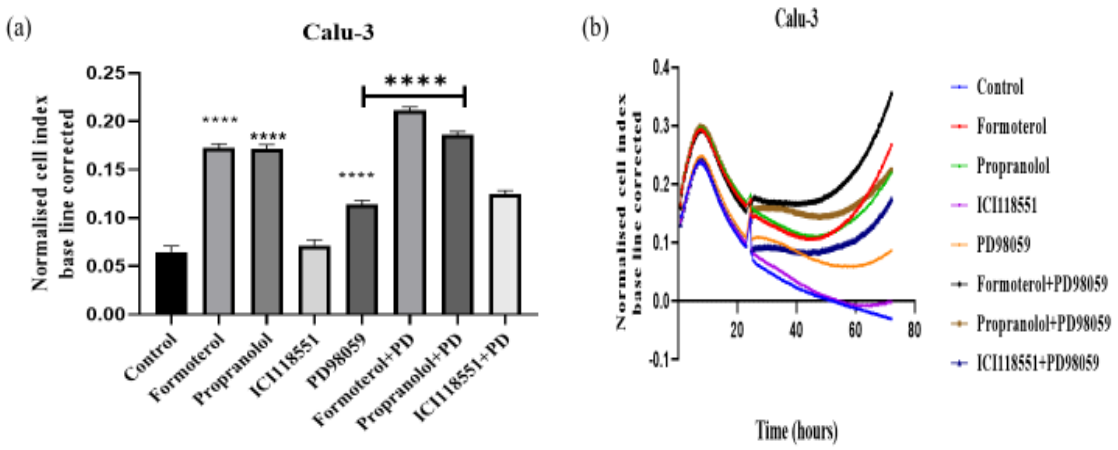


Figure 3.9 Impedance changes in Calu-3 cells in response to PD98059 a MEK1 inhibitor, β2-adrenoceptor agonist and inverse agonists.

(a)The figure shows impedance changes in Calu-3 cells in response to treatments with ligands showing the rapid ascending phase of the racings which reached a maximum response which reached a plateau and slow decaying phase. The black arrow is the point of addition of treatments. Impedance measurements obtained in Calu-3 cells seeded at 250,000 cells/well and treated with β_2-adrenoceptor agonists formoterol (100 nM) and inverse agonists propranolol (1 µM), ICI118551 (1 µM) and PD98059. The effects of formoterol, ICI118551 and propranolol following 30 min pre-treatment with PD98059 are shown. The impedance assay was investigated as described in section 2.2.7. data were normalised to 1 minute prior to addition of treatment and for a duration of 20 minutes after receptor stimulation and recorded for this period. The impedance measurements were recorded every 15 seconds for 20 minutes after addition of treatments. The measurements were then recorded every 15 minutes for 72 hours. The measurements obtained within 20 minutes of addition of treatments displayed significant increase in cellular impedance in formoterol ,propranolol and PD98059,$p \leq 0.0001$ but did produce any significant increase in ICI118551 relative to control.Formoterol+PD98059 and propranolol+PD98059,$p \leq 0.0001$ with no significant increase in impedance in ICI118551+PD98059 relative to PD98059.(b) Impedance tracings for Calu-3 measurements in 72hours. Data are presented as mean ± SEM and represent 3 experiments performed in duplicate ($p<0.05$, $**p<0.01$, analysed using Dunnett's post–hoc test following ANOVA.

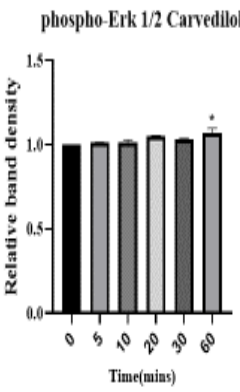
3.2.5 The effects of β-adrenoceptor stimulation on ERK ½ phosphorylation in Human airway cells

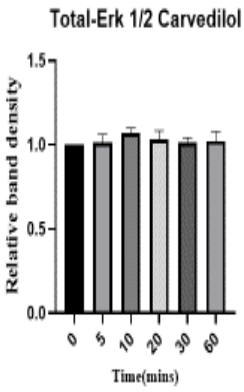
In the initial part of the study the effects of short-term exposure of β-adrenoceptor agonists and inverse agonists were investigated in CHO-β_2 for up to 60minutes(figure 3.10).The phosphorylation of phospho-ERK by carvedilol was significantly increased ,($p \leq 0.05$) 60minutes after stimulation .The phosphorylation of ERK and normalised ERK did not produce any significant effect in carvedilol as shown in (figure 3.10A) relative to control

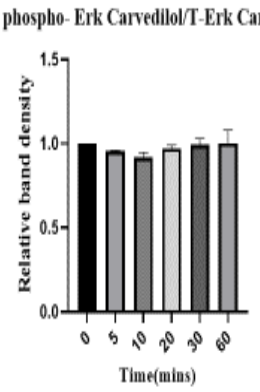
The phosphorylation of phospho-ERK by formoterol was significantly increased 60 minutes after stimulation,$p \leq 0.05$.However,the phosphorylation of total ERK and normalised ERK did not produce any significant effect with formoterol(figure 3.10B). Furthermore, the phosphorylation of PMA was significantly reduced after 10($p \leq 0.05$),20($p \leq 0.01$)and 30 ($p \leq 0.001$)minutes after stimulation(3.10C) relative to control. There was no significant effect on total-ERK phosphorylation or normalised ERK phosphorylation relative to control

Propranolol did not significantly affect phosphorylation of phospho-ERK, total-ERK or normalised phospho-ERK/total ERK(figure 3.10D) relative.

(A)


phospho-Erk 1/2 Carvedilol
Relative band density
1.5
1.0
0.5
0.0
*
0 5 10 20 30 60
Time(mins)


Total-Erk 1/2 Carvedilol
Relative band density
1.5
1.0
0.5
0.0
0 5 10 20 30 60
Time(mins)


phospho- Erk Carvedilol/T-Erk Carvedilol
Relative band density
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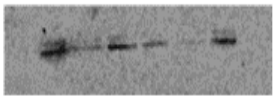
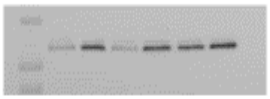
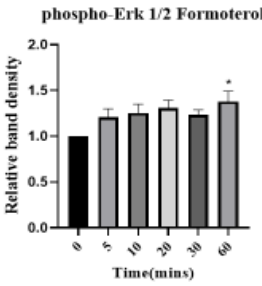
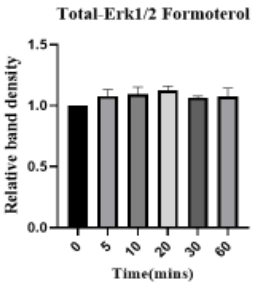
1.0
0.5
0.0
0 5 10 20 30 60
Time(mins)





(B)


phospho-Erk 1/2 Formoterol
Relative band density
2.0
1.5
1.0
0.5
0.0
*
0 5 10 20 30 60
Time(mins)


Total-Erk1/2 Formoterol
Relative band density
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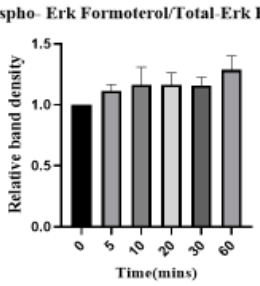
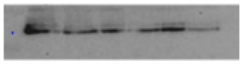
1.0
0.5
0.0
0 5 10 20 30 60
Time(mins)


phospho- Erk Formoterol/Total-Erk Formoterol
Relative band density
1.5
1.0
0.5
0.0
0 5 10 20 30 60
Time(mins)



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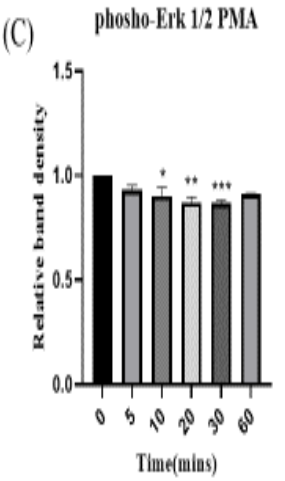
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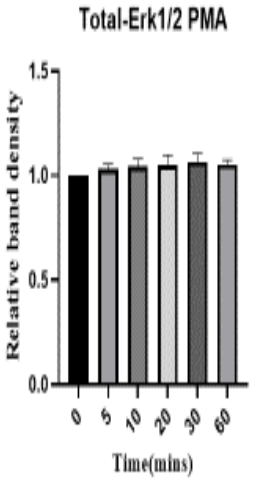
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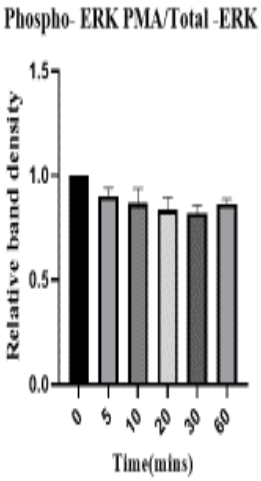
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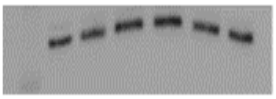

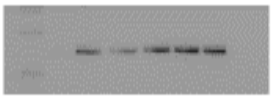

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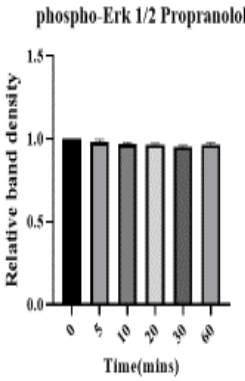
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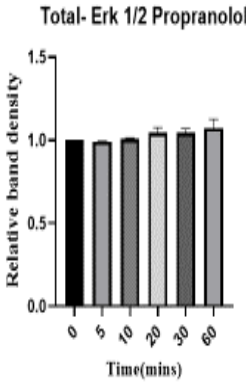
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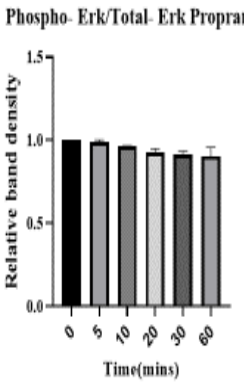
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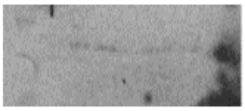


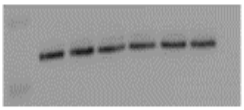


Figure 3.10 ERK 1/2 Phosphorylation in CHO-β2 cells.

Where indicated CHO-β_2 cells were treated with propranolol(1μM), formoterol(100nM), carvedilol(1μM) and phorbol 12 myristate 13 acetate (PMA) (1μM) for a period of 5,10,20,30 and 60mins.Controls were at 0 min. PMA served as a positive control for ERK phosphorylation. Following treatment cells were lysed with RIPA buffer and protease inhibitor for 30mins cell lysates were subjected to SDS page and western blot analysis as mentioned in section 2.2.7 in methods. The blots were probed for ERK and phospho ERK as shown.. The densitometry of 3 blots from each independent experiment was performed using AIDA. Data are presented as mean ± SEM and represent 3 experiments performed in triplicates ($p<0.05$, $**p<0.01$, analysed using Dunnett's post–hoc test following ANOVA)

The phosphorylation of ERK 1/2 by stimulation of the β_2-adrenoceptor did not produce any significant change with reference to the control in Calu-3 cells as shown (figure 3.11).Furthermore ,PD98059+Propranolol did not significantly affect the phosphorylation of phosphor ERK ,total ERK and normalised phosphor/total ERK relative to PD98059(figure 3.11).

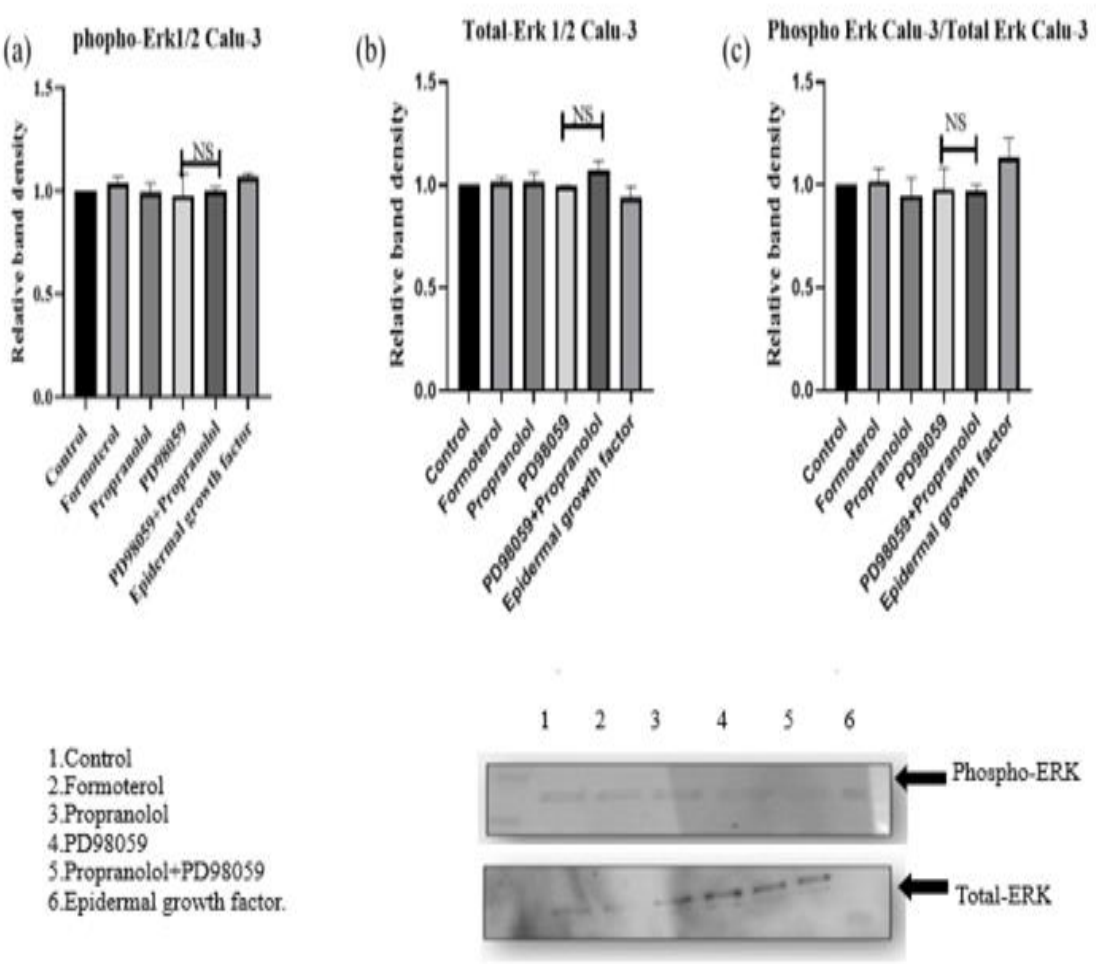


Figure 3.11 ERK1/2 Phosphorylation by Calu-3 cells.

*Where indicated Calu-3 cells were treated with formoterol(100nM),propranolol(1μM), PD98059(10μM) and EGF (5ng/ml) for 30mins. The Propranolol+PD98059 were pre-treated with PD98059 prior to addition of propranolol. Following treatment cells were lysed with RIPA buffer and protease inhibitor for 30mins cell lysates were subjected to SDS page and western blot analysis as mentioned in section 2.2.7 in methods. The blots were probed for ERK and phosphor- ERK and viewed using chemiluminescence as shown. The densitometry from each independent experiment was performed using AIDA software. Data are presented as mean ± SEM and represent 2 experiments (p<0.05, **p<0.01, analysed using Dunnett's post–hoc test following ANOVA).*

3.3 Discussion

In this chapter the production of cyclic AMP through stimulation of the β_2-adrenoceptor was investigated in human airway epithelial cell lines, Calu-3 cells and BEAS2R-1 cells. Western blotting analysis showed the cell signalling pathway involved in the activation of the β_2-adrenoceptor. Real-time analysis of cellular impedance in relation to β_2-adrenoceptor stimulation using the xCELLigence RTCA system in Calu-3, BEAS2R-1 and CHO-β cells was investigated, which provided an integrative assessment of β_2-adrenerceptor stimulation. The analysis of β_2-adrenerceptor stimulation by both agonists and inverse agonists revealed the contribution of both G-protein and non-G protein dependent coupling ,signalling and cyclic AMP production and ERK 1/2 activation. The impedance reduction responses by formoterol ,propranolol,ICI118551 and PD98059 relative to control and increase in responses in propranolol+PD98059 relative to PD98059 shows the involvement of both G-protein and non-Gprotein signalling within 20 minutes of stimulation(figure 3.5). Furthermore, the analysis of this response within 24-48hours showed increase in responses with formoterol+PD98059 and propranolol+PD98059 and reduction of responses with ICI118551+PD98059(figure 3.6).

The observation that some parts of the impedance response that were decreased following treatment with ICI118551+PD98059 relative to PD98059(figure 3.6a)but, responses were increased in formoterol+PD98059and propranolol+PD98059 (figure 3.6) which could not be reiterated by short term stimulation of cyclic AMP or ERK1/2 pathways suggests the contribution of both G-protein and non G-protein dependent signalling. However, the possibility that long-term inhibition of G-proteins leads to cellular changes that can indirectly affect cellular impedance. Our investigation also showed that both cyclic AMP and ERK converge on a common downstream effector which mediates their contribution to

impedance response. The results obtained displayed production of cyclic AMP by β_2-adrenerceptor agonists in Calu-3 cells.

3.3.1 β_2-adrenoceptor activation and real-time analysis

To investigate the activation and/or inhibition of G-protein coupled receptors, cell-based assays are the assays of choice in studying GPCRs, such as cyclic AMP assays and cyclic AMP ELISA assays. The conventional methods such as cyclic AMP assay used in assessing GPCR activation in cell-based assays often involve the use of invasive methods which are laborious to perform. The xCELLigence RTCA system makes continuous monitoring cellular changes in cells possible.

The β_2-adrenoceptor is present in normal lung epithelial and smooth muscle cells (Carstairs et al.,1985) and previous studies have reported the expression of the β_2-adrenoceptor in human airway epithelial, Calu-3 cells (Abraham et al,2004) and BEAS2BR-1 cells (Amstad *et al.,* 1988, Kelsen *et al.,* 1997). The epithelial cells are involved in the pathophysiology and treatment of airway diseases such as asthma because, they release pro and anti-inflammatory mediators (Nijkamp *et al.,* 1982). Therefore, because of these, the β_2-adrenoceptors represent an important target of β_2-adrenergic drugs (Salathe *et al.,* 2002). The constitutive activity of the β_2-adrenoceptor which is sensitive to inhibition by β_2-adrenoceptor inverse agonists has been characterised in constitutively active mutant receptors (Hopkinson *et al.,* 2000) and receptors of β_2-adrenoceptor (Hoffmann *et al.,* 2015).

The cyclic AMP accumulation assays measure cellular levels of cyclic AMP which depends on activity of adenylyl cyclase system, which is regulated by the GPCRs that are coupled to Gs or Ga $_{i/o}$ proteins. The production of cyclic AMP occurs via the stimulation of Gs protein. The results obtained from the experiments were from cells that expressed the receptor under investigation, the β_2-adrenoceptor. The monitoring of the assay in real-time allows the observation of the entire record of events during the assay period. The monitoring of the

entire observations is not possible in a targeted study such as the cyclic AMP assay or cyclic AMP ELISA assay.

Furthermore, in this study, while investigating the coupling of the β_2-adrenoceptor through the cyclic AMP measurement, the β_2-adrenoceptors in Calu-3 and BEAS2BR-1 were functional. Stimulation of the β_2-adrenoceptors in Calu-3 with β_2-adrenoceptor agonists, isoprenaline and formoterol produced cyclic AMP(figure 3.1).The effect of isoprenaline on cyclic AMP accumulation is comparable with previous findings which reported increase cyclic AMP accumulation by isoprenaline in in HEK-293 cells (Wisler *et al.*,2007).

The xCELLigence impedance assay was used to investigate impedance changes in human airways because, it has an advantage of being non-invasive` which allows the continues monitoring of cells.(Xi *et al.*,2008).

The impedance responses were generated after stimulation of the β_2- adrenoceptor in response to inhibition by the MEK 1 inhibitor PD98059 in Calu-3 cells, displayed significant responses in formoterol+PD98059,Propranolol+PD98059 which increased the impedance responses in Calu-3 cell within 20minutes stimulation (figure 3.9a).Also, the CHO-β_2 cells showed (figure 3.7) the impedance responses in PD98059 treated cells were significantly increased 20 minutes after in formoterol+PD,propranolol+PD98059 and ICI118551+PD relative to PD98059 and 24-48hours after stimulation (figure 3.8).The effect of PD98059 on impedance investigated in BEAS2BR-1 cells displayed a significant increase in impedance with propranolol +PD98059 relative to PD98059(figure 3.5).The 24-48hours analysis of the impedance response in BEAS2BR-1 cells after treatment significantly increased impedance in formoterol+PD98059,propranolol+PD98059 and decreased in ICI118551+PD98059 relative to PD98059(figure 3.6) . The data obtained in the study displayed the effect of PD98059 (Figure 3.6) on cellular impedance and the contribution of both cyclic AMP and ERK1/2 pathway activation. The results suggest that the PD98059 partially blocked the ERK activation and the remaining responses may be due to cyclic AMP

activation. It also showed that the inhibition of G_s signalling produces changes in cellular responses. Previously PD98059 was investigated for its role in inhibition of ERK 1/2 phosphorylation in 16HBE14o- human airway epithelial cells. (Steven *et al.*, 2005).Furthermore,PD98059 was also shown to reduce the expression of nitric oxide production by ERK pathway in human airway epithelial cells A549 (Baek *et al.*,2009).

The significant increase with forskolin in BEAS2B-R1 cells suggest stimulation of the of adenylyl cyclase (Figure 3.4). Furthermore, the isoprenaline significantly increased cellular impedance and cell index suggesting stimulation of the cyclic AMP pathway(figure 3.4). The effect of isoprenaline on cellular impedance was previously observed in HEK293S, which was reduced in the presence of both an adenylyl cyclase inhibitor and a MEK 1 inhibitor using the cellular impedance assay (Stallaert *et al.*,2012).The findings in this study are consistent with what was previously observed.

The activation of GPCR mediated cell signalling triggered distinct cellular responses in 3 cell lines which were monitored by the RTCA system. The data obtained from these cell lines, which express the GPCR ,shows the sensitivity of the impedance cell-based assay. The assay is non-invasive and multiple experiments can be performed on the same cell line in real-time. The RTCA system also monitors cellular attachment and cell morphology. This makes the RTCA system a valuable tool for optimizing ligands and time of stimulation of receptors.

3.3.2 The role of β_2 -adrenoceptor stimulation in phosphorylation of ERK1/2 .

Carvedilol was previously demonstrated to bind to a β-adrenoceptor and cause phosphorylation of ERK 1/2 (Drake *et al.*,2008) .Carvedilol also displayed biased agonism by inhibiting G-protein activation and enhancing β-arrestin dependent signalling(Kim *et al.*,2008).The current study explored the effect of β_2 -adrenoceptor stimulation in phosphorylation of ERK1/2 in CHO-β_2 cell. The findings confirmed that carvedilol stimulates the phosphorylation of ERK1/2(figure 3.10A). The findings are comparable with

previous studies which reported the activation of ERK 1/2 (Kim *et al.*,2008).Furthermore ,formoterol stimulated the phosphorylation of ERK 1/2 (figure 3.10B). Despite being an agonist ,formoterol was previously shown to phosphorylate ERK 1/2 in MDA-MB-453 breast cancer cells(Plummer *et al.*,2004). However, PMA significantly decreased ERK1/2 phosphorylation in CHO-β_2 cells within 10,20 and 30 minutes of stimulation(figure 3.10C).This is in contrast to what was previously obtained where PMA induced ERK 1/2 phosphorylation in human pulmonary epithelial cells ,A549(Chang *et al.*,2004).

3.4. Conclusion

The data presented in this chapter indicate isoprenaline and formoterol,β_2-adrenoceptor agonist stimulated the cyclic AMP in Calu-3 cells and increased cellular impedance in BEAS2BR-1cells. Furthermore, the findings displayed similar effects produced by formoterol and propranolol where cellular impedance was decreased in BEAS2BR-1 and increased in Calu-3 cells and CHO-β_2 cells. The observation that the MEK 1 inhibitor, PD98059 did not decrease cellular impedance in both formoterol and propranolol suggest cross talk between cyclic AMP and ERK1/2pathways.The stimulation of ERK 1/2 phosphorylation by formoterol in CHO-β_2 cells suggests modulation of the β_2-adrenoceptor occurs via ERK 1/2 pathway and cyclic AMP pathways.

Chapter 4

The functional effects of the β_2-adrenoceptor stimulation on airway cell biology in human airway epithelial cells

4.1 Introduction

The β_2-adrenoceptor has been studied in detail and its crystal structure has been characterized (Rasmussen *et al.,* 2007). The β_2-adrenoceptors were found in large density in the respiratory airways. Previously, it was shown that the airway epithelium plays an important role in inflammatory airway diseases such as asthma (Polito and Proud 1998). For example, the respiratory epithelium is a target of injury by noxious agents and infective agents. The respiratory epithelium is also the origin of inflammatory mediators (Forrest *et al.,* 2005).Furthermore the functions and inflammatory responses of the airway epithelium are regulated by the β_2-adrenoceptor . Because of these, epithelial damage in airway diseases and altered β_2-adrenoceptor signalling produce airway hyperresponsiveness (Nijkamp *et al.,* 1992). The respiratory epithelium in asthmatic patients, regardless of the severity, is susceptible to damage. This is not common in other respiratory diseases such as chronic bronchitis and chronic obstructive airway disease (Semlali *et al.,* 2007). The respiratory epithelium of asthmatic patients was reported to be susceptible to injury associated with intrinsic functional abnormalities and defective mechanisms of repair (Holgate *et al.,* 2004). It was also reported that, the alteration of the structural lining of the airways was strongly associated with chronic inflammatory airway diseases such as bronchial asthma and chronic obstructive airway disease (Qi *et al.,* 2019). The remodelling of the airway results from abnormal thickening of the bronchial wall, thickening of the epithelial membrane and hypertrophy of the mucous glands (Girodet *et al.,* 2015). This was previously considered to occur in long term disease which increased with severity and duration of the disease and, was recently discovered to occur independent of inflammation and in short term disease (Grainge *et al.,* 2018).

The airway epithelial cells also produce growth factors that are responsible for the pathogenesis of changes in morphology thickening of the airway (Zhang *et al.,* 1999).Such

as the development of airway hyperactivity which causes airflow obstruction in asthma (Bosquet *et al.,* 2000).

Human airway epithelial cells have been used extensively as in vitro models to study cell proliferation, cytokine release from inflammatory cells and MU5AC mucins in asthma. This is because asthma is associated with abnormalities in mucus production and MU5AC is one of the integral components of mucins (Bonser and Earl 2017). Furthermore, the ciliated epithelial lining of the airway is coated by the mucous gel that traps harmful particles and toxins (Jeffrey and Li 1997).But ineffective clearance of mucus is a cause of airway obstruction in asthma (Evans et al.,2009). The β_2-adrenoceptor agonists are commonly used drugs in the management of bronchoconstriction in asthma and chronic obstructive diseases (Hizawa 2011). They affect airway epithelial cells by upregulating cell growth and proliferation, which occurs most likely due to regular use of these drugs and accumulated increase in the administered drug concentration (Nishimura *et al.,* 2002). Furthermore, β_2-adrenoceptor agonists stimulate production of cyclic AMP and together with other cyclic AMP elevating agents inhibit airway smooth muscle proliferation (Kassel *et al.,* 2007). The findings suggest that modulation of cell proliferation in human airways and are regulated by cell signalling pathways related to the G protein-coupled receptor pathway. It was also reported that the epidermal growth factor (EGF), which is upregulated in asthma, regulates cell proliferation and repair (Puddicombe *et al.,* 2000) through the mitogen-activated protein kinase pathway (Holgate *et al.,* 2004).

The abnormal proliferation of airway epithelial cells was identified as a major cause of airway narrowing that results in the worsening of airway obstruction. In a study conducted in human airway epithelial cells (16-Bronchial epithelial cells and NCI-H292), salbutamol, forskolin and 8-bromoadenosine monophosphate induced cell proliferation, which was inhibited by propranolol. Salbutamol induced phosphorylated mitogen activated protein kinase (MAPK) expression that was inhibited by propranolol, MAP kinase kinase (MEK)

inhibitor PD98059 and RpcAMPS (Nishimura *et al.,* 2002). This suggests that stimulation of cell proliferation and cyclic AMP-increased cell growth occurs through MAPK phosphorylation.

The mitogen activated protein kinase (MAP kinase) has been identified as an important regulator of growth and proliferation of many different cell types. MAP kinase has also been discovered to be activated by a variety of ligands, growth factors and stimulators of G protein coupled receptors (Crespo *et al.*, 1994). This suggests an important role for MAP kinase in regulation of growth and proliferation of cells.

Airway epithelial cells have been reported to be involved in the regulation of inflammation by stimulating the production of inflammatory cytokines. Some of these cytokines include the interleukins IL-5, IL-6 and IL-8 and that have increased levels produced in asthmatic airways. It was also reported that the β_2-adrenergic receptors in the bronchial epithelium mediate the anti-inflammatory effects of β_2-adrenergic agonists (Korn *et al.,* 2001). To investigate the effects of long acting β adrenergic agonists on cytokine production in the human bronchial epithelial cell line A549 (stimulated with tumour necrosis factor alpha), salmeterol and formoterol effects were compared. Both agonists reduced cytokine production, with formoterol being the more potent of the agonists (Chiu *et al.,* 2006).

The effects on the production of inflammatory cytokines in asthma was studied in murine models of asthma. Inverse agonists ICI118551 and nadolol chronically administered for 28 days and reduced the total cell counts in antigen challenged mice. This suggests that the beneficial actions of inverse agonists in asthma are more effective following chronic rather than acute administration (Nguyen *et al.,* 2007).

The administration of β_2-agonists for a long duration worsens the symptoms of asthma, because of continuous activation of the β_2-adrenoceptors. This has also been associated with a reduced ability of β_2-agonists to decrease cytokine release from inflammatory cells and

airway epithelial cells. It was discovered to be related to increase in concentration of cytokines from inflammatory and air way epithelial cells and finally to increase hyperresponsiveness (Oehme *et al.,* 2015). Previously, the effect of β_2-agonist isoprenaline and salbutamol induced IL-6 and IL-8 release from human bronchial epithelial 16HBE14 cells, in a dose dependent manner that was inhibited by the β_2-adrenoceptor antagonist ICI118551 and partially inhibited by propranolol (Oehme *et al.,* 2015). This suggest an important role for β_2-adrenoceptors in regulating cytokine release.

The respiratory epithelium is lined by mucus secreting goblet cells, which confer protection to the respiratory tract. The goblet cells produce mucins MUC5B and MUC5AC, which coat and maintain the integrity of the respiratory system (Evan *et al.,* 2004). For example, in an infection of the airway, goblet cells undergo metaplasia that increases the number of goblet cells as well as mucin secretion. This results in obstruction and narrowing of the airway (Yu *et al.,* 2006). A similar process also occurs following exposure to allergens and bacterial toxins (Ueno-lio et al., 2014). A substantial amount of evidence has been obtained in murine models of asthma for the effects of chronic administration of β_2-adrenoceptor inverse agonists on mucin production. The administration of ICI118551 or Nadolol for 7 days had little effect on mucin production. When the drugs were administered for 28 days, however, mucin production was greatly reduced. This suggests that chronic administration of β_2-adrenoceptor inverse agonists is beneficial in reducing mucin production and mucous hyperplasia in asthma models (Ngyuyen *et al.,* 2008).

The investigations reported in this chapter focussed on the functional effects of β_2-adrenoceptor stimulation on airway cell biology. Such functional effects include cell proliferation/cell viability, cytokine release, and mucin production. The effects of a range of β_2-adrenoceptor agonists and inverse agonists were investigated using an MTT reduction assay, MTT real-time glo assay, cell counting, cytokine release (measured by ELISA) and immunofluorescence visualisation of Mucins. In addition, to investigate the effects on

airway cell biology in real-time, the IncuCyte S3 system was used. Furthermore , the possible effects of stimulators and inhibitors of cell proliferation/viability and inhibitors of the MAPK pathway by MEK inhibitor PD98059, on airway cell biology were investigated.

The cell lines Calu-3 cells, BEAS2BR-1 and CHO-β_2 were studied to investigate the functional effects of β_2-adrenoceptor stimulation in airway cell biology.

4.1.2 Chapter aims

The aims of this chapter were:

- To investigate the functional effects of β_2-adrenoceptor agonists and inverse agonists on airway cell biology
- To investigate the functional effect of epidermal growth factor and 5-flourouracil on cell proliferation.
- To investigate the functional effect of inhibitors of mitogen activated protein kinase(MEK) on cell proliferation.

4.2 Results

4.2.1 β_2-adrenoceptor agonist and inverse agonist effects on cell viability.

In human airway epithelial cells we sought to investigate the functional effects of β_2-adrenoceptor agonists and inverse agonists**,** within 72 hours. In the first part of the experiment Calu-3 and BEAS2B-R1 cells were treated with β_2-adrenoceptor agonist and inverse agonist both in serum supplemented and serum-free medium. Following stimulation of cells for 72 hours, they were assessed for MTT reduction over this time-course. The results of the MTT assay showed a significant increase in MTT reduction in BEAS2BR-1 cells in serum supplemented medium containing media, in response to the β_2-adrenoceptor agonist formoterol ($p<0.01$), β_2-adrenoceptor inverse agonists propranolol ($p<0.05$) and nadolol ($p<0.05$) (Figure 4.1a).The inverse agonist ICI118551, agonist isoprenaline and adenylyl cyclase activator forskolin and 0.1%DMSO (vehicle control) did not significantly increase MTT reduction (Figure 4.1a).

In serum-free medium (Figure4.1b), forskolin showed a significant decrease in MTT reduction ($p<0.05$) (Figure 4.1b) in BEAS2B-R1 cells. However, cells treated with ICI118551, nadolol, propranolol, isoprenaline, formoterol and 0.1% DMSO (vehicle control) did not significantly decrease MTT reduction.

In Calu-3 cells (Figure 4.1c), there was a significant decrease in MTT reduction with IBMX($P<0.05$) in serum supplemented medium with no significant effect on MTT reduction in1%DMSO,forskolin,isoprenaline,formoterol,propranolol,nadolol,ICI118551,formoterol+ IBMX and forskolin +IBMX . However, in serum free medium, (Figure4. 1d) Calu-3 cells showed a significant increase in MTT reduction in cells treated with propranolol ($p<0.05$),nadolol ($p<0.01$) and significant decrease in MTT reduction IBMX with while, forskolin, isoprenaline, formoterol and ICI118551, 1%DMSO and 0.1%DMSO did not produce any significant effect on MT reduction (Figure 4.1d).Subsequent experiments with DMSO and IBMX were not investigated. The effect of 0.1% DMSO showed clear profile in

the investigations and did not cause any significant reduction in MTT response in any data set which did not cause any noticeable inhibition at all. Therefore, it was considered that treatments associated with this level of DMSO exposure could be used without concerns about DMSO effects on viability.

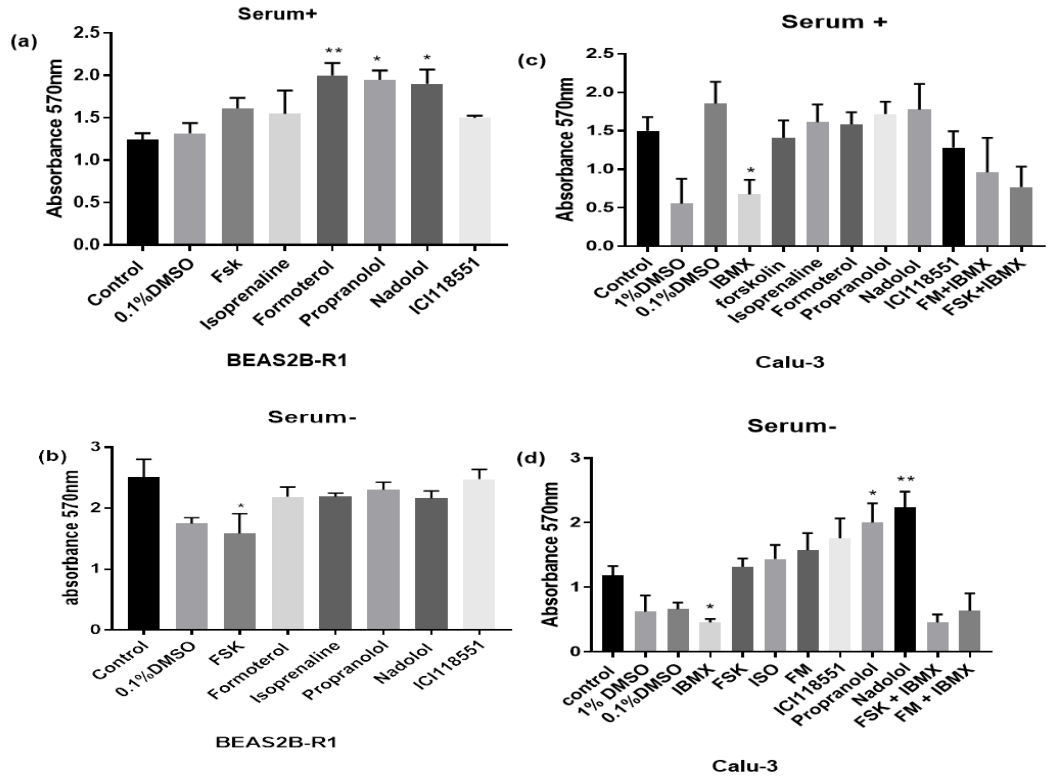


Figure 4.1 The effect of β2adrenoceptor stimulation on MTT reduction in BEAS2B-R1 and Calu-3 cells.

BEAS2B-R1 cells were incubated for 72h in (a) presence and (b) absence of serum supplement medium and treated with: DMSO (0.1 % & 1% vehicle control), forskolin (FSK, 10 μM), isoprenaline (ISO, 1 μM), formoterol (100 nM), ICI118551 (1 μM), propranolol (1 μM) and nadolol (1 μM). (a) Significant increase in MTT reduction with propranolol, nadolol and formoterol and a non-significant in forskolin ,isoprenaline,ICI118551 and 0.1%DMSO.(n=4) (b) Shows a significant decrease in MTT reduction with forskolin $p<0.05$ and a non-significant increase in MTT reduction in isoprenaline,formoterol,nadolol,propranolol,ICI118551 and 0.1%DMSO (n=3). (c) The MTT reduction of in Calu-3 cells was significantly decreased in the presence of IBMX in serum supplemented medium<0.05,with no significant effect in propranolol, nadolol ,isoprenaline,formoterol,ICI118551,formoterol+IBMX,forskolin+IBMX.1%DMSO and o.1%DMSO.,N=9 in control ,N=6 in isoprenaline, formoterol ,N=5 in ICI118551,N=4 in FSK+IBMX and FM+IBMX and N=3 in propranolol and nadolol (d) In the presence serum free media IBMX significantly reduced MTT reduction $p<0.05$ and significant increase in MTT reduction with propranolol $p<0.05$ and nadolol $p<0.01$. There was no significant effect in 0.1%DMSO,1%DMSO,forskolin,isoprenaline,formoterol,forskolin+IBMX,formoterol+IBMX.

*N=12 in control, n=7 in forskolin, isoprenaline, formoterol and ICI118551,n=8 in IBMX=4 in formoterol+IBMX and forskolin +IBMX ,n=5 in propranolol and nadolol=3 in 1%DMSO,n=5,in 0.1%DMSO.Data are represented as mean± SEM, (*p<0.05, **p<0.01, analysed using Dunnett's post –hoc test following ANOVA).*

To validate the results obtained, an MTT Real Time Glo Assay was also used to study the effect of β_2-adrenoceptor agonists and inverse agonists on MTT reduction in Calu-3 and BEAS2BR-1 cells. The real-time assay measures viable cells in real-time which utilizes the luciferase enzyme and a pro-substrate(Duellman et al.,2015). Both the luciferase and pro-substrate were added to treated cells. Viable cells reduce pro-substrate to substrate that is used up by luciferase to generate a luminescence signal . The experiment was conducted as an end-point assay . The results obtained with the MTT real time glo assay indicated no significant effect of MTT reduction with both the β_2-adrenoceptor agonists / inverse agonists in BEAS2BR-1 in the presence of serum supplemented medium (figure 4.2a). In serum-free media BEAS2B-R1 there was also no significant MTT reduction the β_2-adrenoceptor agonists and inverse agonists (figure 4.2b). Furthermore, Calu-3 cells also did not display any significant effect on MTT reduction in both serum supplemented medium and serum free medium (figure4.2c and 4.2d).

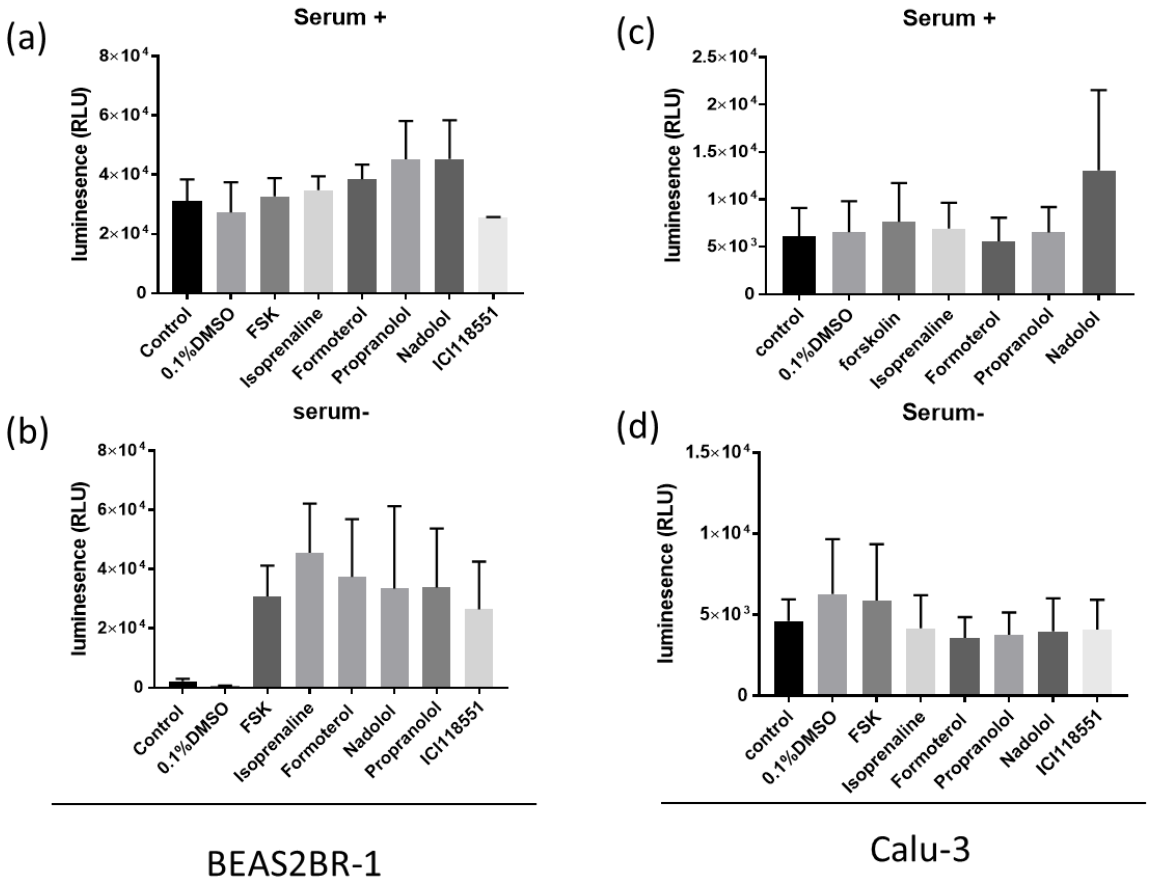


Figure 4.2 The effect of β_2-adrenoceptor stimulation on MTT real time glo assay luminescence in BEAS2B-R1 and Calu-3 cells.

*BEAS2B-R1 and Calu-3 cells were incubated for 72 h in (a) absence and (b) presence of serum supplemented medium with the following treatments: DMSO (0.1 %, vehicle control), forskolin (FSK, 10 μM),isoprenaline (ISO, 1 μM), formoterol (100 nM), ICI118551 (1 μM), propranolol (1 μM) and nadolol (1 μM).Then the pro substrate and luciferase were added according to materials and methods section 2.27. Although no statistically significant increases were observed, the data obtained in the presence of serum supplemented medium (a) showed a trend towards an increase in cell viability in nadolol and propranolol (b)In serum free medium there was no significant increase in cell viability was observed in response to all treatments (c) In serum supplemented media the results showed trends towards increase in cell viability in cells treated with isoprenaline, formoterol and inverse agonists propranolol and nadolol and(d)In serum free media there was an increase in cell viability in the presence of 0.1% DMSO, isoprenaline, forskolin and nadolol. N=3. Data are represented as mean± SEM, (*p<0.05, **p<0.01, analysed using Dunnett's post –hoc test following ANOVA).*

4.2.2 Live-cell imaging analysis of cell proliferation

The next set of experiments were investigated using the IncuCyte cell proliferation assay in Calu-3 cells, CHO-β_2 and BEAS2B-R1.This was investigated as described in section 2.2.3.

Real time assessment of cell proliferation in Calu-3 cells.

Calu-3 cells were seeded at 10,000 cells per well in 96 well plates in serum free medium.

Cells were incubated for 24 hours prior to treatment with β_2-adrenoceptor agonists and inverse agonists. Using the IncuCyte to assess proliferation, forskolin, propranolol, nadolol and salmeterol highly significantly increased cell proliferation in Calu-3 cells (Figure 4.3b). The propranolol effect was greater than that of salmeterol or forskolin. However, formoterol, isoprenaline and carvedilol highly significantly reduced cell proliferation in Calu-3 cells (Figure 4.3b). Furthermore there was no significant effect on proliferation by ICI118551 and alprenolol (Figure 4.3b).

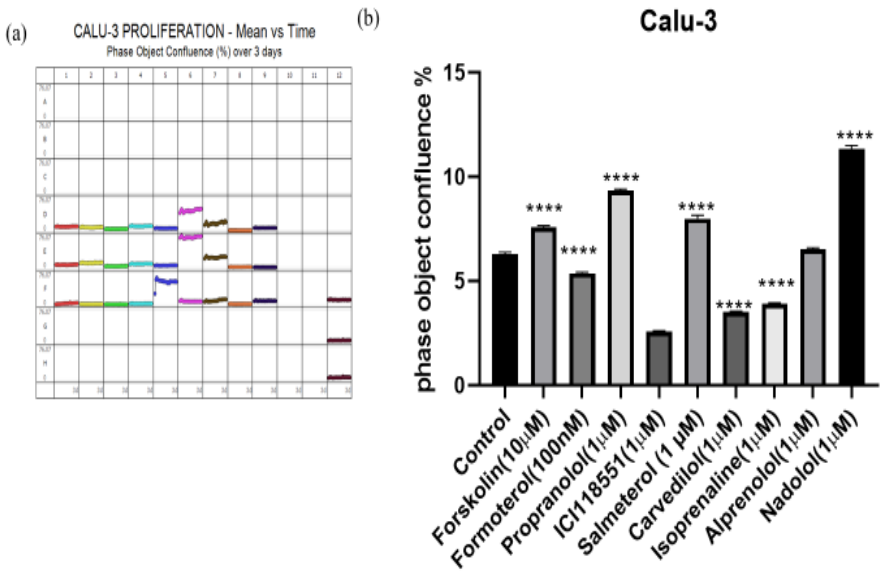


Figure 4.3 Incucyte S3 system assessment of cell proliferation in Calu-3 cells.
*Calu-3 cells were seeded at 10000 cells/well and incubated for 24 hours in serum-free media prior to treatment with forskolin (10 μM),isoprenaline (1 μM), formoterol (100 nM),salmeterol (1μM), alprenolol (1 μM), ICI118551 (1 μM), propranolol (1 μM), carvedilol (1 μM) and nadolol (1 μM) and analysed in the Incucyte S3 system for 72 hours (n=3 for all but ICI118551 (n=2)). Data are represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post–hoc test following ANOVA).*

After 72-hour imaging in the IncuCyte S3 system, Calu-3 cells were assessed for proliferation using the MTT assay (Figure 4.3c).As shown ,there no significant effect on MTT reduction in response to forskolin, formoterol, ICI118551, salmeterol isoprenaline ,nadolol, carvedilol and alprenolol.

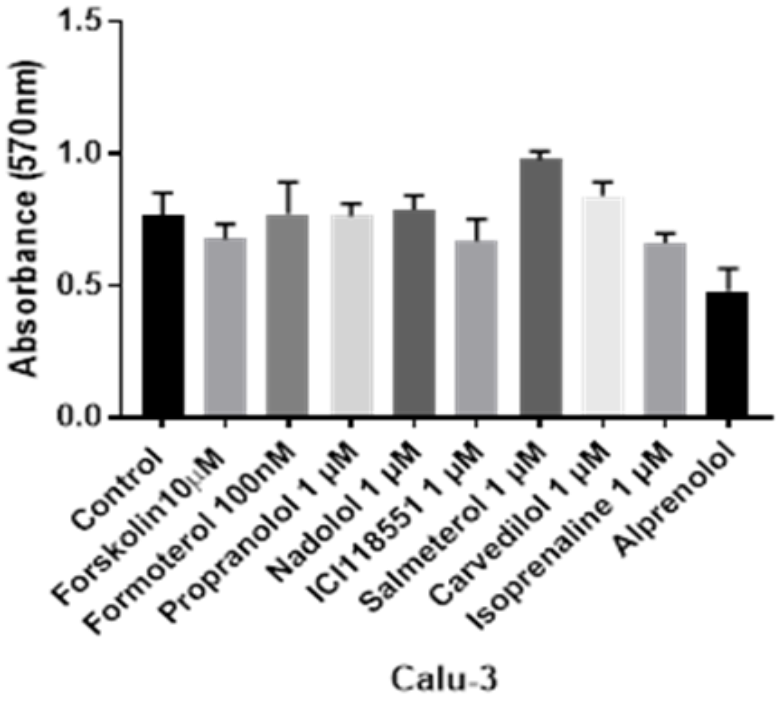


Figure 4.3c The effects of $\beta_2$adrenoceptor stimulation on MTT reduction in Calu-3 cells.

*The Calu-3 cells seeded at 10000 cells per well and incubated for 24hours in serum free containing medium. Cells were stimulated with forskolin(10μM),isoprenaline(1μM),formoterol(100nM),salmeterol(1μM),alprenolol(1μM),ICI118551(1μM),propranolol (1μM),carvedilol(1μM) and nadolol (1μM) and analysed in the incuCyte S3 system for 72hours. The cells were treated with MTT reagent for 4 hours and the absorbance was measured at 570nm. N=3 . Data represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

4.2.3 Real time assessment of proliferation of CHO-β_2 cells

CHO-β_2 cells seeded at a concentration of 5000 cells per well were treated after 24 hours of cellular attachment (Figure 4 .4a, b and c).

Forskolin and Isoprenaline significantly reduced cell proliferation in CHO-β_2 cells, ($p<0.01$).However, formoterol ,nadolol, propranolol,ICI118551 and Carvedilol did not significantly affect cell proliferation(4.4c).

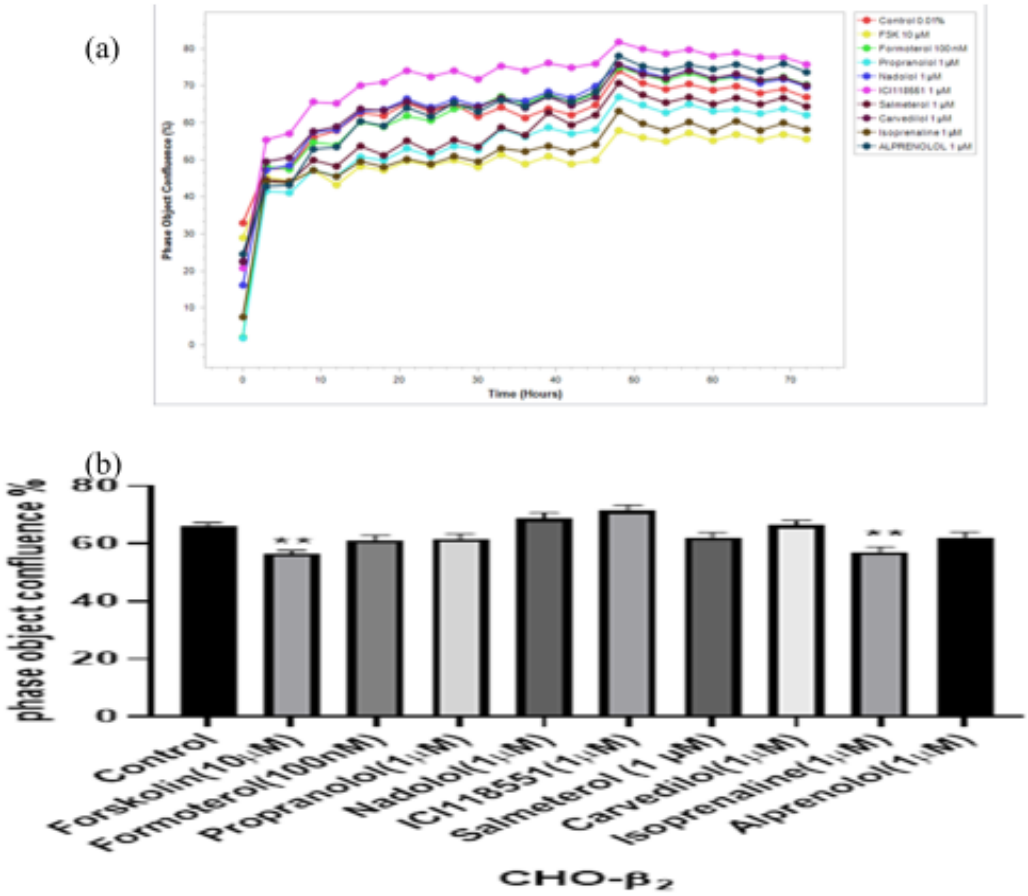


Figure 4.4 Incucyte S3 system assessment of cell proliferation in CHO-β_2 cells.

*a)Shows time course lapse of proliferation rate of 3 experiments and expressed as phase object confluence.(b)CHO-β_2 cells were seeded at 5000 cells/well and incubated for 24 hours in serum free medium prior to treatment with forskolin (10 μM), isoprenaline (1 μM), formoterol (100 nM), salmeterol (1 μM), alprenolol (1 μM), ICI118551 (1 μM), propranolol (1 μM), carvedilol (1 μM) and nadolol (1 μM) and analysed in the IncuCyte S3 system for 72 hours .N=3 for all but ICI118551 and alprenolol, n=2 .(Data are represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

MTT reduction following real-time assessment of cell proliferation .

The MTT reduction of the treated CHO-β_2 cells (Figure 4.4c), which was assessed after the assessment of cell proliferation with the Incucyte S3 system (Figure4. 4b), did not produce any significant change with the treatments.

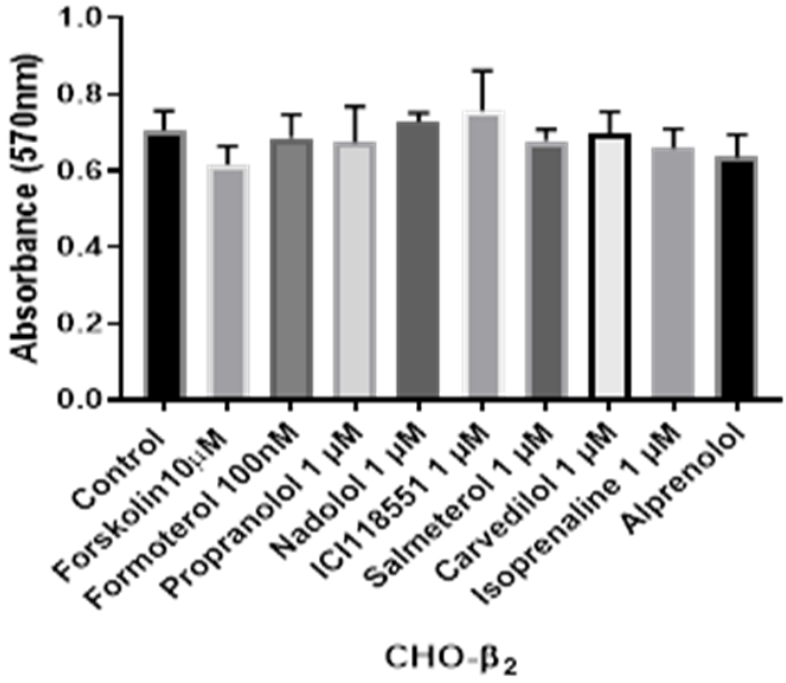


Figure 4.4c The effects of $\beta_2$adrenergic stimulation on MTT reduction in CHO-β_2.

*The CHO-β_2 cells seeded at 5000 cells per well and incubated for 24hours in serum free medium then stimulated with forskolin (10μM), isoprenaline (1μM),formoterol (100nM),salmeterol(1μM),alprenolol(1μM), ICI118551 (1μM), propranolol (1μM),carvedilol(1μM) and nadolol (1μM) and analysed in the incuCyte S3 system for 72hours.The cells were treated with MTT reagent for 4 hours and the absorbance was measured at 570nm. N=3 .Data represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

4.2.4 Real time assessment proliferation of BEAS2BR-1 cells

BEAS2B-R1 cells assessment for the effects of β-adrenoceptor ligands on cell proliferation did not produce any significant change with the control(Figure 4.5c).

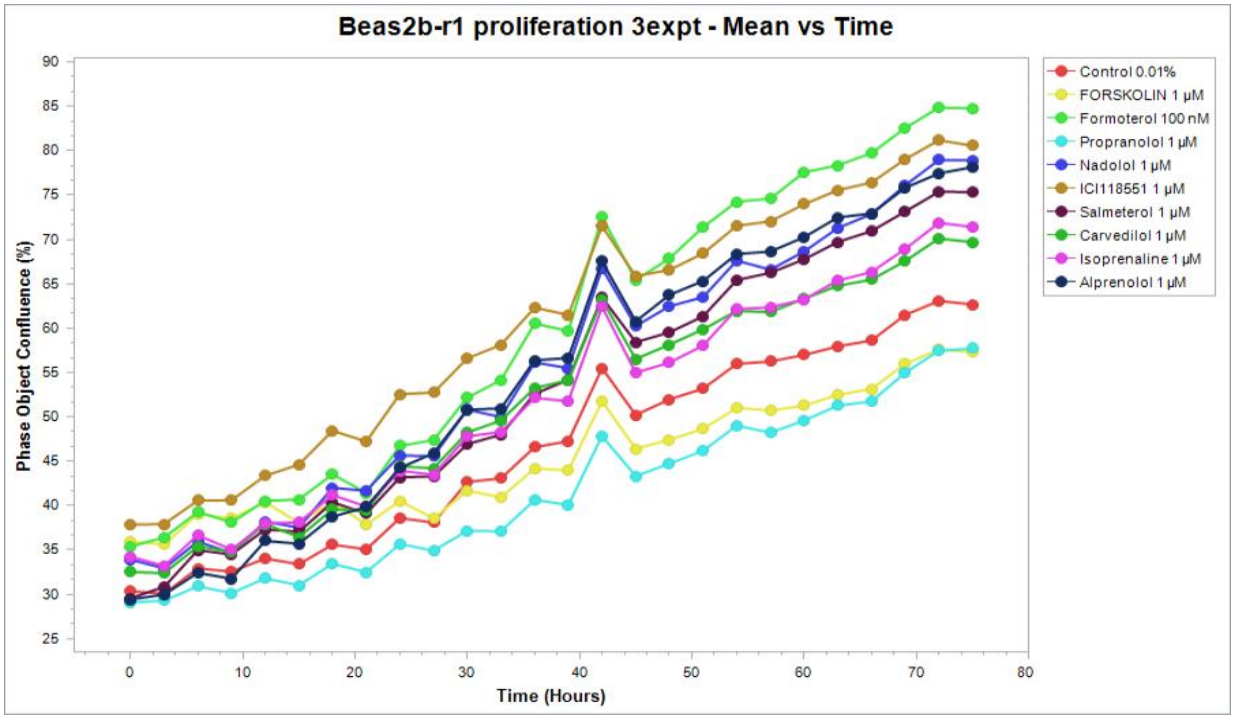


Figure 4.3 (a) IncuCyte cell proliferation Graph in BEAS2BR-1 cells.

IncuCyte cell proliferation in BEAS2BR-1 cells showing the effects of treatments on the cells. The cells were seeded at a concentration of 5000 cells /well and incubated in an incubator for 24hours at 37^0C. Then stimulated with forskolin (10µM), isoprenaline (1µM), formoterol (100nM), salmeterol(1µM), alprenolol (1µM), ICI118551 (1µM), propranolol (1µM), carvedilol(1µM) and nadolol (1µM) were added after cellular attachment. Cells were placed in the incuCyte 3hourly for 72hours.The graph is a representative of 3 independent experiments.

The IncuCyte proliferation assay in BEAS2BR-1 cells (Figure 4.5c).The treatments did not show any significant change compared with the control.

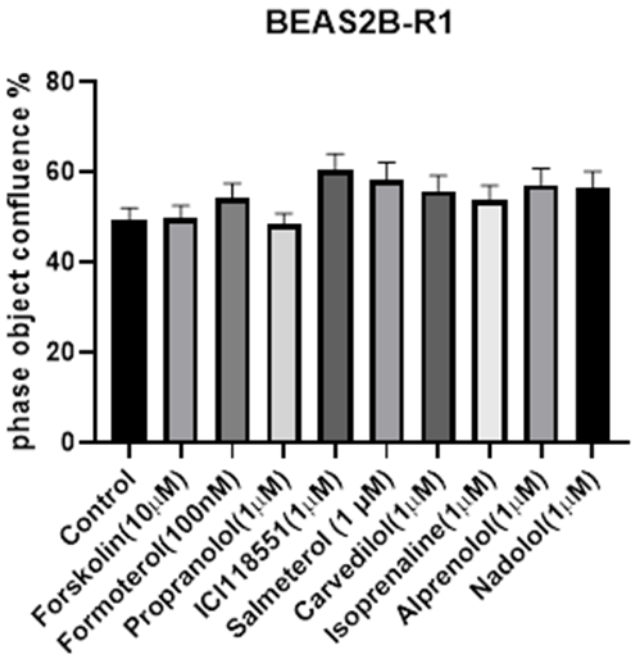


Figure 4.5b. The incuCyte S3 system assessment of cell proliferation in BEAS2BR-1 cells.. *Forskolin(10μM),isoprenaline(1μM),formoterol(100nM),salmeterol(1μM),alprenolol(1μM),ICI118 551 (1μM), propranolol (1μM),carvedilol(1μM) and nadolol (1μM) were added to 5000 cells/well after 24hour incubation in serum free medium and analysed in the incuCyte S3 system 3 hourly for 72hours.N=3 .The treatments did not produce any significant effect on cell proliferation. Data represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post – hoc test following ANOVA).*

The MTT reduction assay in BEAS2BR-1 cells (Figure 4.5c) following real-time assessment of cell proliferation (Figure4.5c), displayed significant increase in MTT reduction in alprenolol ($p<0.01$) and salmeterol($p<0.05$) but did not show any significant increase in forskolin, formoterol, propranolol, nadolol, ICI118551, carvedilol and isoprenaline .

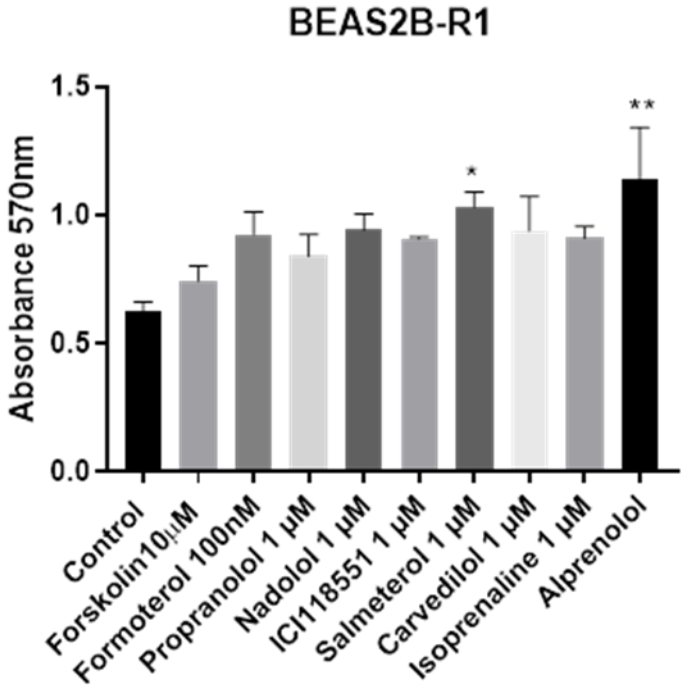


Figure 4.5c The effect of β2adrenergic stimulation on MTT reduction in BEAS2BR-1.

*The BEAS2BR-1 cells seeded at 5000 cells per well and incubated for 24hours in serum free medium prior to treatment with forskolin (10μM),isoprenaline (1μM),formoterol (100nM),salmeterol(1μM) ,alprenololol(1μM), ICI118551 (1μM),propranolol (1μM),carvedilol(1μM) and nadolol (1μM) and analysed in the incuCyte S3 system for 72hours.N=3.Cells were treated with MTT reagent for 4hours and the absorbance was measured at 570nm. Salmeterol and alprenolol significantly increased MTT reduction,p≤0.05 and p≤0.01 respectively. Data represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA.*

4.2.5. The effect of β_2-adrenoceptor activation on pro-inflammatory cytokine release.

The chronic administration of β_2-adrenoceptor agonists in asthma has been observed to worsen the inflammatory response associated with the disease (Broadley, 2006). Therefore, it would be expected that monotherapy with the β_2-adrenoceptor agonists could produce

unwanted pro-inflammatory responses which would limit the use of these drugs in long standing disease (Salpeter *et al.*, 2006). Furthermore, pro-inflammatory cytokine release is enhanced in response to β-agonists treatments and eventually increases airway hyperresponsiveness and decreases mucociliary clearance (Penn *et al.*, 1998). Previously, the release of inflammatory mediators such as IL-6 and IL-8 was studied in bronchial epithelial cells and found to be related to β_2-adrenoceptor agonist-induced cyclic AMP accumulation (Edwards *et al.*, 2007). Also, these inflammatory mediators are produced by the airway epithelium to influence inflammation (Holgate *et al.*, 2007). Therefore, in this study the effect of β_2-adrenoceptor agonists and inverse agonists on IL-6 release was investigated using a cytokine ELISA assay in Calu-3 cells, as described in section 2.2.9. . The samples for the assay were the supernatants removed from treated Calu-3 cells used in Incucyte and MTT reduction experiments (Figure 4.3). Forskolin, formoterol, isoprenaline, alprenolol ,ICI118551 , propranolol, nadolol, salmeterol and carvedilol did not produce any significant effect on IL-6 release in Calu-3 cells (figure 4.6).

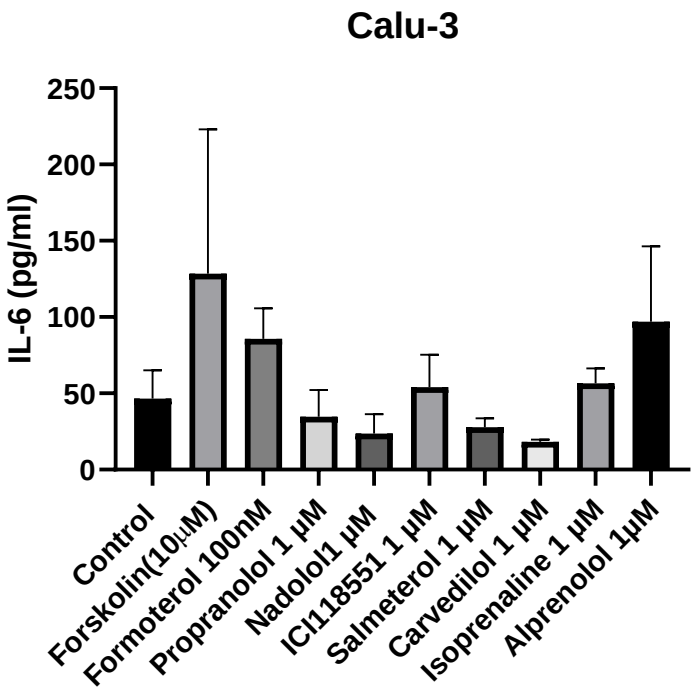


Figure 4.6 .The effect of β-adrenoceptor agonists and inverse agonists on IL-6 secretion after treatment of Calu-3 cells for 72 hours.

*Calu-3 cells were seeded at 10000 cells/well and incubated for 24 hours in serum-free media, prior to treatment with forskolin (10 μM), isoprenaline (1 μM), formoterol (100 nM), salmeterol (1 μM), alprenolol (1 μM), ICI118551 (1 μM), propranolol (1 μM), carvedilol (1 μM) and nadolol (1 μM). Supernatants from triplicates of 3 independent experiments, were removed from the cells after 72 hours and assayed for IL-6 levels, using a Duo Set ELISA kit (Bio Techne).The treatments did not significantly affect the he c Data are represented as mean ± SEM, n=3, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

Cell counting assay in BEAS2B-R1 cells.

The cell assay was investigated at 48 and 72hours.Formoterol and Propranolol did not produce any significant change in cell counts .

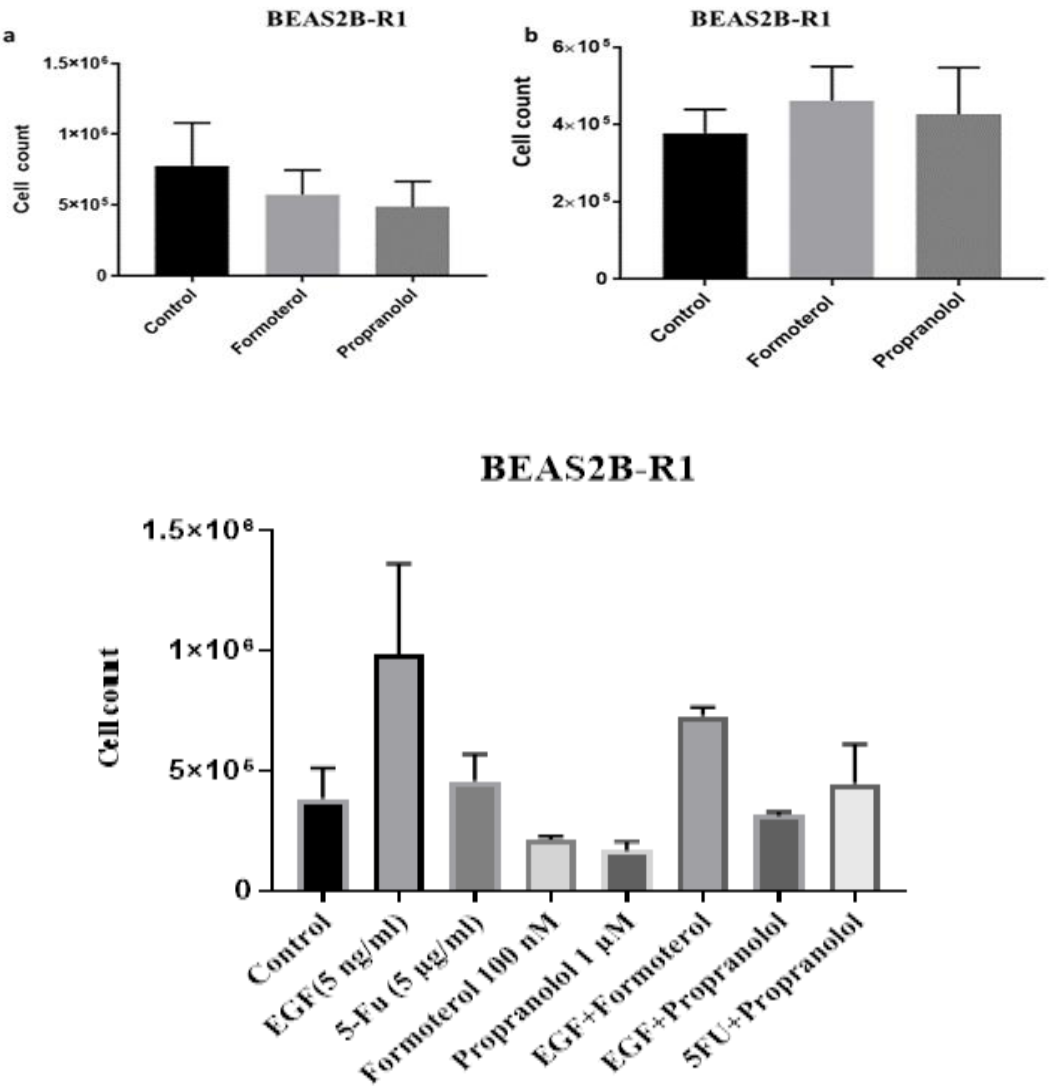


Figure 4.7 Assessment of cell proliferation in BEAS2B-R1 cells using cell counting.

Cells were incubated for 48h(a) and 72h(b) in the presence the following treatments :formoterol (100 nM),propranolol (1µM) (a)48-hour incubation (b) 72h incubation there were trends towards increase in cell proliferation in the presence of propranolol and formoterol .N,=6 for Controls and formoterol and propranolol =3

*(c)Cellsweretreatedafter24hincubationwithEGF(5ng/ml),5flourouracil(5µg/ml),formoterol(100nM) ,propranolol(1µM),EGF+formoterol,EGF+propranolol and 5FU+propranolol.The treatments did have a significant effect ,N=3. Data represented as mean ±SEM, (*p<0.05, **p<0.01, analysed using Dunnett's post –hoc test following ANOVA).*

Real-time analysis of stimulation of cell proliferation in BEAS2BR-1 cells.

The EGF significantly increased cell proliferation in BEAS2BR-1 cells while,LY294002 significantly reduced cell proliferation,p≤0.05. (figure 4.8).

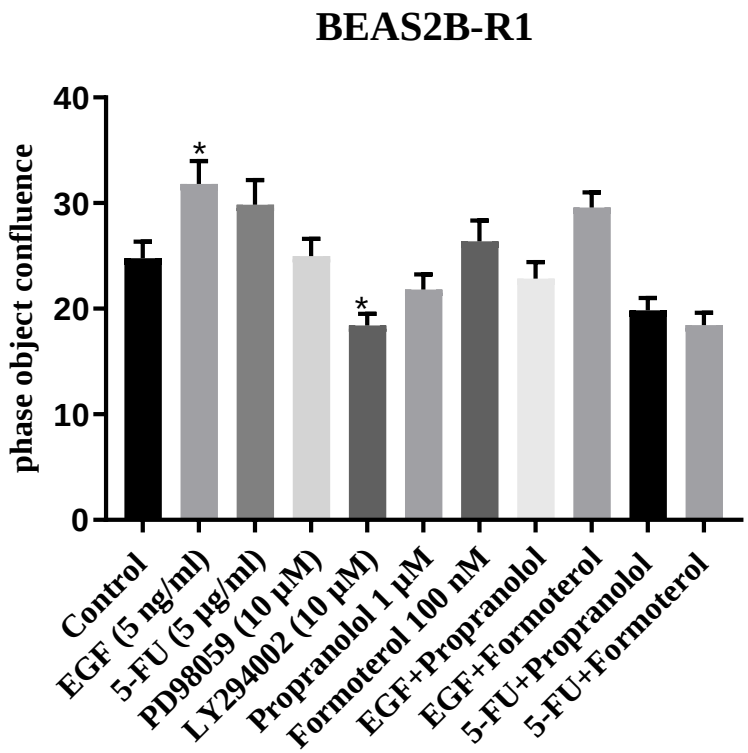


Figure 4. 8. Effects of EGF, 5-Flourouracil and β-adrenoceptor stimulation on cell proliferation in BEAS2B-R1 cells.

*5000 cells/well were seeded and incubated for 24 hours in serum-free media prior to treatment with EGF (5 ng/ml), formoterol (100 nM), propranolol (1 µM), PD98059 (10 µM), 5-Flourouracil (5 µg/ml), LY294002 (10 µM) and analysed using the Incucyte S3 system for 72 hours (n=3 for all conditions). Data are represented as mean ± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

Neutral red assay in Calu-3 and BEAS2BR-1 cells.

The neutral red assay was used to investigate the effect of β-adrenoceptor agonists and inverse agonists on cell viability in human airway cells.

The neutral red assay results in BEAS2BR-1 cells showed significant increase in cell viability with propranolol ,$p<0.0001$ and no significant reduction in cell viability with salmeterol, forskolin ,formoterol and DMSO(Figure 4.9b). There was however no significant effect of forskolin, DMSO ,formoterol, salmeterol and propranolol in Calu-3 cells(Figure 4.9a).

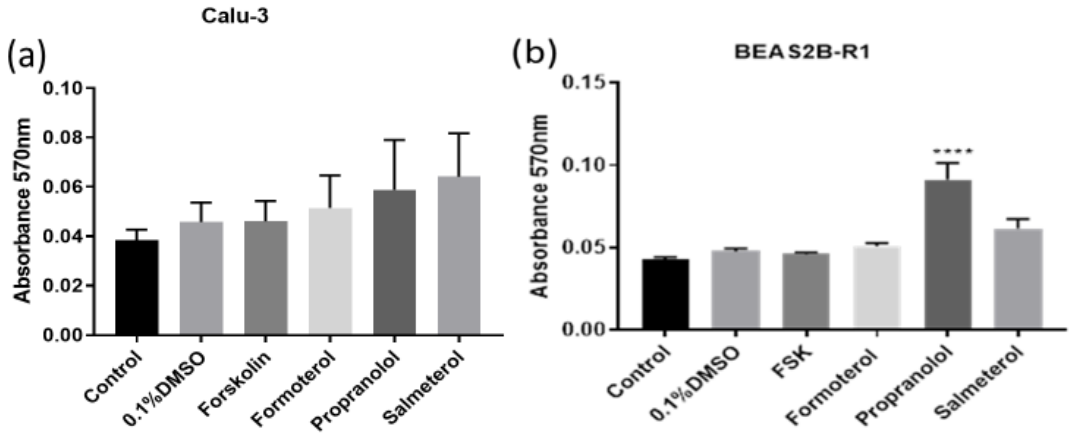


Figure 4.4.Assessment of cell viability using the Neutral red assay in Calu-3 and BEAS2B-R1 cells.

BEAS2B-R1 and Calu-3 cells seeded at 10000cells per well were incubated for 72h in forskolin(10µM),salmeterol(10nM),formoterol(100nM),0.1%DMSO and propranolol(1µM)in serum free media. Treatments were removed and the Neutral red solution was added to wells. This was incubated for 1.30mins,The Neutral red dye was removed using 0.5M NaH2PO4 in 50% ethanol.N=3.There was a statistically significant increase in cell proliferation in cells treated with propranolol in BEAS2B-R1,$p<0.0001$.(Data represented at mean± SEM, ($p<0.05$, **$p<0.01$, analysed using Dunnett's post –hoc test following ANOVA).*

4.3 Discussion

4.3.1 The effects β_2-adrenoceptor stimulation in human airway cells

The abnormal growth of airway epithelial cells, which causes the narrowing of airways was previously studied (Nishimura et al., 2002). Salbutamol (in serum free medium) increased MTT reduction in 16-Human Bronchial epithelial cells and NCI-H292 cells but, not in A459 cells.(Nishimura *et al.*, 2002). In this chapter , multiple techniques were used to investigate the effect of a range of beta -adrenergic stimulation on human airway cells. To assess the effects β-adrenoceptor agonists and inverse agonists on BEAS2B-R1 cells and Calu-3 cells, treatments were applied in serum supplemented medium or serum free medium. Initially, cell viability was investigated using an MTT assay the MTT Real-Time Glo assay by measuring MTT reduction and Neutral assay . The MTT reduction assays are based on the conversion of MTT into formazan crystals by living cells ,which is an indicator of mitochondrial activity. The total mitochondrial activity was previously shown to be related to the number of viable cells(van Meerloo et al.,2011).In this chapter, the data obtained revealed increase in mitochondrial activity as shown by increase in MTT reduction (figure 4.1a) , formoterol, propranolol and nadolol significantly increased MTT reduction in serum supplemented medium in BEASB-R1 cells while, forskolin significantly reduced cell MTT reduction in serum free medium in figure 4.1b. In serum free medium however, Calu-3 cells, displayed a significant increase in MTT reduction in response to nadolol and propranolol and a significant decrease in MTT reduction in IBMX(figure 4.1d). This increase in MTT reduction occurred despite the absence of serum in the treatments. The present finding shows MTT reduction occurred in viable cells which were stimulated with the treatments . In the neutral red assay ,propranolol significantly increased cell viability in BEAS2R-1 cells (figure 4.9 b)which is comparable to propranolol effect on cell viability in MTT reduction assay(figure 4.1a).The data revealed increase in cell viability in Calu-3 and BEAS2BR-1 cells with (β_2-adrenoceptor agonist)formoterol ,propranolol and nadolol (β_2-adrenoceptor inverse agonist)that suggests involvement of cyclic AMP and ERK 1/2 pathway.

Live cell analysis of cell proliferation

Live cell analysis of human airway cells in real-time enable further analysis of cell proliferation in BEAS2B-R1, Calu-3 and CHO-β_2 cells. The incuCyte uses automated imaging to determine cellular confluence at designated intervals over the time course of an experiment as a measure of cell viability. The real-time assay was performed to compliment the endpoint assays. The data in this chapter showed for the first time real-time live cell imaging analysis of proliferation in BEAS2BR-1, Calu-3 and CHO-β_2 cells. The proliferation was measured using phase object confluence. This was investigated to validate the data obtained in the MTT reduction assay. The phase object confluence in Calu-3 cells increased significantly with forskolin, propranolol, salmeterol and nadolol increasing proliferation(Figure 4.3b). The MTT assay in Calu-3 cells in serum free medium showed significant increase in MTT reduction with propranolol and nadolol and IBMX(Figure 4.1).These 2 experiments in Calu-3 cells have shown significant results with both propranolol and nadolol with comparable performance. In contrast to these, proliferation was significantly reduced in response to formoterol, carvedilol and isoprenaline in live cell analysis of Calu-3 cells using the incuCyte S3 system (Figure 4.3b). The real time analysis in CHO-β_2 cells, however, forskolin and isoprenaline on the other hand significantly reduced cell proliferation(Figure 4.4c). This was contrary to the MTT reduction assay with CHO-β_2 cells.

The data in this study showed for the first time, the functional effects of β_2-adrenergic stimulation in 3 cell lines: BEAS2BR-1, Calu-3 and CHO-β_2 cells. Previously, the role of cyclic AMP in stimulation of cell growth has been studied. Salbutamol and isoprenaline stimulated the production of cyclic AMP in human retinal pigment cells (Liu et al.,1992). Also salmeterol and formoterol reduced cell proliferation in airway smooth muscle cells and stimulated an increase in cyclic AMP but the effect of salmeterol was weaker than formoterol (Qi et al., 2019) .Furthermore salbutamol increased cell proliferation in the human bronchial

epithelial cell line (16-HBE) and was inhibited by propranolol. The phospho-MAPK was expressed in salbutamol(16-HBE) treated cells (Nishimura *et al.*, 1998). This suggests activation β_2-adrenoceptor activation mediated activation of MAPK. The β_2-adrenoceptor expressed in airway cells was investigated for their involvement in the prolonged effect of treatment with the β_2-adrenoceptor agonist albuterol which produced desensitization of the receptor and tolerance with albuterol using precision lung cut slices of human lungs. (Cooper and Panettieri,2008). Other studies investigated the effect of IBMX, a phosphodiesterase inhibitor, in asthmatic airway smooth muscle, where it produced a reduction in cyclic AMP with isoproterenol, formoterol and forskolin compared to non-asthmatics. Also, the inhibition of IBMX in asthmatics reduced cell proliferation compared to non-asthmatics. The reduced production of cyclic AMP was responsible for reduced bronchodilation and increases in airway smooth muscle mass (Trian *et al.,* 2011).The findings with IBMX are consistent with the data in this chapter (figure 4.1c and d).Results revealed a decrease in MTT reduction by IBMX in Calu-3 cells which suggests an inhibitory role for IBMX in β_2-adrenoceptors in human airway cells.

The stimulation of airway epithelial cell (A549) proliferation in the presence of salbutamol was also reported (Bonacci *et al.,* 2006). Based on these the present study showed the stimulation and reduction of cell proliferation by β-adrenoceptor agonists and inverse agonists in 3 cell lines. In addition, the live cell imaging and assessment of cell proliferation provided a complete analysis of the functional effects of these drugs for 72 hours. The findings did correspond with some of the effects of β_2-adrenoceptor stimulation in the end point assays(MTT reduction assay and the Neutral red assay). In figure 4.1d which showed a significant increase in MTT reduction in Calu-3 cells in propranolol and nadolol treated cells in serum free medium. When compared with incuCyte real time analysis in Calu-3 cells, propranolol and nadolol significant increased cell proliferation(figure 4.3b).The data showed increase in cell viability in Calu-3 cells suggests a major role for β_2-adrenoceptors

in supporting proliferation in Calu-3 cells . Furthermore ,EGF significantly increased cell viability in BEAS2BR-1 cells while LY294002 decreased cell viability which was assessed by the incuCyte cell proliferation assay(figure 4.8).

The results indicate a major role the for β_2-adrenoceptor activation in the stimulation of cell viability in 3 cell lines. It also showed the sensitivity of the MTT reduction assay and Neutral red assay in assessing cell viability. The live imaging technique using the Incucyte real-time analysis, provided a complete coverage of the effects of the treatment over a prolonged period.

CALU-3 CELLS

Treatment	MTT assay serum-	MTT assay serum+	MTT real-time glo assay	Incucyte proliferation assay	MTT assay following incucyte proliferation assay	Neutral assay
Alprenolol (1µM)	-	-	-	NS	NS	-
Carvedilol (1µM)	-	-	-	NS	NS	-
0.1%DMSO	NS	NS	-	-	-	NS
1%DMSO	NS	NS	-	-	-	-
FM+IBMX	NS	NS	-	-	-	-
FSK+IBMX	NS	NS	-	-	-	-
Forskolin (10µM)	NS	NS	NS	****	NS	NS
Formoterol (100 nM)	NS	NS	NS	****	NS	NS
ICI118551 (1µM)	NS	NS	NS	NS	NS	-
Isoprenaline (1µM)	NS	NS	NS	****	NS	-
IBMX	*	*	-	-	-	-
Nadolol (1µM)	**	NS	NS	****	NS	-
Propranolol (1µM)	*	NS	NS	****	NS	NS
Salmeterol (1µM)	-	-	-	NS	NS	NS

CHO-β_2

Treatment	MTT assay serum-	MTT assay serum+	MTT real-time glo assay	Incucyte proliferation assay	MTT assay following incucyte proliferation assay	Neutral assay
Alprenolol (1μM)	-	-	-	NS	NS	-
Carvedilol (1μM)	-	-	-	NS	NS	-
0.1%DMSO	-	-	-	-	-	-
1%DMSO	-	-	-	-	-	-
FM+IBMX	-	-	-	-	-	-
FSK+IBMX	-	-	-	-	-	-
Forskolin (10μM)	-	-	-	**	NS	-
Formoterol (100 nM)	-	-	-	NS	NS	-
ICI118551 (1μM)	-	-	-	NS	NS	-
Isoprenaline (1μM)	-	-	-	**	NS	-
IBMX	-	-	-	-	-	-
Nadolol (1μM)	-	-	-	NS	NS	-
Propranolol (1μM)	-	-	-	NS	NS	-
Salmeterol (1μM)	-	-	-	NS	NS	-

BEAS2B-R1

Treatment	MTT assay serum-	MTT assay serum+	MTT real-time glo assay	Incucyte proliferation assay	MTT assay following incucyte proliferation assay	Neutral assay
Alprenolol (1µM)	-	-	-	NS	**	-
Carvedilol (1µM)	-	-	-	NS	NS	-
0.1%DMSO	-	-	-	-	-	NS
1%DMSO	-	-	-	-	-	-
FM+IBMX	-	-	-	-	-	-
FSK+IBMX	-	-	-	-	-	-
Forskolin (10µM)	*	NS	NS	NS	NS	NS
Formoterol (100 nM)	NS	**	NS	NS	NS	NS
ICI118551 (1µM)	NS	NS	-	NS	NS	-
Isoprenaline (1µM)	NS	NS	-	NS	NS	-
IBMX	-	-	-	-	-	-
Nadolol (1µM)	NS	*	NS	NS	NS	-
Propranolol (1µM)	NS	*	NS	NS	NS	****
Salmeterol (1µM)	-	-	-	NS	*	NS

Table 4.1 The assessment of cell proliferation in human airway cells using multiple techniques.

*The table summarises the findings of the assessment of cell proliferation in Calu-3, BEAS2B-R1 and CHO-β_2 cells. Cells were treated with: forskolin (10 μM), isoprenaline (1 μM), formoterol (100 nM), salmeterol (1 μM), alprenolol (1 μM), ICI118551 (1 μM), propranolol (1μM), carvedilol(1μM) and nadolol (1μM) as indicated in the table for 72hours. N=3 . Data are represented as mean± SEM, (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA). The effects of these drugs on cell viability were assessed using MTT reduction assay, MTT real-time glo assay and the IncuCyte S3 system.*

4.4 Conclusion

The findings in this chapter indicate that propranolol and nadolol increased cell viability in Calu-3 cells as investigated using MTT assay and real-time analysis of cell proliferation. Furthermore, propranolol increased cell viability in the MTT reduction assay and neutral red assay. These results indicate that the incuCyte real time analysis is well suited for assessing cell proliferation which can be compared with an endpoint assay. The IncuCyte cell proliferation assay is most informative because it provides a complete profile of the events as they happen. Such as increase in the rate of proliferation of epithelial cells which narrow the lumen of the airways could worsen bronchoconstriction.

Chapter 5

The effect β_2-adrenoceptor stimulation on wound healing in bronchial epithelial cells using multiple techniques

5.1 Introduction

The frequent exposure of the human airway to dust particles, infectious agents, microorganisms, allergens and toxins disrupt the integrity of the respiratory epithelium. These affect wound healing and closure after injury and worsen respiratory diseases if (Yarrow *et al.,* 2004).present. Previously, the influence of growth factors on wound healing through migration and proliferation were investigated The investigation of wound healing that produced complete regeneration of the respiratory epithelium was studied previously (Perotin *et al.,* 2014). For these reasons, it was necessary for respiratory airway cells to be investigated as in vitro models for the study of cell migration in wound healing and repair in respiratory disease such as asthma. In chronic asthma, the respiratory airway becomes thickened by up to 300% in fatal disease and by 100% in mild disease and this was supported by radiological evidence of disease severity that increases with the duration of the disease (Tagaya *et al.,* 2007). As β_2-adrenoceptor agonists are the mainstay of treatment, understanding the role of these agonists in wound healing and repair is crucially important.

Salbutamol, a β_2-adrenoceptor agonist, stimulated wound repair in A549 cells, derived from human epithelial basal cells (Perkins *et al.,* 2007). This was also studied in normal human bronchial epithelial cells (NHBE) and Calu-3 cells, using impedance sensing technology after wounding confluent monolayers with a voltage pulse (Peitzman *et al.,* 2015). Salbutamol delayed wound healing and repair in NHBE cells and Calu-3 cells but dobutamine a β_1-agonist, had no effect. NHBE cells treated with propranolol or ICI118551 which were pre-treated with epinephrine showed delay in wound closure. The inhibition was more with ICI118551 than propranolol. This suggests a major role for β_2-adrenoceptors in mediating the action of epinephrine in inhibiting wound closure (Peitzman *et al.,* 2015).

However, in studies conducted in non-respiratory epithelial cells, the results obtained were different. Cells from corneal epithelium and keratinocytes from both dermal and oral origin

displayed a reduction in the rate of wound closure and migration in response to the β_2-adrenoceptor agonist isoproterenol (Steenhius *et al.*, 2011). The β_2-adrenoceptor antagonist did the opposite by improving wound healing and migration in these cells. The inhibition of migration by β_2-adrenoceptor agonist, isoprenaline was an independent cyclic AMP mechanism, which produced dephosphorylation of extracellular signal- regulated kinase (ERK). However, the β_2-adrenoceptor antagonist timolol stimulated wound healing and wound closure by the phosphorylation of ERK (Steenhius *et al.,* 2011).

The Epidermal growth factor (EGF), a member of the epidermal growth factor family, is an important regulator of epithelial wound repair. Its main function is in the stimulation of migration, proliferation and differentiation of epithelial cells (Lawrence *et al.,* 1994). Damage to the airways is a common feature in asthma, especially in mild cases of the disease. It causes a lot of damage to the airway and reduces mucociliary clearance. It also makes the bronchial epithelium vulnerable due to loss of barrier function (Puddicombe *et al.,* 2000).

β-adrenoceptor agonists, antagonists and inverse agonists can influence wound closure by influencing downstream signalling pathways (Spurzem *et al.,* 2002). Cyclic AMP has been reported to influence cell movement and attachment (Spurzem *et al.,* 2002). Indeed, the β_2-adrenoceptor agonist isoprenaline, through activation of cyclic AMP, accelerated migration and wound closure in bovine bronchial epithelial cells (Spurzem *et al.,* 2002). The inhibition of protein kinase A with KT5720 or 4cyano-3-methylisoquinolone prevented the stimulation of wound closure by isoprenaline. This suggests protein kinase A activity is necessary for wound closure to occur (Spurzem *et al.,* 2002).

BEAS2B-R1 and CHO-β_2 adrenoceptor cells reproducibly proliferated faster than Calu-3 cells in cell culture. To validate the data obtained, three different methods of wounding were used. It is also very important to ensure that , the methods were accurately used to generate adequate data for better comparison in the cell lines.

5.1.1 Chapter aims

The aims of this research were:

- To investigate the effects of β-adrenoceptor agonists and inverse agonists on wound healing in human airway epithelial cells.
- To investigate the effects of EGF on wound healing.

This was investigated using a range of techniques commonly used to assess wound healing: a mechanical scratch wound method using a micropipette; a chemical wounding method using sodium hydroxide to create the wounds; and an automated method using the IncuCyte S3 system and associated wound maker. The influence of a variety of β_2-adrenoceptor agonists and inverse agonists and EGF on wound healing in human bronchial epithelial cells (BEAS2B-R1 and Calu-3 cell lines) and CHO cells expressing the human β_2-adrenoceptor was investigated using these approaches.

5.2 Results

5.2.1 The effect of β_2-adrenoceptor stimulation on wound healing in human airway epithelial cells (BEAS2B-R1, Calu3 and CHO-β_2-cells)

5.2.1.1 Assessment of wound healing in BEAS2B-R1 cells using a mechanical scratch wound method.

In the assessment of wound healing using a micropipette, the effects of the β_2-adrenoceptor ligands formoterol and propranolol were investigated in confluent BEAS2B-R1 cells. Shortly after treatment of wounds, the migration and proliferation of cells from the wound edges reduced the distance between the wound edges. This is shown in the images of the wounded areas (Figure 5.1c) at 0, 24 and 48 hours in the absence or presence of treatment. The stimulation of the wounds with β_2-adrenoceptor agonist, formoterol or β_2-adrenoceptor inverse agonist, propranolol, did not significantly reduce the wound distance within 24 hours post wounding. After 48hours of stimulation ,both formoterol and propranolol did not produce any significant effect on the wounds(Figure 5.1 a and b).

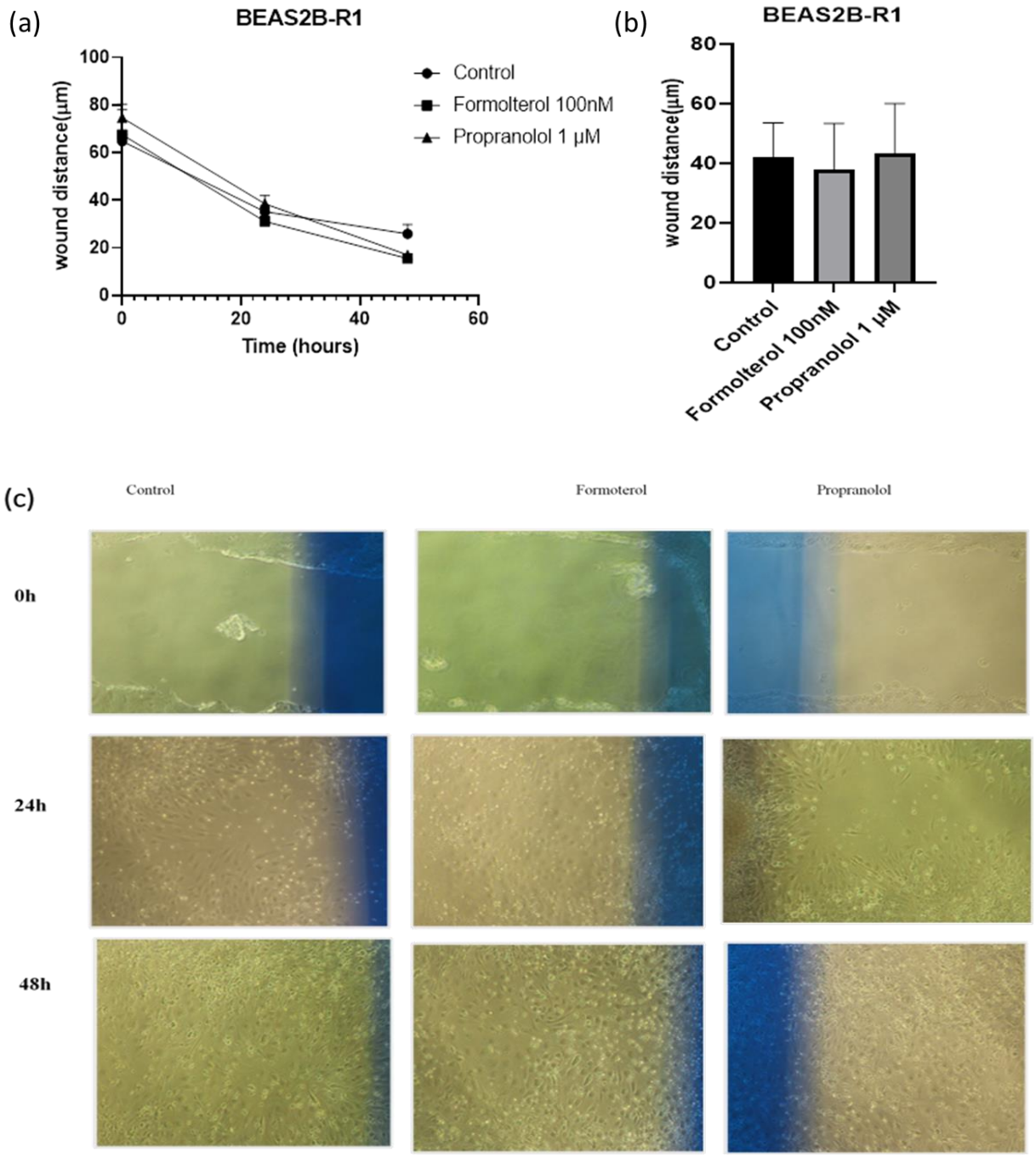


Figure 5.1 β2-adrenoceptor activation in wound healing in BEAS2R-1 cells.

*The bottom of 24 well plates were demarcated with a vertical line. BEAS2B-R1 cells were seeded at 1x10^5 cells/ml. Cells were incubated in a humidified environment at 37^{0}C, 5% carbon dioxide and 95% humidified air. At 100% confluency, a sterile 10µl pipette tip was used to scratch a gap at right angles to the demarcated line. The media was removed, washed with 2ml of PBS and replaced with 900µl of media. Cells were treated with propranolol (1 µM), formoterol (100 nM) or no treatment for Control wells. Images were captured at 0, 24 and 48h. The distances between the wounding centre and the vertical axis of the migrating cells were measured for statistical analysis using the ImageJ software. Data shown are n=4, with each experiment performed in triplicate. (a and b) Shows the graph of wound closure and treatments. Data are represented as mean ±SEM. (*p<0.05, **p<0.01, analysed using Dunnett's post –hoc test following ANOVA). (c) β_2-adrenoceptor activation of in wound healing in BEAS2R-1 cells. Images of the wounded confluent monolayers 0, 24- and 48-hours post wounding. Wounds were created using a sterile 10µl pipette tip.*

5.2.1.2 Assessment of wound healing in BEAS2B-R1 cells using a chemical method of wounding

In the assessment of wound healing using the chemical method to create wounds in confluent monolayers of BEAS2B-R1 cells, within 24 hours the control wound edges narrowed first. The narrowing of wound edges was subsequently followed by Formoterol and then Propranolol treated wounds. The wound edges of the formoterol+propranolol treated wound closure was delayed and on the average the widest at the end of the 48-hour assessment of wound healing. There were, however, no statistically significant differences between any conditions (Figure 5.2).

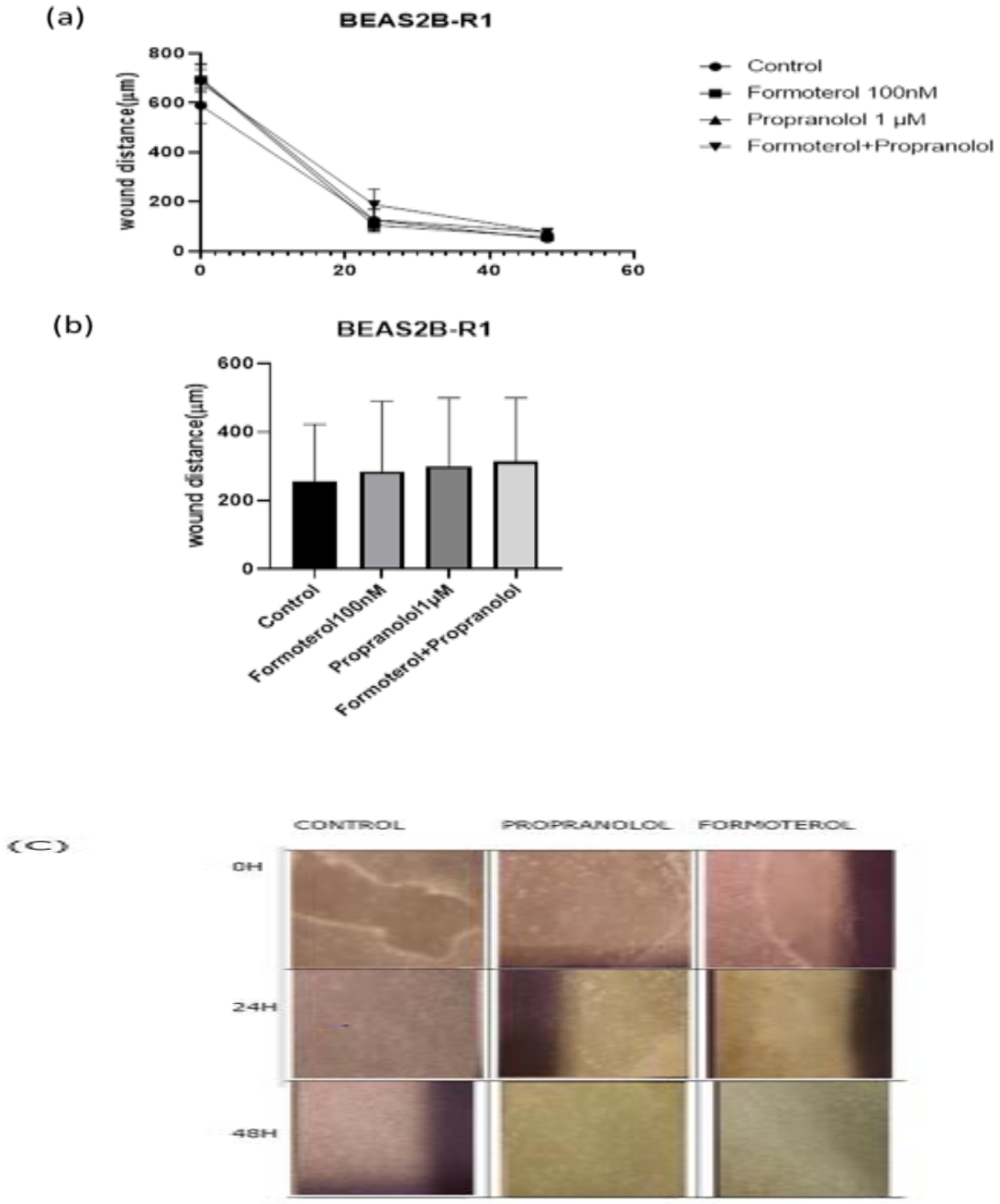


Figure 5.2 β2-adrenoceptor activation in wound healing in BEAS2R-1 cells.

*The bottom of 24 well plates were demarcated with a vertical line. BEAS2B-R1 cells were seeded at 1x10^5 cells/ml and incubated 37^{0}C,5% carbon dioxide and 95% humidified air. At 100% confluency, 10µl 0f 0.5M NaOH was used to create a wound at the centre of each well. The medium was removed washed with 2ml of PBS prior to addition of treatments. The cells were briefly stimulated with propranolol (1µM) and formoterol (100nM),a combination of both formoterol and propranolol. and no treatment for control wells. Images were captured at 0,24&48h.The distances between the wounding centre and the vertical axis of the migrating cells were measured for statistical analysis using image J software. N=4 ,experiment performed in triplicates.(a and b)Show the graphs of wound closure and treatments(c)Images of wounds including treatment. Data represented as mean ±SEM. (*p<0.05, **p<0.01, analysed using Dunnett's post –hoc test following ANOVA).*

5.2.1.3 Assessment of wound healing using an automated method

BEAS2B-R1 cells

In the first part of the investigation, the wound closure was displayed by the presence of cells in the wounded area. The presence of cells in the wounded area closes the gap and as a result the confluency of cells is increased. The number of cells which migrated from the wound edge contribute to the wound confluence percentage. The images of the created wound are shown(figure 5.3.1.a).which were captured 3hourly for 66hours as described in methods section 2.2.4.The data was as expressed as wound confluence percentage which measures the confluence of cells in the wound region.

The wound confluence percentage was significantly reduced in all wounds treated with forskolin($p \leq 0.001$),formoterol,ICI118551,propranolol,carvedilol,($p \leq 0.0001$)andEGF($p \leq 0.001$), relative to control as shown in figure 5.3b.However,the effect of salmeterol on wound confluence was not significantly reduced relative to control. The effect EGF +salmeterol and EGF+ICI118551 did not significantly reduce wound confluence percentage relative to EGF.

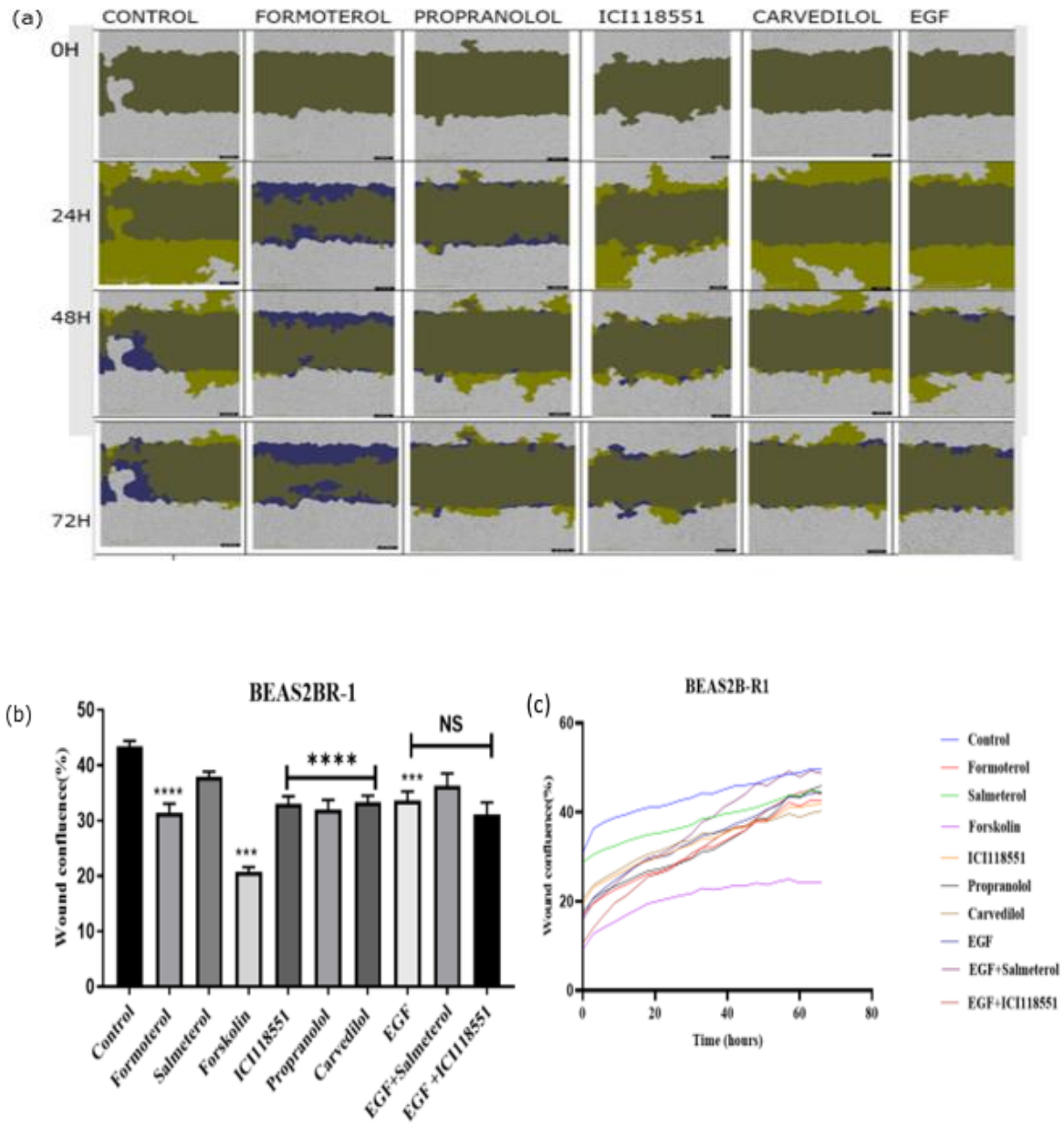


Figure 5.3.1. The effect of β2-adrenoceptor stimulation on wound healing in BEAS2B-R1 cells.*(a)BEAS2B-R1 cells were cultured to confluence in 96 well image lock plates and wounds created using wound maker(see section 2.2.4 for details).The pictures represent wounded confluent monolayers captured in real-time using the incuCyte S3 system The yellow colour on the wounds represents initial wound scratch mask and the blue colour represents extent of wound closure. The treatments were added to triplicate wells after creating wounds. Images were captured 3-hourly for 66 hours using the incuCyte S3 live imaging system and acquired using 10X objective.(b) The wound confluence percentage in treatment groups were significantly decreased in forskolin (10 μM), formoterol (100 nM), ICIII8551 (1 μM), carvedilol (1 μM) and propranolol (1 μM) ($p<0.001$),EGF (5 ng/ml) relative to control. (c) Time course closure of wounds expressed as wound confluence percentage which is a measure of cell confluence within the wound region over time. This is expressed as percentage of wound area occupied by cells. Data normalized to control showing time points. (Data are represented as mean ± SEM, n=3-4, analysed using Dunnett's post–hoc test following ANOVA).*

5.2.1.4 Real-time analysis of wound healing in BEAS2B-R1 cells, assessed by wound width

In addition to confluence of cells in wound region, the wound width was used to investigate wound healing. The wound width measures the distance between the wound edges. As shown in figure 5.3.2,stimulation of the wounds increased the wound width significantly in the presence of forskolin, formoterol, carvedilol and propranolol,$p \leq 0.0001$.The effect of salmeterol,ICI118551 and EGF did not significantly increase the wound width. However EGF+ICI118551 significantly reduced wound width relative to EGF($p \leq 0.001$),while EGF+Salmeterol did not significantly increase wound width relative to EGF.

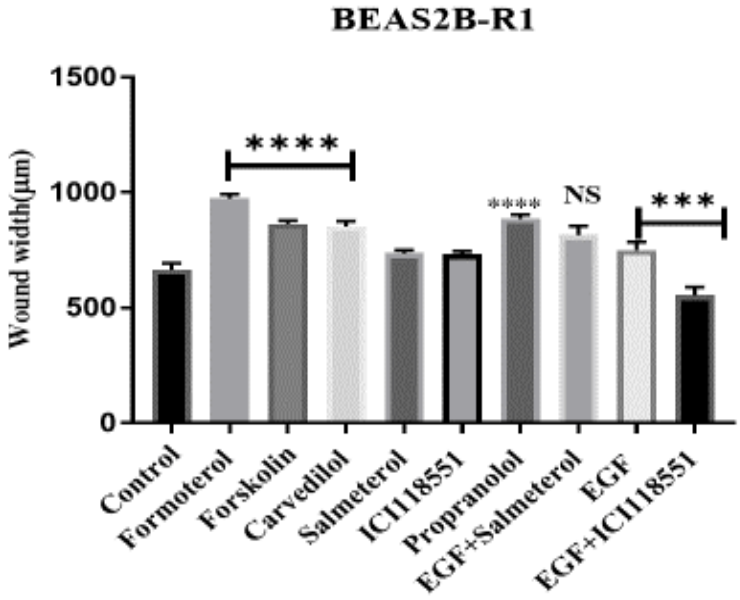


Figure 5.3.2 The effect of β2-adrenoceptor stimulation on wound healing in BEAS2B-R1 cells, assessed by wound width.

*Wound widths were significantly increased in response to forskolin (10 µM), β_2-adrenoceptor agonist formoterol (100 nM), inverse agonists carvedilol (1 µM) and propranolol (1 µM) ($p<0.0001$) and EGF+salmeterol ($p<0.001$) .The modest increases in wound width in response to ICIII8551 (1 µM), salmeterol (1 µM) and EGF (5 ng/ml) were not significantly increased. (*i)IncuCyte image of scratch wounds in BEAS2R-1 cells showing control and formoterol treated wounds at time 0,48,66 hours. (Data are represented as mean ± SEM, n=4, analysed using Dunnett's post–hoc test following ANOVA).*

5.2.1.5 Real-time analysis of wound healing in BEAS2B-R1, assessed using relative wound density

The effect of wound healing in BEAS2B-R1 was also assessed using the relative wound density (RWD). RWD measures the density of the wound region at a particular time point. It also accounts for the cell growth which is expressed as a percentage of wound density relative to the density of cells outside the wounded area ,with respect to the cells in the entire well at every time point. As shown in figure(5.3.3) The RWD in BEAS2BR-1 cells was significantly increased in formoterol , ICI118551 ($p<0.05$), propranolol ($p<0.01$), EGF ($p<0.0001$ relative to control but did not produce any significant effect with forskolin, carvedilol and salmeterol. However, EGF+salmeterol and EGF+ICI11855 did not significantly reduce RWD% relative to EGF.

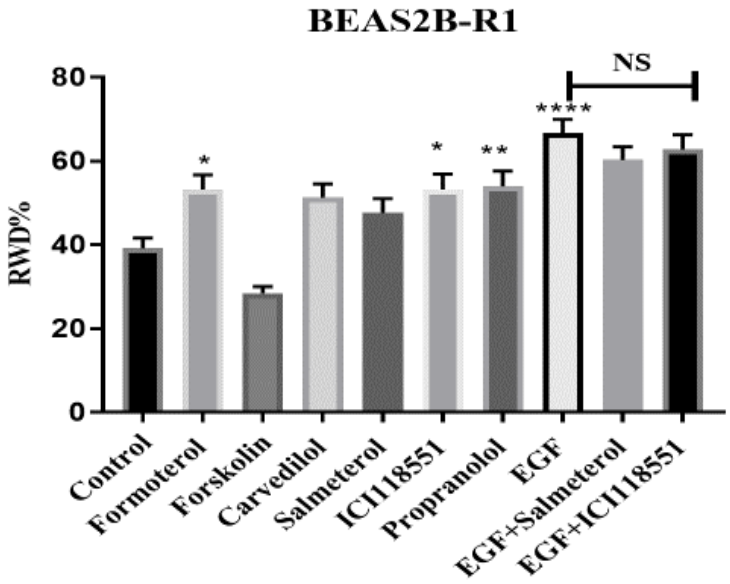


Figure 5.3.3. The effect of β2-adrenoceptor stimulation on wound healing in BEAS2B-R1 cells, assessed using relative wound density. *The assessment of wound healing in BEASBR-1 cells using the relative wound density (RWD) percentage. Images were captured 3hourly for 66hours using the IncuCyte S3systemThe RWD% was increased significantly in formoterol (100 nM)($p<0.05$), ICI118551(1μM),$p<0.05$),propranolol(1μM), $p<0.01$, Epidermal growth factor(5ng/ml),$p<0.0001$) with no significant effect with forskolin, salmeterol and carvedilol relative to control. Also ,EGF+salmeterol and EGF+ICI118551 did not significantly reduce RWD% relative to EGF. (Data represented as mean ± SEM, N=4, analysed using Dunnett's post –hoc test following ANOVA.*

5.2.1.6 Real-time assessment of wound healing in Calu-3 cells

Wound healing in Calu-3 cells was assessed using the automated method over 72hours. Calu-3 cells were seeded at a concentration of $5.0x10^4$ cells/well in 96 well image lock plates coated with poly-l-lysine. Wounds were created using a wound maker, as indicated in section 2.2.4.

5.2.1.7 Real-time analysis of wound healing using wound confluence

The analysis of wound healing by assessing wound confluence is shown in Figure 5.4.1.

The stimulation of the created wounds in confluent monolayers in calu-3 cells showed significant increase in wound confluence in forskolin ,salmeterol ,propranolol, EGF, carvedilol($p<0.0001$) and ICI118551($p\leq0.01$) relative to control. Also, the effect of EGF+ICI118551 and EGF+salmeterol significantly decreased wound confluence relative to EGF. However, the effect of formoterol did not significantly decrease wound confluence percentage in calu-3 cells.

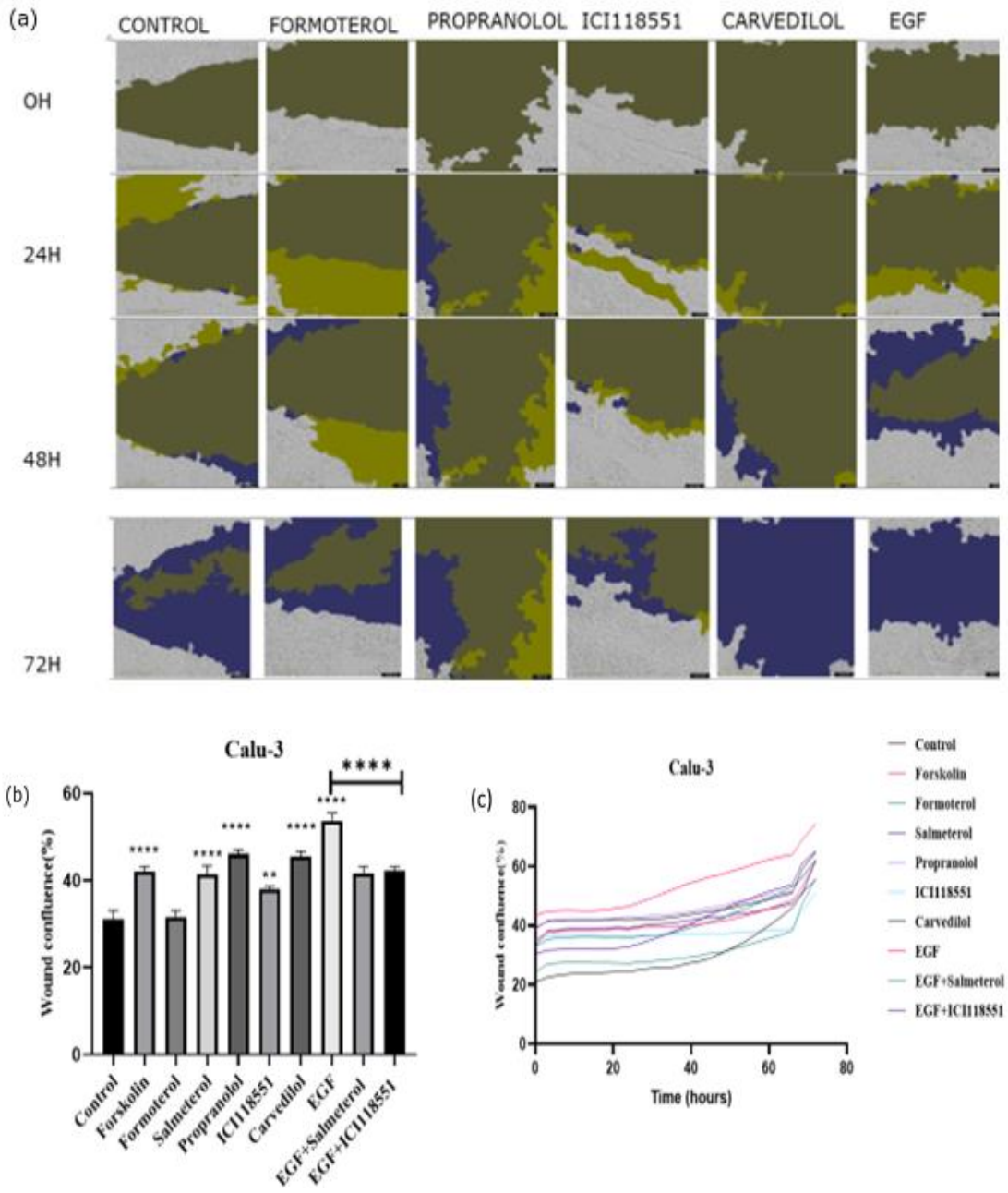


Figure 5.4.1. The effect of β2-adrenoceptor stimulation on wound healing in Calu-3 cells.

(a)Shows Calu-3 cells after wounding with the wound maker and images captured 3hourly for 72hours with the incuCyte S3 system.(b)The wound confluence percent was significantly increased in forskolin(10μM),salmeterol (1μM), carvedilol(1μM) propranolol(1μM),EGF(5ng/ml)($p \leq 0.0001$) and ICI118551($p < 0.0001$) relative to control. The effect of EGF+salmeterol and EGF+1CI118551 on wound confluence percent was significantly decreased relative to EGF while ,the effect of formoterol on wound confluence percentage was not significant relative to control. (c) Time course closure of wounds expressed as wound confluence percentage which is a measure of cell confluence within the wound region over time. This is expressed as percentage of wound area occupied by cells .N=3,Data represented as mean ± SEM, , analysed using Dunnett's post –hoc test following ANOVA.

5.2.1.8 Real-time analysis of wound healing in Calu-3 cells assessed using wound width

The analysis of wound healing using wound width (the distance between wound edges) is shown in Figure 5.4.2.The wound width was significantly decreased in forskolin($p \leq 0.05$)and ICI118551($p \leq 0001$) relative to control. The effect of EGF+ICI118551 on wound width was significantly increased relative to EGF but not with EGF+salmeterol. However there was no significant effect on wound width with forskolin, salmeterol, propranolol and carvedilol relative to control.

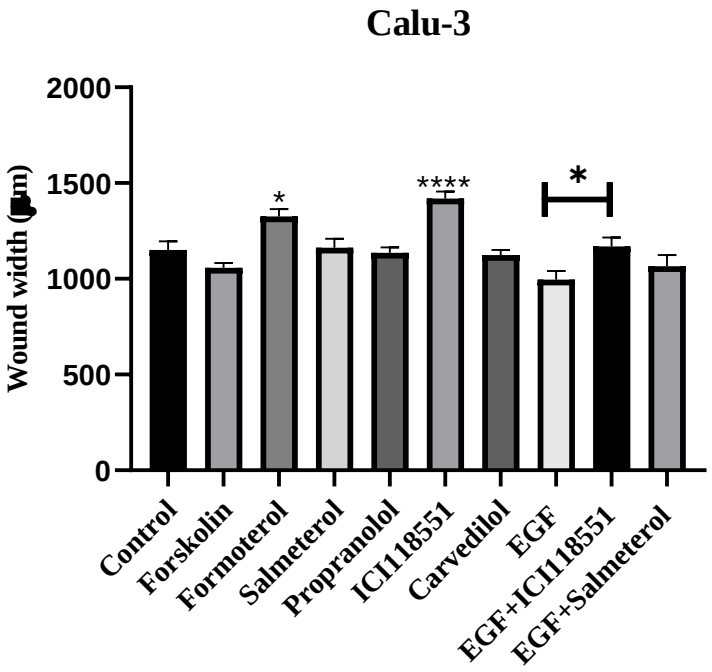


Figure 5.4.2. The effect of β_2-adrenoceptor stimulation on wound healing in Calu-3 cells.

Histogram showing a significant increase in wound width with formoterol($p \leq 0.05$) in Calu-3 cells and ICI118551($p \leq 0.0001$) relative to control. However, the forskolin ,salmeterol, propranolol and carvedilol did not show any significant effect. The effect of EGF+ICI118551 significantly increased wound width relative to EGF but EGF+salmeterol did not show any significant effect .n=3.Data represented as mean ± SEM, , analysed using Dunnett's post –hoc test following ANOVA.

5.2.1.9 Real-time analysis of wound healing in Calu-3 cells using relative wound density

The relative wound density (RWD) of Calu-3 cell wounds treated with β_2-adrenoceptor agonists and inverse agonist were analysed with incuCyte S3 system. As shown in figure 5.4.3, the RWD was significantly decreased with ICI118551,($p \leq 0.01$) relative to control. There was no significant effect on RWD in forskolin ,formoterol ,salmeterol ,propranolol, carvedilol and EGF relative to control . Furthermore , EGF+salmeterol and EGF+ICI118551 did significantly increase RWD relative to EGF.

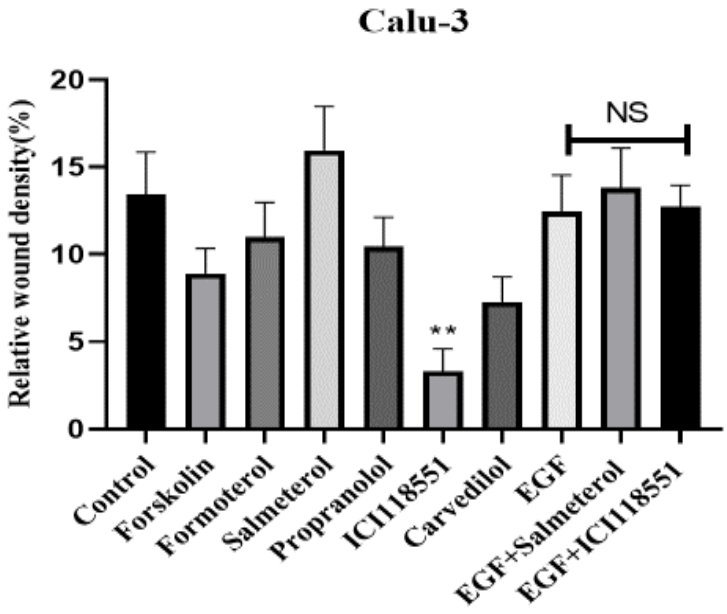


Figure 5.4.3. The effect of β_2-adrenoceptor stimulation on wound healing in Calu-3 cells

Histogram showing significant reduction in RWD with ICI118551,($p \leq 0.01$) with no *significant effect on RWD with forskolin,formoterol,salmeterol,carvedilol,propranolol and EGF relative to control. Also EGF+salmeterol and EGF+ICI118551 did significantly reduce RWD relative to EGF. Data represented as mean ± SEM, , analysed using Dunnett's post –hoc test following ANOVA.*

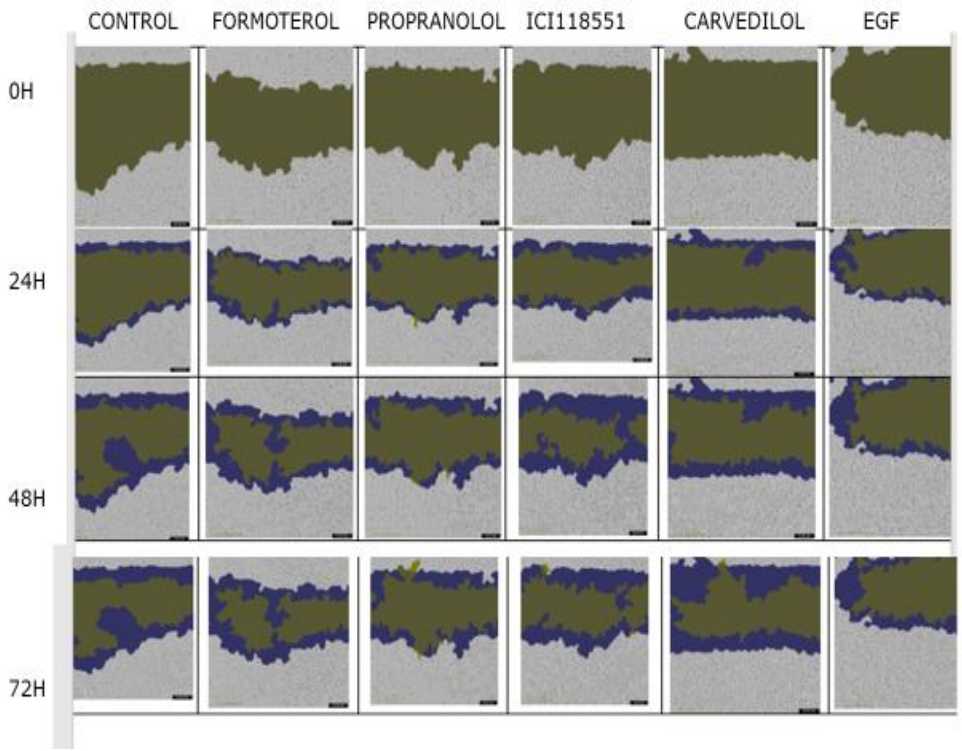
5.2.1.10 Real-time assessment of wound healing in CHO-β_2-cells

Wound healing in CHO-β_2-cells using the automated method was investigated over 66 hours. CHO-β_2-cells were seeded at a concentration of 5.0×10^4 cells/well in 96 well image lock

plates, which were coated with poly-l-lysine. Wounds were created using a wound maker as indicated in section 2.2.4.

5.2.1.11 Real-time analysis of wound healing in CHO-β_2-cells using wound confluence percentage

The created wounds from confluent monolayers of the CHO cells as shown in figure 5.5.1.a,were assessed using wound confluence percentage. The wound confluence percentage was significantly increased in Carvedilol ($p<0.01$) and EGF ($p<0.05$) treated wounds relative to control(figure 5.5.1.b) but did not produce any significant change in wound confluence percentage in forskolin ,formoterol ,salmeterol ,propranolol and ICI11851 relative to control. Also ,EGF+salmeterol and EGF+ ICI118551 did not produce any significant effect on wound confluence percentage relative to EGF(figure 5.5.1.b).



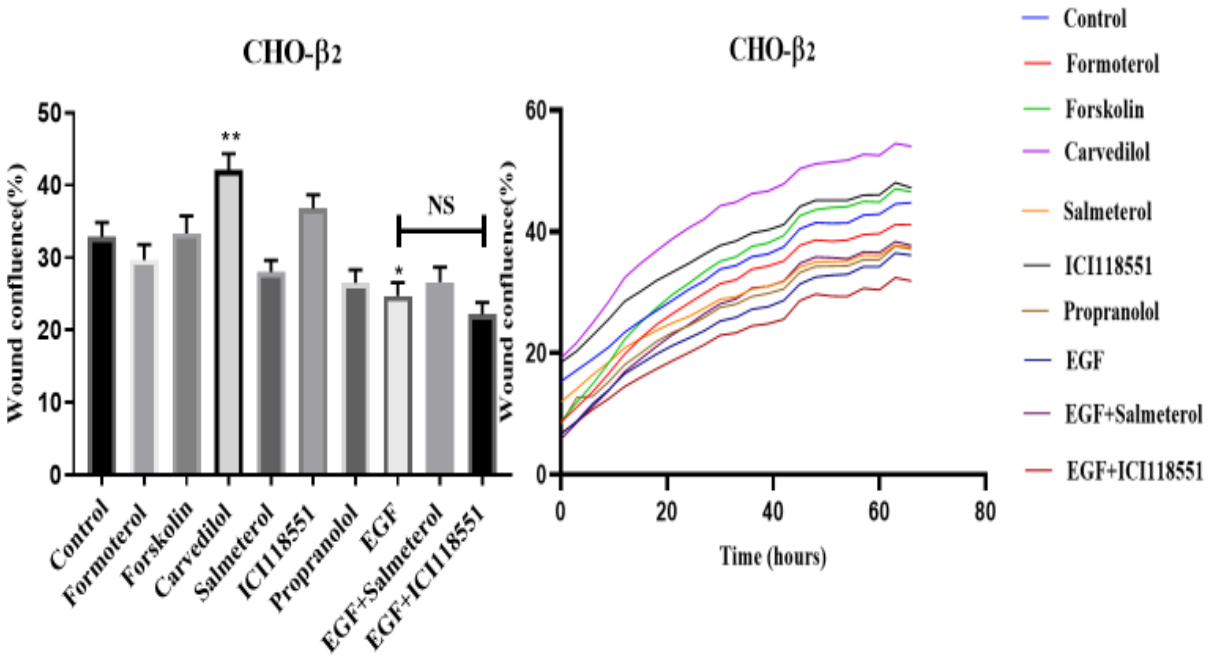


Figure 5.5.1. The effect of β2-adrenoceptor stimulation on wound healing in CHO-β2 cells.

(a) Histogram illustrating wounded confluent monolayers of CHO-β_2 cells.(b) The effect of β_2-adrenoceptor stimulation *wound confluence percentage was significantly increased in Carvedilol(1 μM) ($p<0.01$) and EGF(0.05) relative to control. However there was no significant effect on wound confluence percentage in forskolin(1μM),formoterol(100nM),salmeterol(1μM),propranolol(1 μM) and ICI11855(1μM relative to control. Time course closure of wounds expressed as wound confluence percentage which is a measure of cell confluence within the wound region over time. This is expressed as percentage of wound area occupied by cells. Images were captured 3hourly for 66hours using the IncuCyte S3 system. Data represented as mean ± SEM, analysed using Dunnett's post –hoc test following ANOVA.*

5.2.1.12 Real-time analysis of wound healing using relative wound density in CHO-β_2 cells.

The analysis of the wound healing assay using relative wound density (RWD) showed significant increase in RWD% in response to Forskolin($p<0.05$),figure 5.5.2 while,formoterol,carvedilol,salmeterol,ICI118551 and propranolol did not significantly affect RWD percentage relative to control. Furthermore , EGF+salmeterol significantly increased RWD percentage ($p\leq0.001$) relative to EGF. However , EGF+ICI118551 did not significantly reduce RWD percentage relative to EGF.

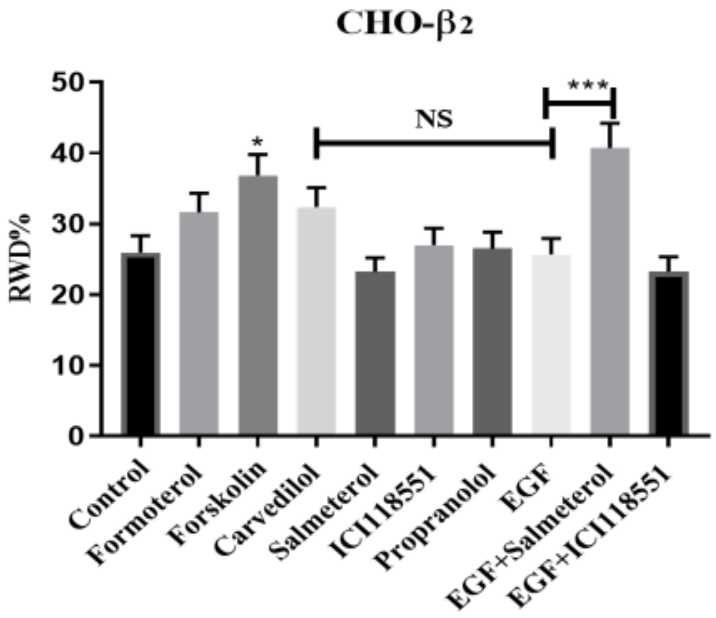


Figure 5.5.2 .The analysis of wound healing using Relative wound density in CHO-β2 cells.

The RWD percentage in CHO-β2 was significantly increased in forskolin ($p\leq0.05$)relative to control but, was not significantly increased in salmeterol, formoterol,carvedilol,ICI118551,EGF and propranolol. The EGF+salmeterol significantly increased RWD percentage($p\leq0.001$)relative to EGF but was not significantly reduced with EGF+ICI118551. N=3.Data represented as mean ± SEM, , analysed using Dunnett's post –hoc test following ANOVA.

5.2.1.13 Real-time analysis of wound healing in CHO-β_2 cells, assessed using wound width.

The real-time analysis of wound healing in CHO-β_2 cells using wound width showed the effect of β-adrenoceptor stimulation on wound edges. As shown in Figure 5.5.3, the wound width was significantly increased in salmeterol ($p \leq 0.0001$) and propranolol ($p < 0.05$) relative to control .There was no significant effect on wound width in formoterol, forskolin, carvedilol, ICI118551 or EGF relative to EGF.

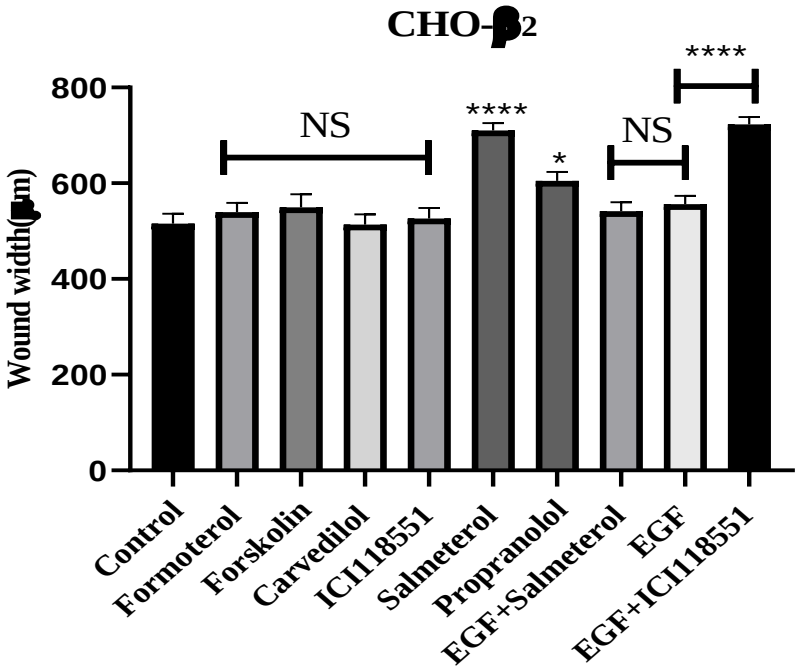


Figure 5.5.3. The analysis of wound healing using the wound width in CHO-β2 cells.

The wound width was significantly increased in salmeterol ($p \leq 0.0001$ and propranolol($p \leq 0.05$) but, was not significantly increased in formoterol,forskolin,ICI118551,carvedilol and EGF relative to control. EGF +ICI118551 significantly increased wound width($p \leq 0.0001$)but, did not reduce wound width relative to EGF. N=3.Data represented as mean ± SEM, , analysed using Dunnett's post –hoc test following ANOVA.

5.3 Discussion

Previously the alteration of wound healing by β -adrenoceptor agonists and inverse agonists was investigated in an ex vivo human skin model and in vivo murine tail wounding model, where β-adrenoceptor agonists were found to delay wound closure (Pullar *et al.,* 2006). β-adrenoceptor agonists have also been found to delay migration of keratinocytes and corneal epithelial cells (Ghoghawala *et al.,* 2008).The β-adrenoceptor agonist, isoprenaline delayed epidermal wound closure in adult newt limbs(Donaldson *et al.,* 1984).In contrast to these β-adrenoceptor antagonists, however ,improved wound healing by increasing the rate of wound repair in an ex vivo human skin model and increase the phosphorylation of ERK1/2 in the skin cells which increased migration and proliferation of keratinocytes (Pullar *et al.,*2007).In the management of wounds in burns patients , β-adrenoceptor antagonists healed their wounds at a faster rate(Arabi *et al.,*2007).The effects of β-adrenoceptor stimulation in wound healing in airway epithelial cells was previously investigated in normal human bronchial epithelial cells and Calu-3 cells. Salbutamol significantly decreased wound migration and pre-treatment of wounds with β-adrenoceptor antagonist had no effect on migration of cells (Peitzman *et al.,* 2015).

The closure of wounds occurs by two main physiological processes of cell migration and proliferation as previously investigated (Eming *et al.,* 2007).In the previous chapter4, the assessment of β-adrenoceptor agonists and inverse agonists showed significant effects with MTT reduction assay and IncuCyte S3 system analysis. This was observed in the significant on MTT reduction in Calu-3 and BEAS2BR-1 cells with propranolol and nadolol (figure 4.1). Furthermore, the neutral red assay in BEAS2B-R1 cells showed a significant increase in cell viability with propranolol treated cells(figure 4.9). To improve the wound healing assay , even sized wounds which might improve consistency of data sets using the automated method of the IncuCyte wound maker and IncuCyte S3 system as described in section 2.2.7

In this chapter (5) propranolol increased wound confluence percentage in Calu-3 cells(figure 5.4.1) .The effect of propranolol in 3 techniques suggests the proliferative effect of propranolol in wound healing in Calu-3 cells. However, in this chapter ,propranolol reduced wound confluence percentage(5.3.1)and increased wound width (5.3.2)in BEAS2BR-1 cells . As a result of this, wound healing was delayed despite the increase in RWD by propranolol(5.3.3) in BEAS2BR-1 cells . Furthermore ,propranolol increased wound width in CHO-β_2 cells and delayed wound healing (5.5.3).These findings have demonstrated the effect of effect of β-adrenoceptor stimulation by showing different results across 3 cell lines.

The chapter aimed to increase the understanding of the role of β-adrenoceptor stimulation in wound healing in human airway cells. In BEAS2BR-1 cells for instance formoterol, forskolin ,carvedilol and propranolol,ICI118551 and EGF significantly delayed wound healing because the wound confluence percentage was reduced (5.3.1) and wound width was increased at the end of the experiment relative to control(figure 5.3.2). The significant effect of forskolin in the delay in wound healing suggest that the effect of formoterol was cyclic AMP mediated. Furthermore, despite the significant increase in RWD in formoterol,ICI118551,propranolol and EGF, wound healing was delayed in BEAS2BR-1 cells(5.3.3).

In Calu-3 cells, β_2-adrenoceptor inverse agonist ICI118551 significantly delayed wound healing as shown by the significant increase in wound width(figure 5.4.2). The significant reduction of RWD in ICI118851(figure 5.4.3) confirmed the significant delay in wound healing, despite the significant increase in wound confluence percentage in ICI118551(Figure 5.4.1).The Epidermal growth factor significantly increased wound confluence percentage(Figure 5.4.1).However,EGF+ICI118551significantly increased wound width relative to EGF(5.4.2) and delayed wound healing. The increase in wound width in EGF+ICI118551 could be due to the effect of ICI118551(5.4.2) .Forskolin

significantly increased wound confluence percentage(figure 5.4.1) with no significant effect in wound width or RWD percentage. In view of this, the role cyclic AMP in wound healing in Calu-3 cells could not be confirmed.

The β_2-adrenoceptor agonists salmeterol and β_2-adrenoceptor inverse agonist propranolol significantly delayed wound healing by increasing the wound width in CHO-β_2 cells relative to control (5.5.3). Carvedilol significantly increased wound confluence and EGF significantly decreased wound confluence (5.5.1). The RWD was however significantly increased with forskolin in CHO-β_2 cells and EGF+salmeterol (5.5.2).The wound with was significantly increased in salmeterol and propranolol relative to control. The wound width was also increased in EGF+ICI118551 relative to EGF(5.5.3). From the effect of the β_2-adrenoceptor stimulation in the CHO-β_2 cells, none of the treatments significantly affected wound healing when analysed using the all the 3 parameters.

Previously the ability of the respiratory epithelium to repair after injury by cell proliferation and migration was studied in human surface epithelial cells wounded by NaOH solution.(Zahm *et al.,*1997).In the management of patients with acute lung injury ,salbutamol ehanced wound repair within 72 hours of treatment which was in contrast to animal studies.(Perkins *et al.,*2007). Furthermore isoprenaline and salbutamol reduced keratinocyte cell migration via ERK1/2 and p38 dependent mechanisms(Steenhuis et *al.,*2011).However, in human dermal fibroblast cells, isoprenaline enhanced wound migration(Pullar and Isseroff 2006).The EGF has been studied as a promotor of wound repair in airway epithelial cells(Burgel and Nadal 2004).In contrast to these findings, EGF did not enhance wound healing in all the 3 cell lines despite producing a significant increase in wound confluence in Calu-3 cells(figure 5.4.1) Furthermore, the reproducibility of most wound scratch assays are difficult to investigate because of the methods used to create the wounds(Kraimer *et al.,* 2013) .An approach which involved using a 2 dimensional method is routinely used to investigate the ability of cells at wounded regions to migrate into the wounded region. The

most common methods of wounding are the removal of a section of a confluent monolayer using instruments(Ashby and Zilstra 2012).

In the IncuCyte real time imaging analysis has better rate of reproducibility than previous conventional methods of using pipettes, razors or chemicals to create wounds. This was previously investigated in PC-3 (Prostrate cancer cells) in which cell migration was studied to determine the best outcome of the scratch assay by reporting the initial starting density of cells in the wounded region(Jin *et al .,*2016).

The analysis of data obtained from the study showed different responses to β_2-adrenoceptor stimulation in the three cell lines. Based on the 3 types of analyses of the result, the wound width provided the most suitable method of analysing wounds. This is because the wounds were created with a wound maker and the reduction or increase in wound size gave the most precise method of measuring wound size. The wound confluence percent showed the confluence of cells of the wounded region which may not have migrated to all the areas of the wounded region. The RWD provides the density of the cells that migrated to the wounded region. Therefore, the migration of the cells might not be evenly distributed across the healed area.

Activation of the β_2-adrenoceptor in Calu-3 cells by the selective β_2-adrenoceptor agonist formoterol significantly delayed wound healing and repair as shown by the increase in wound width by formoterol relative to control(figure 5.4.2). This delay in wound healing was previously reported with β_2-adrenoceptor agonist salbutamol in Calu-3 cells (Peitzman *et al.,* 2015).The treatment of Calu-3 cells with β_2-adrenoceptor inverse agonist propranolol, and ICI118551 significantly reduced wound width in Calu-3 cells which is consistent with previous experiments with these drugs, in which addition of β_2-adrenoceptor antagonists delayed wound healing (Pullar *et al.,* 2008). A similar finding was observed in this present study , which showed that ICI118551 delayed wound healing across all the 3 analysis methods in Calu-3 cells(figure 5.4.2).The β_2-adrenoceptor inverse agonist propranolol also

delayed wound healing in BEAS2BR-1 cells as shown by decrease in wound confluence percentage(figure 5.3.1), wound width(5.3.2) and relative density(figure 5.3.3) .In CHO-β_2 cells, propranolol also significantly increased wound width and delayed wound healing as shown(figure 5.5.3) . Furthermore, the effect of carvedilol in wound healing, a β_2-adrenoceptor antagonist was also investigated in this chapter. The wound confluence percentage was significantly increased in CHO- β_2 cells (5.5.1) and Calu-3 cells (5.4.1). However, in BEASBR-1cells,carvedilol significantly reduced wound confluence percentage(figure 5.3.1) and increased wound width (figure 5.3.2). The delay in wound healing by carvedilol could not be confirmed in CHO- β_2 and Calu-3 cells but ,in BEAS2R-1 cells carvedilol significantly delayed wound healing. This is because, carvedilol significantly reduced wound confluence percentage and wound width. This is consistent with previous studies .For example ,carvedilol, a β_2-adrenoceptor antagonist known to inhibit the cyclic AMP pathway and facilitate β-arrestin-dependent signalling (Kim *et al.,* 2008), was previously reported to inhibit wound repair in airway epithelial cells. The β_2-adrenoceptor agonist salbutamol also inhibited airway epithelial cell wound repair (Peitzman *et al.,* 2015). In order to investigate the mechanisms which regulate bronchial epithelial repair in the bronchial mucosa, the effect of EGF on wound healing was studied in this chapter. Previously the effect of EGF in broncho epithelial repair was investigated in asthmatic bronchial mucosa and, was discovered to initiate wound repair (Puddicombe *et al.,* 2000). In view of this, the effect of EGF on wound healing was studied in all three cell lines. In this chapter, the wound confluence percentage was significantly increased by EGF in Calu-3 cells (relative to control) , significantly reduced by EGF+salmeterol and EGF+ICI118551 relative to EGF (figure 5.4.1).The wound width was reduced in Calu-3 cells in EGF+ICI118551 relative to EGF(figure 5.4.2).In CHO- β_2 cells ,EGF significantly reduced wound confluence percentage relative to control while EGF+salmeterol increased RWD relative to EGF(figure 5.5.2).The wound width was significantly increased in

EGF+ICI118551 relative to EGF(figure 5.5.3) in CHO- β_2 cells . In BEASBR-1 cells however, EGF significantly increased RWD (figure 5.3.3) and significantly decreased the wound confluence percentage relative to control(figure 5.3.1). Furthermore, the wound width was significantly decreased in EGF+ICI118551(figure 5.3.2) relative to EGF.

5.4 Conclusion

The findings from this study are consistent with previous data which showed the inhibitory effects of β_2-adrenoceptor stimulation on wound healing and repair. The effect of carvedilol acting a β_2-adrenoceptor antagonist, as a bias ligand by producing similar results as the β_2-adrenoceptor agonist salmeterol ,suggests that the delay in wound repair is cyclic AMP dependent. Also ,the significant increase in wound confluence forskolin in Calu-3 cells suggests a cyclic AMP dependent role in wound healing and repair (figure 5.4.1).

Overall the effects of the a β_2-adrenoceptor agonists and inverse agonists/antagonists suggest a possible unwanted effect of these drugs in asthma therapy would be a delay in wound healing. Also, given the reported positive effect of EGF in asthma, the findings presented in the present study support the use of EGF in the management of asthma because it significantly increased wound confluence in Calu-3(figure 5.4.1) but, reduced the wound confluence BEAS2BR-1 cells (figure 5.3.1) and CHO- β_2 cells(figure 5.5.1) .

BEAS2BR-1

(a)

β2-adenergic agonist/inverse agonist/EGF	Wound confluence(%)	Relative wound density %	Wound width(μM)
Forskolin (10μM)	*** ↓	NS	**** ↑
Formoterol (100nM)	**** ↓	*	**** ↑
Salmeterol(1μM)	NS	NS	NS
Propranolol(1μM)	**** ↓	NS	**** ↑
Carvedilol (1μM)	**** ↓	NS	**** ↑
ICI118551 (1μM)	**** ↓	*	NS
EGF (5ng/ml)	*** ↓	****	NS
EGF+ salmeterol	NS	NS	NS
EGF+ICI118551	NS	NS	**** ↓

Calu-3 cells

(b)

β2-adenergic agonist/inverse agonist/EGF	Wound confluence(%)	Relative wound density %	Wound width(μM)
Forskolin (10μM)	**** ↑	NS	NS
Formoterol (100nM)	NS	NS	- ↑
Salmeterol(1μM)	**** ↑	NS	NS
Propranolol(1μM)	**** ↑	NS	NS
Carvedilol (1μM)	**** ↑	NS	NS
ICI118551 (1μM)	** ↑	** ↓	**** ↑
EGF (5ng/ml)	**** ↑	NS	NS
EGF+ salmeterol	**** ↓	NS	NS
EGF+ICI118551	**** ↓	NS	**** ↑

CHO-β2 cells

(c)

β2-adenergic agonist/inverse agonist/EGF	Wound confluence(%)	Relative wound density %	Wound width(μM)
Forskolin (10μM)	NS	*	NS
Formoterol (100nM)	NS	NS	NS
Salmeterol(1μM)	NS	NS	**** ↑
Propranolol(1μM)	NS	NS	* ↑
Carvedilol (1μM)	**	NS	NS
ICI118551 (1μM)		NS	NS
EGF (5ng/ml)	*	NS	NS
EGF+ salmeterol	NS	*** ↑	NS
EGF+ICI118551	NS	NS	**** ↑

Table 5.1 Summary of IncuCyte wound healing assay real-time analysis results.

The table (a)Shows BEAS2-R1 cells(b)Calu-3 and (c) CHO-β_2 cells wound healing analysis showing the effects of the β-adrenoceptor stimulation in wound healing, using wound confluence percentage, RWD and wound width.

Chapter 6
Air liquid interface experiments

6.1 Introduction

Air liquid interface experiments previously investigated involved the culture of cells on micro porous membranes which permit the addition of cell culture media in the basal side of the membrane. The apical part, however, is in direct contact with cells and the humidified air. These partitions mimic the physiological condition s of the human respiratory system (Rasmussen *et al.*, 1984).Also the air liquid interface represents in vivo exposure conditions of lungs better than in submerged cell culture(Paur *et al.,* 2011).The air liquid interface experiments have been used to investigate cell morphology, fluid and ion transport, inflammation and cell signalling (Pezzulo *et al.,* 2010). Airway epithelial cells line the respiratory airways in the form of pseudo epithelial cells which only some of them reach the luminal surface of the basement membrane. Also, these cells form barriers in the form of tight junctions which separate apical from basal surfaces of cells. The apical cell surface only requires small amount of fluid which separate the cells from gases of the lumen (Pezzullo *et al.,* 2010). Previously, forskolin was used to investigate cystic fibrosis transmembrane conductance regulator (CFTR) of chloride ion transport channels in airway epithelial cells in vitro cultured in air liquid interface (Zabner *et al.,* 1998). The effect of isoproterenol on Chloride ion transport was also investigated in CFTR using short circuit measurements for measuring transepithelial differences(Mizuno et al.,2000). In this chapter cells were grown on 12 well trans well inserts until confluent then medium in the apical compartment was removed and cells were raised to air liquid interface for 3 days. Measurements of Transepithelial electrical resistance were recorded using chopstick electrodes and an EVOM voltmeter(STX-2) and Evom G, World Precision Instruments, Stevenage UK).

The adenocarcinoma derived human bronchial epithelial cell line; Calu-3 has been investigated as one of the few cell lines that are able to form tight junctions in vitro. This has made it possible for Calu-3 cells to be used in air way modelling research (Grainger *et al.*,

2006). The Calu-3 cells when studied under air liquid interface (ALI) assume a differentiated and functional form making the cells suitable for drug delivery and toxicological research (Forbes *et al.,* 2005). Other bronchial epithelial cell line lines cultured at ALI and differences in cell morphology, biology and biochemistry were previously investigated and identified in rat tracheal epithelial cells. The cells differentiated into ciliated cells similar in number with basal and goblet cells. The number of ciliated cells was reduced in submerged cultured cells (Kaartinen *et al.,* 1993). Cells cultured under ALI were exposed to oxygen which is attributed to increase in the number of ciliated cells (Ehrhardt *et al.,* 2002). Changes in cellular morphology were investigated in Calu-3 cells, which formed tight junctions, secreted more mucous, produced more columnar epithelium and rugged topography in air liquid interface than in liquid culture (Grainger *et al.,* 2006). The BEAS-2B cells differentiate into a squamous phenotype but, do not form ciliated cells, tight junctions or secrete mucus (Penn *et al.,* 2005). The 16HBE14$_{O}$ form cilia and tight junctions but do not produce mucus (Forbes *et al.,* 2003) while the normal human bronchial epithelial cells (NHBE) form tight junctions but culturing them in the laboratory is labour intensive (Gray *et al.,* 2007). As stated in these studies, the Calu-3 cells are the most suitable cells to investigate the presence and formation of tight junctions and mucous secretion at air liquid interface. This was previously investigated using transepithelial electrical resistance measurements, microscopy and permeability. Calu-3 cells grown in ALI produced a model morphologically similar to the airway epithelium (Grainger *et al.,* 2006).It has also been reported that NHBE cultured under ALI produced a maximum Transepithelial resistance measurement of 766 ±154Ωcm^{-2} on the 8th day of measurement .The morphological characteristics were observed using light microscopy and the formation of tight junctions which were cultured at ALI were evaluated for fitness as suitable models for airway research by measuring transepithelial electrical resistance.

The aims of the chapter are;

To investigate the whether Calu-3 cells are suitable models for asthma cultured using the ALI system.

To investigate the effects of β_2-adrenoceptor stimulation on transepithelial electrical measurements in Calu-3 cells.

To investigate the effects of β_2-adrenoceptor stimulation on mucin production in Calu-3 cells.

6.2 Results

6.2.1 Transepithelial electrical resistance (TEER) measurements in Calu-3 cells

Calu-3 cells were cultured to confluence between passages 12 and 26 in 10-14 days. The ALI experiments were performed at passages 13-35.

At the time of the investigations the Calu-3 cells achieved confluency within 5 days at passages 33-35 and in passages 12-15 confluency was achieved within 15-21 days. Also, the Calu-3 cells produced mucus which was visibly seen from the 14^{th} day when the apical compartment was washed with Hank's balanced salt solution. This indicates Calu-3 cells at higher passages of 33-35 attained confluency faster than Calu-3 cells at lower passages of 12-15.

When seeded in 12 well inserts (12mm in diameter and 0.4μm pore size), the Calu-3 cells formed tight epithelial monolayers post ALI after 21 days. To investigate the integrity of the monolayers of Calu-3 cells, TEER measurements were obtained when the cells were raised to air liquid interface. This was achieved when the apical medium of the inserts was removed. The TEER measurements showed the Calu-3 cells seeded at 250000 cells per well produced a measurable TEER from the 3^{rd} day of measurement and onwards. Also on visual inspection the Calu-3 cells, tightly packed monolayers were observed. In Figure 6.1,confluent monolayers developed increasing TEER measurement of $>1000\Omega/cm^2$ ($p\leq0.0001$) relative day 0, which was achieved on the 10^{th} day post ALI as shown. A non-significant reduction in TEER was recorded on days 11,15 and 19.On the 14th ,17th and 21^{st} days of measurement the TEER significantly increases relative to day 0 with TEER values of $855\Omega/cm^2$($p\leq0.001$),$1003\Omega/cm^2$ ($p\leq0.0001$)and $917\Omega/cm^2$ ($p\leq0.0001$) respectively. However the TEER on the 14^{th} day of measurement was significantly reduced ,($p \leq0.05$)relative to the TEER measured on day 10^{th} day of measurement.

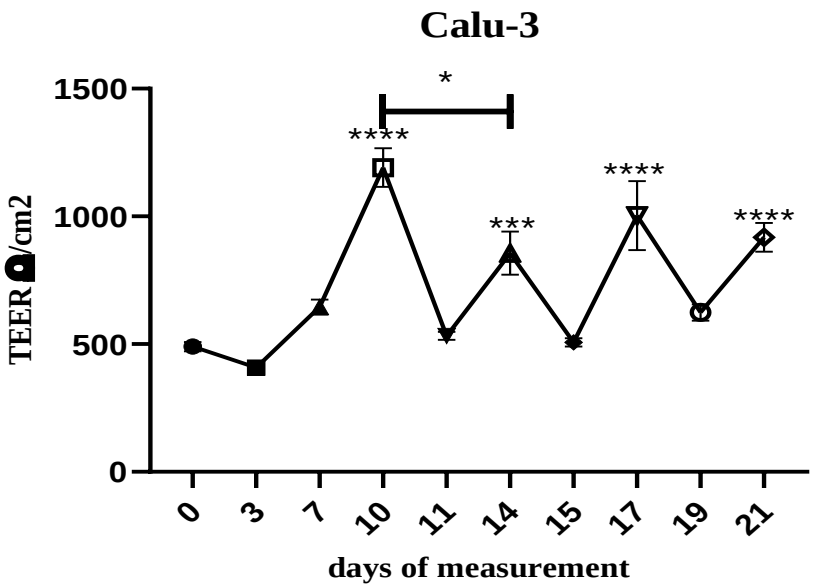


Figure 6.1. Sequential changes in transepithelial electrical resistance in Calu-3 cells.

*Calu-3 cells were seeded in inserts at 2.50x10^{5} cells per well until 100% confluent(as described in section 2.3.8 for details) .Then raised to air liquid interface for 3 days during which the medium in the apical compartment of the trans wells was removed. Measurements were recorded on days shown and plotted as a function of time from 6 independent experiments showing sequential changes in TEER . The data represents Mean ± standard error of the mean. (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

6.2.2 The effects of β_2-adrenoceptor ligands on tight junction formation

To investigate the whether β_2-adrenoceptor ligands have any effect that would alter tight junction formation, Calu-3 cells were treated with formoterol and propranolol on day 21 of ALI culture. After the attainment of the TEER of peak values(Figure 6.1) ,the Calu-3 cells were stimulated with the β_2-adrenoceptor agonists, inverse agonists as displayed in (Fig6.2) The treatments were added to the apical compartment of the trans wells while the basolateral compartment only had cell culture media.

In the initial part of the experiment, the β_2-adrenoceptor agonist formoterol and the β_2-adrenoceptor inverse agonist propranolol significantly decreased TEER ($p \leq 0.05$) and ($p \leq 0.01$) after 30 min stimulation relative to control(Fig 6.2a). To determine the effect of the treatments beyond this 30-minute stimulation, TEER was recorded after 24 h (Fig 6.2b). The data showed a non-significant decrease in TEER cells treated with propranolol and formoterol.

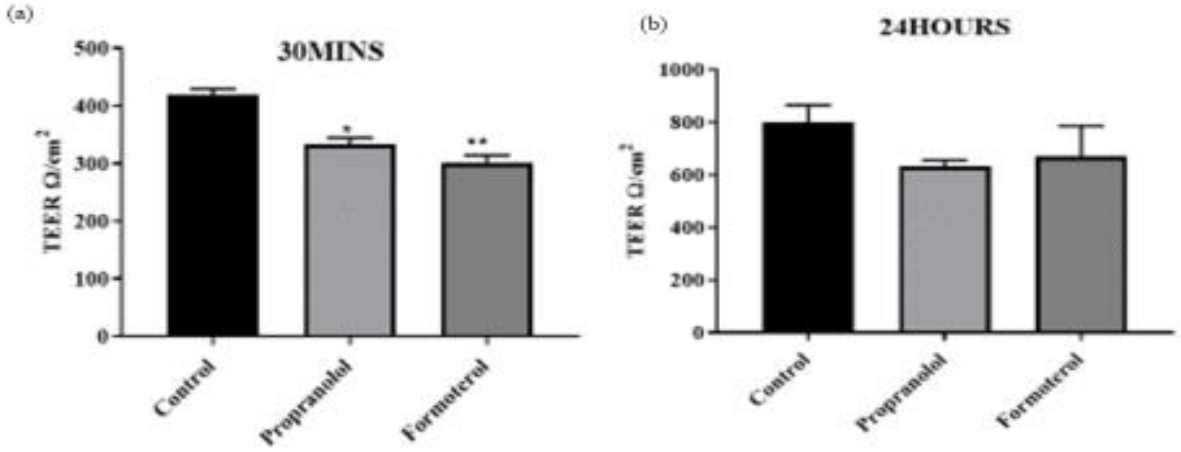


Figure 6.2 The effects of β2-adrenoceptor agonist and β2-adrenoceptor inverse agonist on TEER measurements in Calu-3 cells. *Calu-3 cells were seeded in inserts at 2.50x10^5 cells per well until 100% confluent, then raised to air liquid interface for 3 days after removal of the medial in apical compartment of the trans wells. Treatments were added after day 21measurements for 30 mins (a) and 24h (b)Propranolol (1μM) and Formoterol (100nM) were added to duplicate wells. N=3The data represents Mean ± standard error of the mean. (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

To determine the effect of the β_2-adrenoceptor agonist and inverse agonist over 7 days, the treatment was prolonged and a significant increase in the measured TEER was obtained in the formoterol and propranolol relative to control ($p \leq 0.05$)(Fig 6.3).

During the duration of treatment, the medium in the basal compartment of the trans well inserts was not changed (Fig 6.3).

To investigate whether the inverse agonist blocked the activity of the β_2-adrenoceptor agonist, the formoterol and propranolol were added in combination. Propranolol was added to the cells for 30 min prior to addition of formoterol.

A significant decrease in the TEER was displayed in the Formoterol+propranolol combination relative to formoterol,($p \leq 0.05$)(Fig6.3b). Furthermore, effect of Formoterol+propranolol on TEER was also significantly reduced relative to propranolol($p \leq 0.05$), (figure 6.3c).

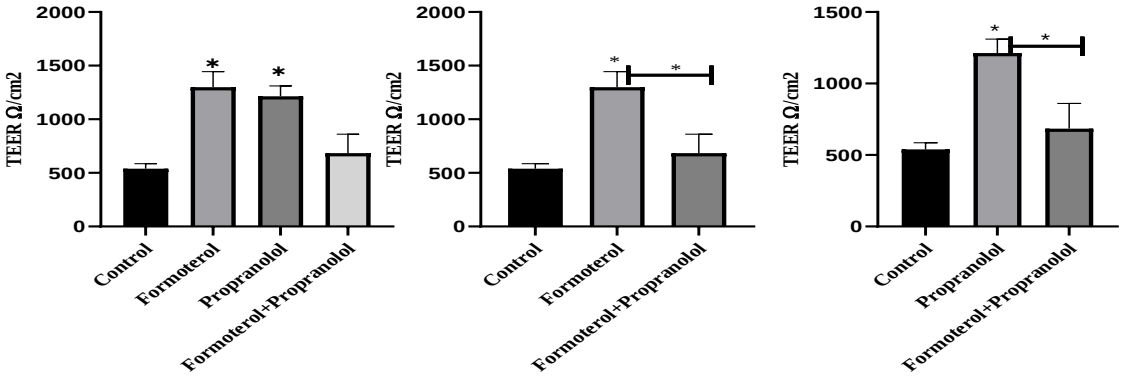


Figure 6.3 The effects of β2-adrenoceptor agonists and β2-adrenoceptor inverse agonists on TEER measurements in Calu-3 cells over 7 days.

Calu-3 cells cultured at air liquid interface were treated with formoterol (100nM) and propranolol (1μM). The treated cells were incubated at 37^{0}C for 7 days and TEER measurements were recorded. TEER measurements in formoterol treated cells increased significantly ($p<0.01$) relative to control. The measurements also increased significantly in propranolol-treated cells ($p<0.05$). In cells stimulated with both formoterol and propranolol, the TEER significantly decreased relative to formoterol and significantly reduced relative to propranolol. Data represent 3 independent experiments, Mean ± SEM. ($p<0.05$, **$p<0.01$, ****$p<0.0001$ analysed using Dunnett's post –hoc test following ANOVA).*

The effect of β_2-adrenoceptor agonists, inverse agonists and EGF were investigated. The measurements as shown(Figure 6.4) displayed significant reduction of TEER relative to day 0 ($p \leq 0.0001$) on days3,7,11 and 15. Then, the β_2-adrenoceptor agonist formoterol ,inverse agonists propranolol,ICI118551 and EGF were added to the apical compartment of the trans wells in duplicates. In this experiment the medium in basal compartment of the trans well inserts were changed after 3 days. The experiment was performed within passages 33-35 from 3 different frozen vials.

The Calu-3 cells developed a non-significant TEER of 762Ω/cm^2 on day 0.This further declined highly significantly to 355Ω/cm^2 on day 3 to 305Ω/cm^2 on day 7, 349Ω/cm^2 on day 11and 561Ω/cm^2 on day 15 ($p<0.0001$) relative to day 0(Figure 6.4a). Then the confluent monolayers were stimulated with propranolol, formoterol and a combination of both while the untreated wells were the controls. As shown (Figure 6.4b)The formoterol and propranolol produced a non-significant increase in TEER relative to control. However, the formoterol + propranolol treated wells produced a non-significant reduction in TEER relative to formoterol and propranolol while,ICI118551 and EGF treated wells produced a non-significant reduction in TEER.

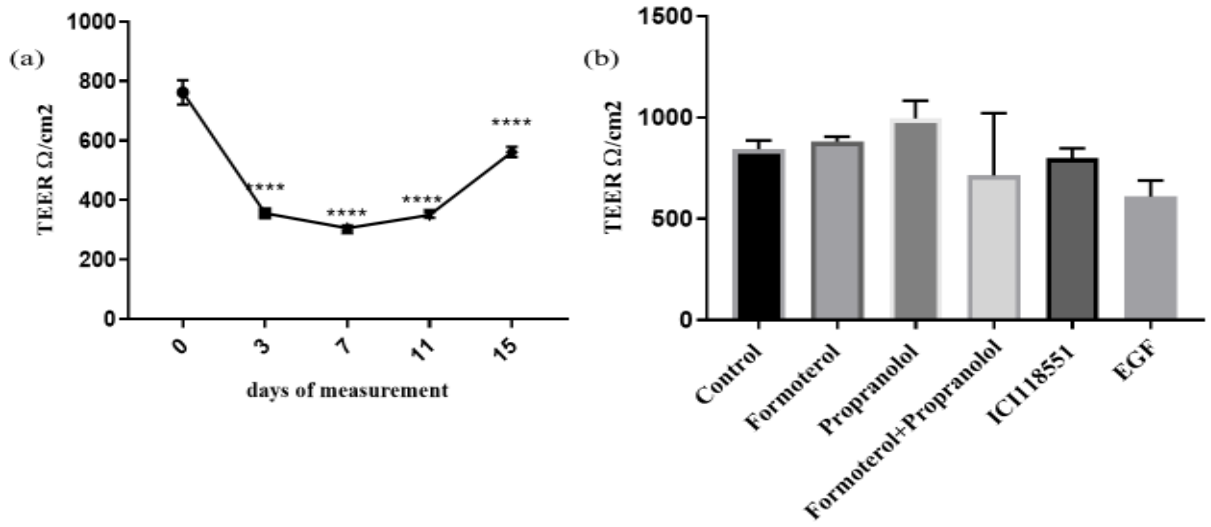


Figure 6.4 Transepithelial electrical resistance measurements in Calu-3 cells using Air liquid interface.

*Calu-3 cells were seeded in 12 well trans well inserts at 2.50x10^{5} cells per well until 100% confluent, then raised to air liquid interface. Measurement were recorded on days shown plotted as a function of time from 3 independent experiments (a). The data represents Mean ±Standard error of mean (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).Following TEER measurements, the confluent monolayers were stimulated with formoterol (100nM), propranolol (1µM), ICI118551(1µM), EGF or combinations of treatments in duplicate in each case. The treated cells were incubated at 37^{0}C for 7 days and TEER measurements were recorded. The medium in the basolateral compartment of the trans well inserts after was removed for the period of treatment .The TEER measurements in propranolol and formoterol treated cells produced non-significant increase relative to control . Non-significant reduction in TEER were recorded in EGF,ICI118551 relative to control while formoterol +propranolol did not significantly produce a reduction in TEER relative to formoterol or propranolol .. Data represent 3 independent experiments, Mean ± SEM. (*p<0.05, **p<0.01, ****p<0.0001 analysed using Dunnett's post –hoc test following ANOVA).*

6.2.3 The effects of β_2-adrenoceotor agonists and inverse agonists on Mucin production in Air liquid interface.

To investigate whether the β_2-adrenoceptor agonist formoterol and the β_2-adrenoceptor inverse agonists propranolol affect mucus production in Calu-3 cells, cultured at ALI. After sequential measurements of TEER ,the treatments were added to basal compartment of the trans wells while the medium for 7days while ,the medium in the apical compartments was removed . The relative intensity of MUC5AC did not produce any significant effect in both formoterol and propranolol treated wells(Figure 6.5 a and b).

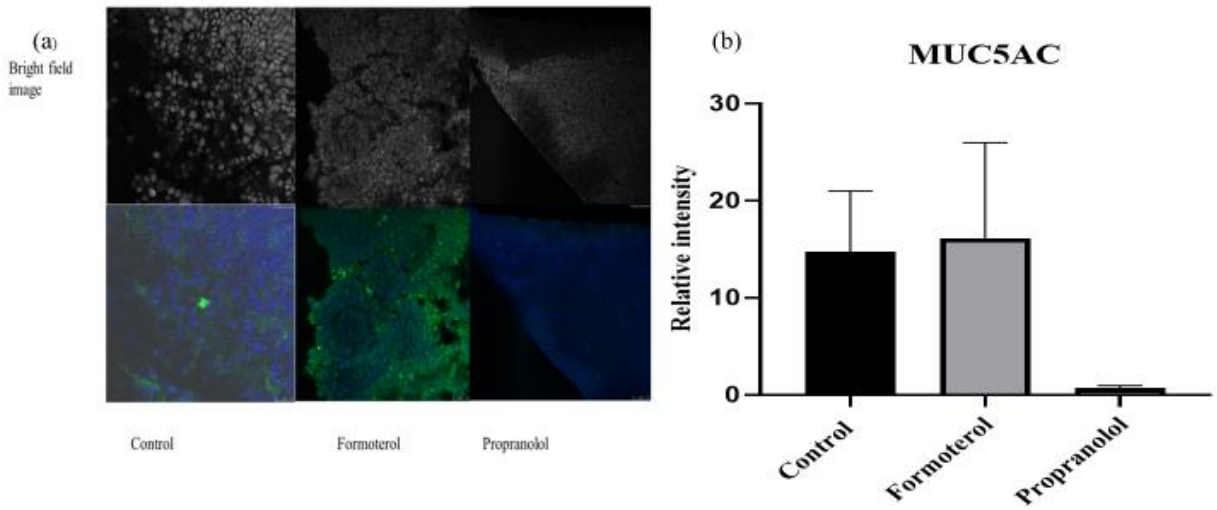


Figure 6.5 The effects of β2-adrenoceptor ligands in Mucin production Calu-3 cells raised to Air liquid interface.

The Calu-3 cells cultured at air liquid interface were stimulated with formoterol (100nM), propranolol(1µM) for 7 days and incubated at 37^{0}C for 7 days and TEER measurements were recorded. The treatments were added to the basolateral compartment of the trans wells while the media in the apical compartment was removed. Then the cells were washed with PBS and Fixed with 95% ice cold methanol(v/v) at -20^{0}C for 10mins. Then the cells were permeabilised with 0.1% Triton X at room temperature for 10mins. Cells were then incubated in the primary antibody (Mucin) for 24 hours .The cells were then incubation secondary antibody(FITC) for 1 hour (blue) and washed with PBS and counter stained with DAPI(green). The experiments was performed as in section 2.2.7 in the methods sections. Images were captured using confocal microscopy sp5 .The representative images of 2 independent experiments are shown including their corresponding bright field images.(b)Quantification of MUC5AC intensity expressed as the percentage intensity of mean of MUC5AC in each well to the mean of the control well ,data presented as relative intensity relative to control. The relative intensity in formoterol treated wells did not significantly increase MUC5AC expression and did not significantly reduce MUC5AC expression in propranolol treated wells.

6.3 Discussion

In this chapter, the human bronchial epithelial cell line Calu-3 cell was evaluated as ALI model for asthma research. Functional properties of confluent monolayers of Calu-3 cells in ALI were investigated using TEER measurements. The TEER measurements were used to investigate the formation of the tight junctional protein ,zonula occludens-1(ZO-1). Using the β_2-adrenoceptor agonist formoterol and β_2-adrenoceptor inverse agonist propranolol ,the

effect of these drugs on the integrity of tight junctions in Calu-3 cells at ALI were determined. Both formoterol and propranolol significantly increased TEER. The effects of β_2-adrenoceptor agonist formoterol and β_2-adrenoceptor inverse propranolol on the of goblet cells marker,MUC5AC was investigated using immunofluorescence techniques. MUC5AC is a mucous protein expressed by goblet cells in lung epithelium(Rodgers 2003), while the zonula occludens -1 (ZO-1) is one of the markers of tight junction formation and barrier integrity. The main outcomes of the results showed the establishment of tight junctions indicated by the TEER measurements(figure 6.1) that showed passage number variations in the investigations(figure 6.1 and figure 6.4). The MUC5AC is major gene expression in mucin in the goblet cells in bronchial asthma. Formoterol was previously investigated for its ability to increase the production of mucous in NHBE cells. Formoterol enhanced the production of mucous by mimicking the effect of epinephrine(Al Sawalha et al., 2014).Excessive production of mucin is the hallmark of asthma in the airways. In the management of asthma chronic bronchodilator use has been associated with worsening of symptoms which results in narrowing of the airways and obstruction.

6.3.1. Modulation of Tight Junctions in Calu-3 cells raised in Air liquid interface.

Establishment of tight junctions in Calu-3 cells

The formation of tight junctions in Calu-3 cells has been reported when the cells were cultured in ALI, which produced a cell layer similar to the airway epithelial morphology and electrical resistance in vivo (Krouse et al.,2004). Although there is no agreed method for

culturing Calu-3 cells for the purpose of ALI experiments, differences in cell morphology and functionality of cell layers have been observed which could be due to different cell culture conditions and passage numbers (Grainger *et al.*,2006). The development of a confluent monolayer which formed tight junctions was associated with increasing TEER in Calu-3 cells .This was previously investigated in Calu-3 cells in which a peak TEER value was displayed that later gradually declined (Cereijido *et al.*,1983). This was suggested to be due to the increase in number of cells which accumulate on the monolayer and make it over confluent because, increase in the length of cells perimeter per unit area would cause a decrease in the TEER. The absence of TEER reduction was previously reported in growth of arrested growth of cells with an increase in perimeter of monolayer (Francis *et al*,1999; (Wan *et al.,* 2000). The main outcome of the study showed variation of TEER with experiment and passage number (figures 6.1 and 6.4). The measured TEER significantly reached peak values on day 10 relative to day zero TEER (Figure 6.1). The measurement on day 14 was significantly decreased relative to day 10 TEER. The TEER on day 14,19 and 21 significantly increased relative to day 0(Figure 6.1). The initial results indicated the formation of tight junctions as displayed with the increase in TEER measurements. The TEER measurements of Calu-3 cells were previously reported to have increased from 327Ωcm^2 from 6th day and declined to 120Ωcm^2 on the 14th day of measurements (Wan *et al.*, 2000). However, from the present study the measured TEER on the 10th day was1191Ω/ cm^2 and 917 Ω/cm^2 on day 21st day (Fig 6.1). It is important to note from these findings that the measurements from this study could be due to variations in passage number of Calu-3 cells used in the study or other interlaboratory conditions that may exist .In this study Calu-3 cells were used from passages 12-35. Cells culture in the ALI experiments at higher passages(33-35) attained confluency faster in the 12 well inserts within 5 days(figure 6.4a) than cells obtained from lower passages(12-15) which became confluent within 15-21

days(figure 6.1).The TEER measurements significantly decreased relative to day 0 TEER and attained a significant peak TEER on day 15 relative to day 0 (Figure 6.4a).

6.3.2. The role of β_2-adrenoceptor agonists and inverse agonists in tight junction formation

In this chapter, ALI experiments were investigated in Calu-3 cells as demonstrated by the establishment of tight junction formation. The TEER measurements and immunohistochemical staining of MUC5A to investigate the expression of mucin protein in goblet cells Calu-3 cells.

The use of Calu-3 cells in this investigation revealed insights into mechanisms of tight junction formation in human airway cells. The results obtained showed the effects of the β_2--adrenoceptor agonist, inverse agonists and epidermal growth factor(EGF) on TEER measurements. Previously, the effect of EGF on the disruption of tight junctions in asthmatic airway epithelial cells cultured in ALI was investigated (Xiao *et al* ., 2011) . The epithelial airway of asthmatics raised to air liquid interface was reported to increase cytokine production when exposed to viral agents and mechanical injury more than in non-asthmatic airway epithelium. This suggests that an abnormal inflammatory response due to altered epithelial lining may be behind the abnormal increase of cytokines in asthmatics (Hackett et al., 2011).

In the present study, the stimulation of Calu-3 cells on the apical compartment of the trans wells (Fig 6.2 b), showed a significant decrease in TEER after 30min stimulation of confluent monolayers with formoterol and propranolol relative to control. However when the confluent monolayers were stimulated for 7 days (without changing of the media in the basolateral compartment), showed significant increase in a measured TEER in formoterol and propranolol of $> 1000\ \Omega\ /cm^2$ relative to control (Fig 6.3a). The possible explanation for formoterol increase in TEER could be the agonist stimulation of the β_2-adrenoceptor .

The increase in TEER in Calu-3 cells treated with the β_2-adrenoceptor agonists stimulated the β_2-adrenoceptor. The β_2-adrenoceptor increases cyclic AMP via the adenylate cyclase system and reduction of cyclic AMP production by β_2-adrenoceptor inverse agonists by binding to the inactive state of the β_2-adrenoceptor. The ability of growth factors such as EGF to restore tight junction formation and improve barrier function after injury have been studied in epithelial cell cultures from asthmatics both in vivo and in vitro(Basuroy *et al.*,2006) . Furthermore, the addition of EGF in the basal compartment of the trans wells enhanced barrier function and was reported to have stimulated the EGFR/Rac/JNK pathway (Terakado *et al* 2011) . It was also suggested that the apical addition of EGF to trans wells improved barrier function without affecting proliferation of cell cultures of asthmatics while the basolateral addition of EGF affected proliferation without affecting barrier function (Xiao *et al.,* 2011).

The human bronchial epithelial cell line Calu-3 has been used for permeability studies and the absorption of drugs have been compared with absorption of drugs in lungs both in vivo and in vitro (Mathias *et al.*, 2002). The permeability of formoterol and propranolol in Calu-3 cells raised to air liquid interface, were investigated with formoterol producing greater rate of absorption than propranolol (Bosquillon *et al.,* 2017). The β_2-adrenoceptor being the predominant subtype of the receptor in the respiratory epithelium (Davis *et al.,* 1990) was investigated for its involvement in mucus production in bronchial asthma. The inverse agonist, ICI118551 reduced mucin gene expression, while formoterol increased mucin production in normal human airway epithelial cells (Al-Sawalha *et al.,* 2015). This suggests involvement of the β-$_2$ adrenoceptor in Mucin production in airway epithelial cells. The increase in TEER by both formoterol and propranolol in this study suggest the involvement of both cyclic AMP and non-cyclic AMP pathway such as the ERK 1/2 pathway.

6.4 Conclusion

The investigations in this chapter demonstrated the suitability of the Calu-3 cells as cell culture models for bronchial asthma research. The findings demonstrated formation of tight junctions as displayed by the sequential changes in TEER .The findings highlight the modulation of the β_2-adrenoceptor is carried out at molecular level via cyclic AMP and ERK1/2 dependent pathways.

Chapter 7
General Discussion

7.1 General Discussion

The focus of this research is to investigate the inverse agonist signalling in Human airway cells. It aimed at assessing whether the β_2-adrenoceptor population in human airway cells show any form of constitutive activity that is sensitive to inhibition by inverse agonists. In

addition, the research investigated the functional effects of β_2-adrenoceptor stimulation on airway cell biology and the suitability of Human airway cells for drug delivery and toxicological research.

The βadrenoceptors are members of the 7 transmembrane receptor of the G protein coupled receptor family(Dickenson et al., 2012) .Three main types successfully identified ; $\beta 1$(heart), β_2(lungs, kidney, liver, uterus and peripheral vessels)andβ_3(adipose tissue),(Ferguson and Feldman, 2014).The use of β_2-adrenoceptor agonists in bronchial asthma is the mainstay of treatment (Barnes 1999). However, chronic administration the β_2-adrenoceptor was previously investigated as a cause of worsening disease symptoms in bronchial asthma(Broadley 2006).Furthermore, the chronic activation of the β_2-adrenoceptor was the cause of the deleterious effects of the β_2-adrenoceptor agonists that limit monotherapy in long standing disease(Ngyuyen *et al.*,2009) which lead the search for new and better drugs. Previously airway epithelial cells were investigated to be an additional target of β_2-agonists are involved in the pathophysiology of asthma and are also modified by β_2-agonists(Salathe 2002).The airway epithelium is also involved in the production of anti-inflammatory mediators and inactivation pro inflammatory mediators which influence inflammatory reactions by recruiting inflammatory cells ,cell-cell adhesion and therefore control the severity of respiratory airway diseases(Abraham et al.,2004).The activities of the inflammatory mediators are mediated by cyclic AMP via the β_2-adrenoceptor activation(Nijikamp et al.,1992).The protection of the epithelium was previously studied to be conferred on the airway epithelial cells by the β_2-adrenoceptor agonists where the counteract the effect of bacterial invasion and epithelial airway wound repair(Salathe 2002)..Furthermore congestive cardiac failure and asthma were discovered to have similarities in modalities of treatment (Bond et al.,2003).As a result studies were conducted in murine models of asthma with β_2-adrenoceptor antagonists and inverse when administered chronically and produced beneficial results(Callaerts-Vegh et al., 2004).The

β_2-adrenoceptor antagonists blocked the receptor while the β_2-adrenoceptor inverse agonists reduced the activity of the receptor which caused a reduction in receptor dependent signalling(Nguyen et al., 2008).

The β_2-adrenoceptor were also shown to be involved in the abnormal up regulation of airway epithelial cell growth through the activation of mitogen activated kinase protein cascade(Nishimura et al.,2002).The β_2-adrenoceptor antagonists and inverse were discovered to signal via the non-cyclic AMP pathway or the β-arrestins pathway stimulating ERK phosphorylation .They can also activation both pathways and serve as biased agonists(Baker et al.,2011).The β_2-adrenoceptor activation by agonists, antagonists and inverse agonists was also shown to activate multiple signalling pathways using the xCELLigence impedance assay(Stallaert et al.,2011).

The findings of the previous studies conducted on the β_2-adrenoceptor have linked β_2-adrenoceptor to cyclic AMP accumulation, cell proliferation/viability, cytokine production ,wound healing and cell signalling.

Summary of findings

7.1.2 .Modulation of the β_2-adrenoceptor by inverse agonists.

The Calu-3 cell line has been reported to be suitable for use in cell signalling pathways and suitable models to study function and regulation of β-adrenoceptor signalling in the respiratory system(Abraham *et al.*,2004).They have also been reported to stimulate β_2-adrenoceptor activation of cyclic AMP, up regulate cell growth through activation of mitogen activated protein kinase(Nishimura *et al.*,2002).The BEAS2B cells have been previously been investigated as the only permanent lung cell culture model for expression of β_2-adrenoceptor expression and function(Amstad *et al.*,1988) while, the BEAS2B-R1 cells were previously investigated for HTR4 expression(Hodge *et al.*,2013).HEK293 cells were investigated for multiple signalling events by stimulation of β_2-adrenoceptor (Stallaert *et al* ., 2012).

The Calu-3 ,BEAS2BR-1 and CHO-β_2 cells were used to validate results obtained previously in Calu-3 ,BEAS2B and HEK293 cells. The findings in this study were mostly comparable with some of the results obtained in Calu-3 cells except for some minor variations that could be attributed to cell line-related responses. Isoprenaline and formoterol stimulated cyclic AMP production in Calu-3 cells .The generation of cyclic AMP was confirmed with increased cellular impedance in BEAS2BR-1 cells. Subsequent experiments confirmed formoterol and propranolol increased impedance Calu-3 and CHO-β_2 cells but decreased impedance in BEAS2BR-1 cells. In addition the MEK 1 inhibitor did not decrease impedance in formoterol and propranolol treated cells. Furthermore, western blot analysis showed that formoterol and carvedilol stimulated ERK phosphorylation in CHO-β_2 cells . Overall, the stimulation of ERK phosphorylation by an agonist which was shown to stimulate cyclic AMP production suggest that both cyclic AMP and non-cyclic AMP involvement via ERK pathway in involved.

7.13.The functional effects of β_2-adrenoceptor stimulation on airway cell biology in human airway epithelial cells.

This part of the study evaluated the possible role β_2-adrenoceptor stimulation in airway cell biology after exposure of Calu-3 ,BEAS2BR-1 and CHO-β_2 cells to treatments.

The data generated with MTT reduction showed increased cell viability in Calu-3 cells and BEAS2B-R1cells.However,neutral red assay increased cell viability in BEAS2BR-1cells.

Furthermore, the treatment of Calu-3 and CHO-β_2 significantly increased cell viability assessed by incuCyte cell proliferation assay. These findings suggest that the β_2-adrenoceptor has a prominent role in the functional effects of airway cell biology.

7.14.The effect β_2-adrenoceptor stimulation on wound healing in bronchial epithelial cells using multiple techniques.

Exposure to dust, mites, toxins and chemicals have been reported to cause injury to the airway epithelium in bronchial asthma and worsen symptoms (Yarrow *et al.,* 2004).Previous studies in human airway epithelial cells have investigated the stimulation of wound healing and repair by isoproterenol by a cyclic AMP dependent mechanism(Peitzman et al.,2015).In this part of the study an automated system of creating wounds and imaging of wounded monolayers, the incuCyte S3 system to evaluate the role of β_2-adrenoceptor in wound healing. This automated system permitted the real time analysis of the effect of β_2-adrenoceptor stimulation in human airway cells. Furthermore, the data showed the inhibitory effect of β_2-adrenoceptor stimulation on wound healing in Calu-3 ,BEAS2BR-1 and CHO-β cells. Considering the role of the β_2-adrenoceptor in stimulation cell viability by both β_2-adrenoceptor agonists and inverse agonists and the inhibitory effect on wound healing by β_2-adrenoceptor agonists and inverse agonists, it may be suggested that the observed response may have a common mechanism. Furthermore, the effect of β_2-adrenoceptor increased cell viability which is what is required in the management of asthma because viable cells would enhance epithelial cell proliferation after damage. Furthermore, the data in this study revealed the inhibitory effect of β_2-adrenoceptor stimulation on wound healing and repair which is most likely cyclic AMP dependent via ERK1/2 pathway.

7.15. Air liquid interface experiments

The main site of drug deposition in the lungs is the respiratory airway (Patton *et al.*,2000) and is a major barrier to drug absorption(Fiegel *et al.*,2003). Therefore an effective model for predicting the fate of drugs delivered to the lungs during drug formulation is necessary and a cell culture model that can show the extent of drug permeability in vivo is important (Haghi *et al.,* 2010). Calu-3 cells have been reported to be a reliable cell model for drug delivery in the respiratory airway (Forbes *et al.,* 2005). The last part of the current study involved culturing Calu-3 cells at air liquid interface condition on trans well inserts. The functional properties of the monolayers were studied by measurements of TEER, investigated for the effects of β_2-adrenoceptor on TEER and mucin secretion. The stimulation β_2-adrenoceptor by both a β_2-adrenoceptor agonist and inverse agonist stimulation significantly increased TEER in Calu-3 cells. Based on these findings and considering the role of β_2-adrenoceptor stimulation in increasing epithelial cell proliferation ,it may be suggested that the observed response exhibit a similar mechanism .Such as the presence of biased agonism ,where an agonists or inverse agonist preferentially activates a cell signalling pathway. Furthermore, the findings indicate a prominent role of the β_2-adrenoceptor modulation via both the cyclic AMP and cyclic AMP pathway via ERK1/2 .

Chapter 8
Limitations and Future work

8.1 Limitations of the study

In this study, an automated wound maker was used to create wounds in confluent monolayers in contrast to using chemical or mechanical methods which are common in many literature. However, further assessment could be done to investigate the inflammatory cytokines released after injury to bronchial epithelial cells. This is because injury to the bronchial epithelium in asthma is associated with the release of inflammatory cytokines such as IL-6 and IL-8 to assess the role of inflammatory cytokines in airway inflammation.

The air liquid interface experiments focused on assessing the formation of tight Junctions in airway epithelial cells using trans epithelial electrical measurements (TEER) and the assessment of mucin expression in Calu-3 cells. Increase in mucin content in Mucous accumulates in the airways that reduces the effective airway diameter and increase airway resistance. The tight junctional proteins constitute a dynamic and functional structure, which consists of transmembrane proteins such as occludin and cytoplasmic proteins such as Zonula occludens-1 (ZO-1).The assessment of ZO-1 using confocal imaging techniques would provide a broad additional information on the integrity of tight junction formation, which TEER measurements alone could not provide.

The real-time cellular impedance assay using the xCELLigence RTCA system clearly indicated that cyclic AMP and ERK1/2 activation are not sufficient to explain the entire impedance response in both β_2-adrenergic agonists and inverse agonists. The β_2-adrenoceptor couples both Gs and Gi and the inhibition of the Gs with cholera toxin selectively downregulates Gs. The use of the cholera toxin in the impedance assay would provide more information on the effect of cholera toxin on cellular impedance.

Furthermore, additional investigation into the effect of EGF and Kinase inhibitors of cell proliferation, is required in human airway cells to validate the data obtained using the real-time incuCyte S3 system, such as MTT assay and cell counting .

8.2 Future work

The recommendations for future based on the findings of the study are;

The study investigated modulation of the of the β_2-adrenoceptor by inverse agonists. The assessment of cyclic AMP production through Gs component of the β_2-adrenoceptor was used to evaluate the effect the receptor activation. This was achieved was achieved without much success .The assessment of cyclic AMP production through Gi component of the β_2-adrenoceptor would be of interest in the future to assess the potential of the Gi to modulate the activity of the β_2-adrenoceptor.Since 2 cells lines were used to assess the production of cyclic AMP, it would be of great interest to evaluate the possible modulation of the β_2-adrenoceptor via the Gi component in these cell lines. The β_2-adrenoceptor preferentially couples the stimulatory Gs component of the G-protein which ,stimulate cyclic AMP and the inhibitory component Gi/o .which inhibits adenylyl cyclase(Xiao et al.,2001).The conformational changes observed in Gs and Gi coupling have been reported to be similar but, some distinct differences were detected in intercellular loop 2 of the GPCR which could be the reason for the G protein selectivity(Ma et al.,2020).

The results obtained suggests the activation of β_2-adrenoceptor was achieved β_2-adrenergic agonists isoprenaline and formoterol as obtained in the cyclic AMP assay and xCELLigence impedance assay. However the effect of the β_2-adrenoceptor inverse agonists in the xCELLigence impedance assay did not precisely indicate the signalling pathway involved in the impedance changes .Previously it was assumed that one GPCR activated a single cell pathway signalling pathway but, now it has been suggested that multiple signalling events could be activated by a receptor(Kanekin *et al.,* 2010).Furthermore, some β-adrenoceptor agonists and inverse agonist preferentially activate one signalling pathway over another i.e. show functional selectivity or exhibit biased signally. Previously, it was suggested that functionally selective drugs at a single receptor may provide more clinical benefits over drugs acting on the same receptor(Noma *et al.*,2007). The design of drugs with functional

selectivity would provide therapeutic benefit with reduced unwanted effects. Therefore further investigation to asses this functional selectivity using the real time cellular impedance assay would be very interesting in drug development.

The investigation of wound healing using multiple techniques assessed the role β-adrenoceptor ligands wound closure which focused on migration and proliferations of cells to the wounded region. The study assessed the effects of β-adrenoceptor agonists, inverse agonists and Epidermal growth factor. However, further assessment could be done to investigate the role kinase inhibitors in wound healing. For example, the inhibition of ERK1/2 with PD98059 or MAPK using SB203580 or JNK with SP600125 could substantially inhibit cell migration by preventing the migration of cells into the wounded region. It would be interesting to investigate the effect of the kinase inhibitors on the β-adrenoceptor ligands in wound healing. Further assessment with combination of both β-adrenoceptor agonists and inverse agonists would provide information about the signalling pathway involved. In addition gene knock out techniques could help in confirming the pathway involved in promoting or reducing wound closure. The effect of stress activated protein kinases were investigated for their role in wound migration in human bronchial epithelial cell line 16HBE14o-(Steven *et al.*,2004).A similar approach would provide insight into the effect of stress activated protein kinases in human airway epithelial cells.

The ALI experiments in Calu-3 cells revealed increase in TEER in the presence β_2-adrenoceptor stimulation. However, the assessment of permeability would be useful in drug delivery because peptide and protein drugs show poor absorption via the oral route but show increased absorption via the nasal route **(**Wang *et al.,* 2003**,** Mahesh *et al.,* 2004, Surendrakumar *et al*.,2003).

The Calu-3 cells were reported as drug absorption models using air liquid interface experiments and was successful (Borchard *et al.*,2002, Florea *et al.*,2001, Foster *et al.*,2000). Furthermore, this aerosol drug formulations penetrate airway epithelia and provide optimal

therapeutic effect.Therefore,further study would provide more information on the absorption barrier in the respiratory epithelium. The tracheobronchial deposition of drugs was previously reported to be greater than in alveolar deposition. Hence the understanding of absorption barrier in the airways would be useful in the development of drug formulations.

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