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A Pharmacodynamics Analysis of Glucocorticoid Receptor-Mediated Gene Expression in BEAS-2B Human Airway Epithelial Cells

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A Pharmacodynamics Analysis of Glucocorticoid Receptor-Mediated Gene Expression in BEAS-2B Human Airway Epithelial Cells

by

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A THESIS

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Abstract

International treatment guidelines recommend that inhaled glucocorticoids be used as a monotherapy to control mild-to-moderate asthma. However, a combination of an inhaled glucocorticoid with a long-acting β_2-adrenoceptor agonist is recommended for managing moderate-to-severe asthma, which cannot be controlled by glucocorticoids alone. Increasing evidence supports the idea that glucocorticoids acting through the glucocorticoid receptor (GR) can attenuate inflammatory responses either by inducing anti-inflammatory genes (a process called transactivation) or by suppressing pro-inflammatory genes (a process called transrepression). Gene transactivation by glucocorticoids was initially thought to be responsible for causing the metabolic side-effects and, hence, is relatively understudied when compared to transrepression. However, it has become increasingly clear that transactivation plays an important role in the anti-inflammatory actions of glucocorticoids. Moreover, the extent to which clinically-relevant glucocorticoids are equivalent in their ability to promote gene expression is unclear.

This thesis describes the first pharmacodynamic approach to evaluate the transactivation potential of a panel of glucocorticoids alone and in combination with indacaterol (Ind), a long-acting β_2-adrenoceptor agonist. Pharmacodynamic analyses showed that magnitude of luciferase gene induction was agonist dependent (i.e. seven different glucocorticoids tested displayed varying degrees of agonism). In addition, there were significant differences in agonist potency and, more importantly, the relationship between GR occupancy and response. To complement the reporter studies, similar analyses were performed on four glucocorticoid-inducible candidate genes. Three of these (*GILZ, p57^{kip2}* and *CRISPLD2)* are genes with potential anti-inflammatory activity and a fourth gene, *PDK4* is predicted to promote metabolic side-effects. Similar to the luciferase reporter system, the expression of these genes was agonist-dependent and displayed markedly different GR

occupancy-response relationships. Furthermore, Ind, when combined with the seven GR agonists tested, synergistically enhanced transactivation, the magnitude of which was agonist and gene dependent. These studies demonstrate that when gene transactivation is used as a functional output, glucocorticoids used to treat asthma are not biologically-equivalent. It is proposed that these differences may be exploited to therapeutic advantage. Thus, the generation of gene expression 'fingerprints' in target and off-target human tissues may allow new GR agonists to be rationally designed for asthma with an improved therapeutic index.

Preface

Most of the data presented in this thesis have been published in the following manuscripts:

Taruna Joshi, Malcolm Johnson Robert Newton & Mark A. Giembycz (2015). An analysis of glucocorticoid receptor-mediated transcription in human airway epithelial cells identifies unique, ligand-directed, gene expression fingerprints with implications for asthma therapeutics. *British Journal of Pharmacology*, **172** (5), 1360–1378.

Taruna Joshi, Malcolm Johnson, Robert Newton & Mark A. Giembycz (2015). The long-acting β_2-adrenoceptor agonist, indacaterol, enhances glucocorticoid receptor-mediated transcription in human airway epithelial cells in a gene- and agonist-dependent manner. *British Journal of Pharmacology*, **172** (10), 2634–2653.

During PhD I worked on multiple research projects and thus, I have authored as well as co-authored other manuscripts as follows:

Taruna Joshi, Dong Yan, Omar Hamed, Michael Salmon, Stacey L. Tannheimer, Gary B. Phillips, Ruby Z. Cai, Clifford D. Wright, Robert Newton & Mark A. Giembycz (2016). GS-5759, a Heterobifunctional Phosphodiesterase 4 Inhibitor and Bivalent β2-Adrenoceptor Agonist for Chronic Obstructive Pulmonary Disease: Effects on Gene Expression in Human Airway Epithelial Cells. (Under Review *Mol. Pharmacology*).

Stephanie Greer, Cara P. Page, **Taruna Joshi**, Dong Yan, Robert Newton, Mark A. Giembycz (2013). Concurrent activation of the adenosine A_{2B}- and glucocorticoid receptors in human airway epithelial cells cooperatively induces genes with anti-inflammatory potential: a novel approach to treat COPD. *J Pharmacol Exp Ther*., **346** (3), 473-85.

Thunicia Moodley, Sylvia M. Wilson, **Taruna Joshi**, Christopher F. Rider, Pawan Sharma, Dong Yan, Robert Newton & Mark A. Giembycz (2013). Phosphodiesterase 4 inhibitors augment the ability of formoterol to enhance glucocorticoid-dependent gene transcription in human airway epithelial cells: a novel mechanism for the clinical efficacy of roflumilast in severe COPD. *Mol. Pharmacology*, **83**(4), 894-906.

During the course of my PhD I have also presented much of my work at the annual meetings of the *American Thoracic Society* and *European Respiratory Society* as follows:

Taruna Joshi, Robert Newton & Mark A. Giembycz. (2014). A panel of clinically-relevant glucocorticoids display distinct gene expression profiles in human airway epithelial cells. *Am.J.Respir.Crit.Care Med.*, **189**, A4892.

Taruna Joshi, Robert Newton & Mark A. Giembycz. (2013). Relationship between the intrinsic activity of a glucocorticoid receptor ligand and the ability of long-acting β_2-adrenoceptor full agonists to enhance GRE-dependent transcription. *Am.J.Respir.Crit.Care Med.*, **187**, A1839.

Taruna Joshi, Robert Newton & Mark A. Giembycz. (2013). Glucocorticoid regulated gene expression profiling in human bronchial epithelial cells. *European Respiratory Journal*, **42** (Suppl 57), P677.

Taruna Joshi, Sylvia M. Wilson, Stacey L. Tannheimer, Clifford D. Wright, Michael Salmon, Robert Newton & Mark A. Giembycz. (2012). GS-5759, a novel bi-functional phosphodiesterase 4 inhibitor and long-acting β_2-adrenoceptor agonist, augments GRE-dependent transcription in human airway epithelial cells. *Am.J.Respir.Crit.Care Med.,* **185**, A5693.

Sylvia M. Wilson, **Taruna Joshi**, Clifford D. Wright, Stacey Tannheimer, Michael Salmon, Robert Newton & Mark A. Giembycz. (2012) GS-5759, A novel bi-functional phosphodiesterase 4 inhibitor and long-acting β_2-adrenoceptor agonist, inhibits CXCL9 and CXCL10 release from human airway epithelial cells. *Am.J.Respir.Crit.Care Med.,* **185**, A5692.

Thunicia Moodley, **Taruna Joshi**, Sylvia M. Wilson, Pawan Sharma, Christopher F. Rider, Robert Newton & Mark A. Giembycz. (2012). Phosphodiesterase 4 inhibitors enhance glucocorticoid-dependent gene transcription in human airway epithelial cells: a mechanism for the clinical efficacy of roflumilast in severe chronic obstructive pulmonary disease. *Am.J.Respir.Crit.Care Med.,* **185**, A5691.

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I am indebted to my committee members Dr. Derek McKay and Dr. Pierre-Yves von der Weid for their guidance, time and valuable feedbacks. My gratitude flows towards Dr. Don Welsh and Dr. Andrew Braun for being exemplary graduate program coordinators. They not only nominated me for multiple graduate awards and conferences, but also continually guided me to shape my overall professional development as a PhD student.

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But most of all, I want to thank my family, specially my mother Nayan, my father Pradeep, my brother Tarun, my sister Tanvi and my brother-in-law Abhishek, along with my grandparents, cousins, uncles and aunts, whose love carried me through every single day. My large family is God's special blessing to me.

Dedication

I dedicate this thesis to my parents, Nayan and Pradeep, who always believed in me. My parents are the source of my strength and have always provided me unconditional love and support throughout the years.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic releasing hormone
AF	Activator function
ANOVA	Analysis of variance
AP-1	Activator protein-1
ASM	Airway smooth muscle
ATF	Activating transcription factor
β_2-AR	β_2-adrenoceptor
C/EBP	CCAAT/enhancer binding protein
cAMP	Cyclic adenosine-3',5'-monophosphate
CBX	Carbenoxolone
CCL	Chemokine ligand
CDKN	Cyclin dependent kinase inhibitor
cDNA	Complementary DNA
COX	Cyclooxygenase
CRE	cAMP-response element
CREB	cAMP-response element binding
CRH	Corticotrophin releasing hormone
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2
CXCL	Chemokine (CXC motif) ligand
DAG	Diacylglycerol
DBD	DNA binding domain
DC	Des-isobutyryl ciclesonide
Dex	Dexamethasone
Dex-Mes	Dexamethasone 21-mesylate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DUSP	Dual specificity phosphatase
EGF	Epidermal growth factor
EPAC	Exchange protein activated by cAMP
ERK	Extracellular signal regulated kinase
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in 1 second
FF	Fluticasone furoate
FKBP	FK506 binding protein

Fsk	Forskolin
G-418	Geneticin sulfate
GC	Glucocorticoid
GDP	Guanosine diphosphate
GILZ	Glucocorticoid inducible leucine zipper
GINA	Global initiative for asthma
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GR/NR3C1	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSK	GSK9027
GTP	Guanine triphosphate
GW	GW870086X
HC	Hydrocortisone
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ind	Indacaterol
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IRF	Interferon regulatory factor
JNK	c-jun *N*-terminal kinase
LABA	Long-acting β_2-adrenoceptor agonist
LBD	Ligand binding domain
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Mif	Mifepristone
MKP	Mitogen-activated protein kinase phosphatase
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear factor *kappa*-light-chain-enhancer of activated B cells
NS	Not stimulated

Org	Org34517
PDC	Pyruvate dehydrogenase complex
PDE	Phosphodiesterase
PDK4	Pyruvate dehydrogenase kinase isoenzyme 4
PDP	Pyruvate dehydrogenase phosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKIα	cAMP protein kinase inhibitor alpha
POMC	Pro-opiomelanocortin
RE	Response element
RGS	Regulator of G-protein signalling
SABA	Short-acting β_2-adrenoceptor agonist
SFM	Serum free medium
SLPI	Secretory leukocyte protease inhibitor
TAT	Tyrosine amino transferase
TBP	TATA binding protein
TF	Transcription factor
TFRE	Transcription factor response element
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TTP	Tristetraprolin
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor

Epigraph

If physiology is concerned with the function, anatomy with the structure, and biochemistry with the chemistry of the living body, then pharmacology is concerned with the changes in function, structure and chemical properties of the body brought about by chemical substances.

-W.D.M. Paton (1986)

Chapter One: **Introduction**

1.1 Asthma: General Features

Asthma is a complex, chronic inflammatory disorder of the airways, characterized by shortness of breath, wheezing and chest tightness (Douglas and Elword, 2011; Masoli et al., 2004; Wenzel, 2006). Chronic inflammatory processes in asthma cause variable airflow limitation, bronchial hyper-responsiveness and remodeling (Holgate, 2011; Holgate and Polosa, 2006). Moreover, asthma pathology involves eosinophil and/or neutrophil infiltration into the airways, increased mast cell numbers, smooth muscle cell hypertrophy (increased cell size) and hyperplasia (increased cell proliferation and muscle fibres), airways obstruction due to thick mucus plugs and loss of the bronchial epithelium (Busse and Lemanske, 2001; Douglas and Elword, 2011). Consequently, asthma studies including the examination of induced sputum, bronchoalveolar lavage (BAL) fluid, lung function as well as performing bronchoscopy have proven extremely useful in disease diagnosis (Douglas and Elword, 2011; Holgate and Polosa, 2006).

Globally, it is estimated that 300 million people have asthma and it appears to be more prevalent in developed countries. It is reported that 15 million disability-adjusted life years (DALYs) are lost due to asthma each year (Bateman et al., 2008). According to the World Health Organization about 1 in every 250 deaths per year is ascribed to asthma (Douglas and Elword, 2011; Masoli et al., 2004). The incidence of asthma has increased in the past 25 years worldwide and it is predicted that by 2025, an additional 100 million people will have asthma. Asthma causes a tremendous global economic burden, resulting from hospital admission costs, medication expenses, time lost from work and premature morbidity (Braman, 2006; Masoli et al., 2004). Moreover, asthma is a heterogeneous disease, which cannot simply be diagnosed by a set of

assigned symptoms, and involves multiple phenotypes or endotypes. A phenotype is defined by the visible characteristics or symptoms of an organism resulting from interaction of its genotype and environment. If the specific biological pathway responsible for the generation of a particular phenotype is identified it is known as an endotype (Wenzel, 2006, 2012). However, despite all the approaches based on allergens, biomarkers, symptoms, genetic history, inflammatory responses and association of comorbidities, allocation of asthma phenotypes is still evolving. Several asthma phenotypes were suggested in the past including exercise-induced, smoking-related, obesity-associated, eosinophilic (allergic), neutrophilic (non-allergic) and aspirin-induced, but none of them truly depicted a clear association with all the disease characteristics (Cohn et al., 2004; Wenzel, 2006, 2012)

Asthma triggers are recognized as allergic and non-allergic. House dust mite, cat or dog dander, fungal spores, cockroaches, grass/tree pollens, peanuts, are some of the more common allergens (Jahnsen et al., 2006). In contrast, non-allergic exacerbators include viruses, exercise, cigarette smoke, emotional stress, thunderstorms and air as well as bio-fuel pollution. Atopy is the genetic predisposition to develop an allergy (Douglas and Elword, 2011). Asthma, eczema and allergic rhinitis are some of the allergic diseases which are associated with atopy. Immunoglobulin E (IgE) serum levels and skin prick tests are used to confirm an atopic state (Douglas and Elword, 2011). Bronchial hypersensitivity is one of the characteristic features of asthma and is a result of loss of immune tolerance towards common dormant antigens such as pollens or animal dander (Jahnsen et al., 2006). Hypersensitivity encompasses dysregulated recruitment and excessive activation of mast cells, eosinophils, dendritic cells and macrophages; this results in increased levels of IgEs leading to severe inflammatory reactions against common innocuous aero-allergens (Galli and Tsai, 2012).

1.2 Asthma's Major League Players: Involvement of Structural and Immune Cells

The asthmatic immune response can be divided into early and late phases (Buc et al., 2009). The early response involves mast cells and the release of inflammatory mediators from them (Figure 1.1). Inflammatory mediators including cytokines released during the early phase can orchestrate the late response by activating and recruiting eosinophils, basophils, neutrophils, dendritic cells and T cells (Figure 1.1) (Gern and Busse, 2002). The asthmatic condition is worsened with the release of inflammatory mediators from these secondary cells, including the myriad of cytokines, chemokines, growth factors, prostaglandins, leukotrienes, histamine and nitric oxide (NO), which induce contraction of airways smooth muscle (ASM), mucous secretion, and vasodilatation (Lambrecht and Hammad, 2015). In addition to infiltrating inflammatory cells, airway structural cells such as epithelial cells, smooth muscle cells, endothelial cells, fibroblasts and myofibroblasts are also involved in the pathogenesis of asthma, through their ability to induce and regulate the release of different pro-inflammatory mediators (Figure 1.1) (Braman, 2006; Dekkers et al., 2009; Masoli et al., 2004). The release of these inflammatory mediators accelerates changes in the airways that include bronchoconstriction, mucus secretion, airway hyper responsiveness (AHR) and remodeling (Dekkers et al., 2009). Some of these changes worsen with disease severity and can become irreversible.

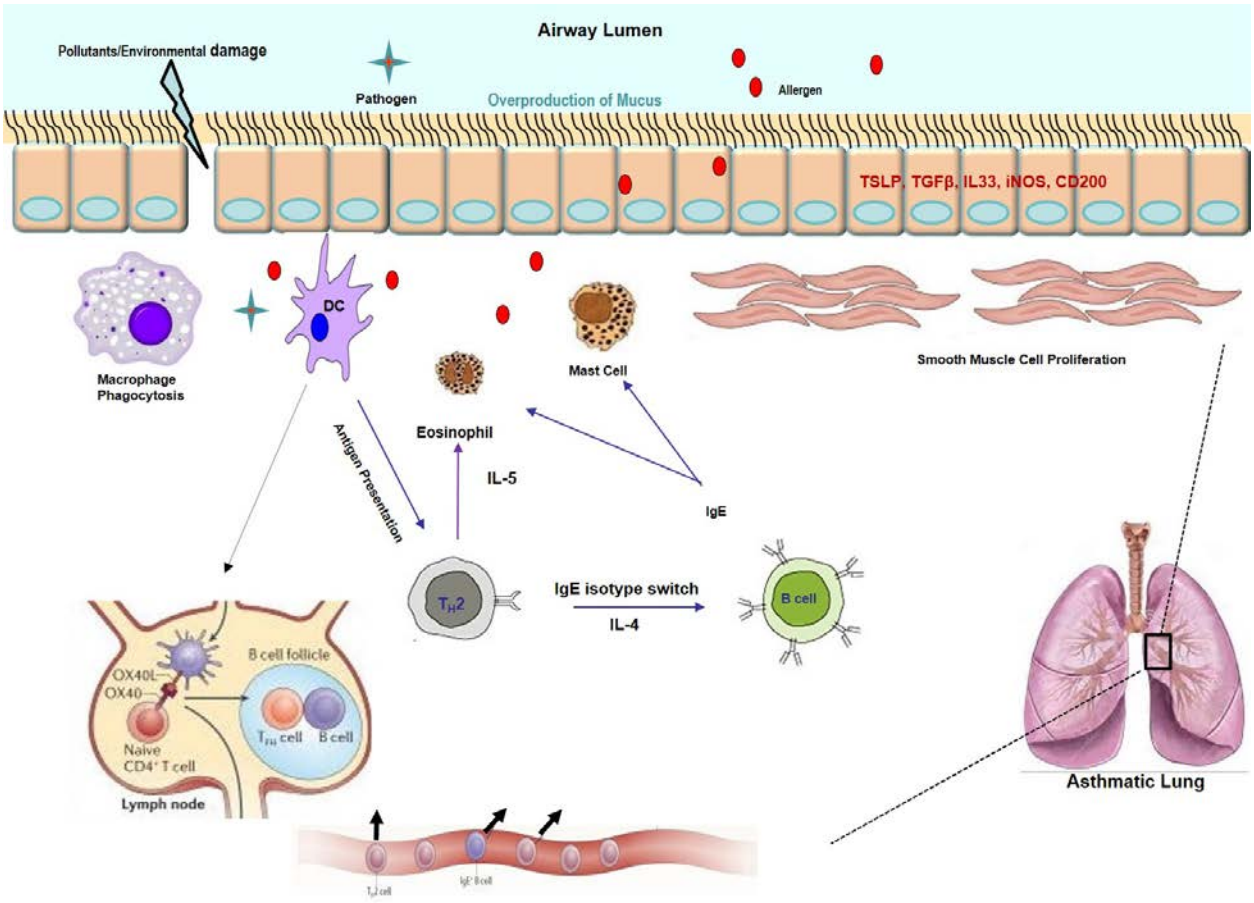


Figure 1.1 Inflammation in asthma.
In atopic asthmatics, antigen presenting cells, such as dendritic cells, on allergen exposure lead to the activation of T_H2 cells and IgE synthesis. Subsequent exposures to allergen results in the release of inflammatory mediators from mast cells, macrophages and eosinophils causing an early, or acute, asthmatic response. Mast cell degranulation releases histamine and other bronchoconstrictive mediators. Chemokines and cytokines released during the early phase lead to inflammatory-cell recruitment and activation, which is responsible for the late phase along with airway structural changes. Release of epithelial cell cytokines, particularly IL-33 and TSLP, induces the expression of OX40 ligand (OX40L) on dendritic cells to initiate their migration to local draining lymph nodes where they activate naïve CD4+ T cells. These activated IL-4 secreting T cells then migrate to the B cell zone in the lymph node and differentiate into follicular T helper cells (T_{FH} cells), which then potentiate IgE class-switching in B cells (Adapted from Wenzel, 2012).

1.2.1 *Epithelial Cells*

Airway epithelia have a seminal, dual role in asthma pathogenesis: as a physical barrier and as first point of contact for inhaled allergens or pathogens. Epithelial cells have three unique characteristics: i) they are tightly juxtaposed and adhere to one another via specialised cell junctions; ii) they exhibit polarity in function as well as morphology (apical, basal and lateral surfaces); and iii) they sit on a basement membrane (Ross and Pawlina, 2006). Tracheobronchial apical epithelial cells carry ciliary processes for fluid/mucus and particle movement as well as clearance. The epithelial lateral domain possesses cell adhesion molecules which make inter-epithelial junctions (Figure 1.2). Three types of junctional complexes have been identified in the epithelium: occluding junctions or tight junctions, which act as barriers and limit the movement of macromolecules across the cells; anchoring junctions, which maintain the structural integrity of the epithelium, and communicating junctions or gap junctions, which allow the movement of ions, amino acids, second messengers and sugars, between epithelial cells (Ross and Pawlina, 2006).

Human airways are divided into two zones: a) a conducting zone, which includes the nasal cavity, pharynx, larynx, bronchi and bronchioles; and b) a respiratory zone including respiratory bronchioles, alveolar ducts and alveolar sacs (Ross and Pawlina, 2006). A continuous layer of multiple types of epithelial cells line the entire human airway. The conducting zone up until the bronchi is mainly lined with pseudostratified columnar ciliated epithelium interspersed with non-ciliated basal cells and secretory cells (Ross and Pawlina, 2006). Columnar ciliated epithelial cells maintain unidirectional upward mucus flow from the lungs towards the throat, *via* mitochondria-powered beating cilia (Ross and Pawlina, 2006). Basal cells are found throughout the conducting zone as a monolayer attached to the basement membrane (Ross and Pawlina, 2006). Basal cells

are responsible for giving the conducting zone epithelia a pseudostratified appearance because of the way these are interspersed within ciliated columnar epithelial cells (Ross and Pawlina, 2006). Basal cells are reported to be the progenitor cells for goblet and ciliated epithelial cells (Proud, 2008). Interestingly, even ciliated epithelial cells can transdifferentiate to other airway cell types which suggest plasticity as well as extremely high regenerative capacity of airway epithelia in response to any kind of injury (Ross and Pawlina, 2006).

Mucous producing goblet cells are present in the small epithelial pockets formed by submucosal glands. Goblet cells contain acidic mucin glycoprotein granules which when released into the airways form mucous layer on top of the ciliated epithelial cells (Proud, 2008). Mucus, in addition to keeping airways moist, also entraps pathogens and other particulate matter, which are propelled out of airways (Proud, 2008). Mucus production is enhanced under inflammatory conditions or due to inhalation of airway irritants and noxious gases such as tobacco smoke (Proud, 2008).

In bronchioles, the epithelium transitions from pseudostratified to simple squamous type containing ciliated simple squamous epithelial cells, non-ciliated basal cells but the major cell type are non-ciliated club cells, which also act as progenitor cells (Proud, 2008). Club cells are also involved in airways defence as these can secrete club cell 10 kD protein (CC10 or CCSP) which can inhibit phospholipase A_2 (PLA_2) and is shown to be anti-inflammatory (Proud, 2008). Club cells can also secrete pulmonary surfactants and anti-proteases (Proud, 2008).

In the respiratory zone, the epithelium is replaced with alveolar type I and type II cells. Type I cells are large squamous cells and are majorly involved with the gas exchange due to their thin membranes (Ross and Pawlina, 2006). Type II cells are cuboidal in shape and exhibit progenitor capability. Primary role of type II cells is the production of pulmonary surfactant type

A, B, C and D (Ross and Pawlina, 2006). Surfactant is a mixture of proteins and phospholipid which reduces the surface tension and prevents alveoli from collapsing (Ross and Pawlina, 2006).

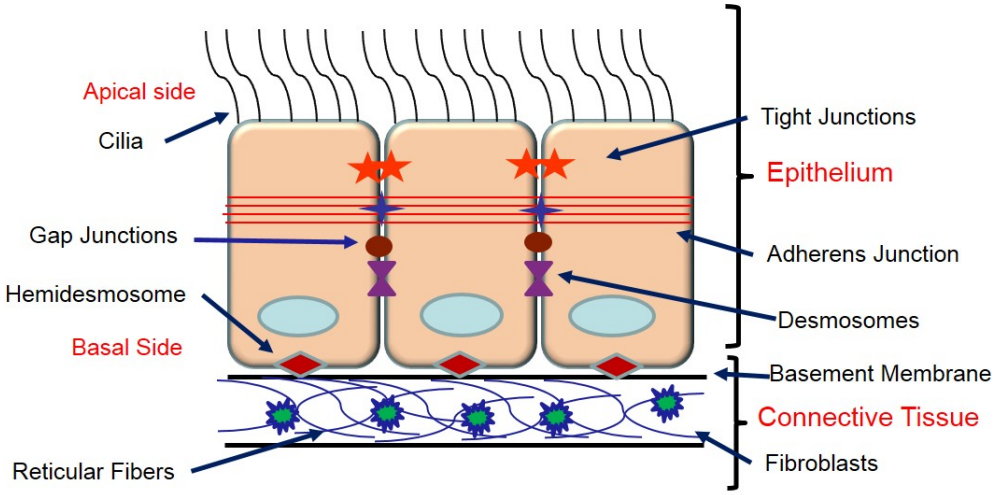


Figure 1.2 Schematic diagram showing junctional complexes and structural elements of the bronchial epithelium.
The figure shows polarity of the epithelial cells, which comprises apical, lateral and basal sides. Cilia are motile cytoplasmic structures, which are responsible for clearance of mucus and inhaled particulates. Gap junctions permit direct exchange of ions, small metabolites and regulatory molecules among epithelial cells. Tight junctions or occluding junctions form the primary diffusion barrier and selectively allow the passage of substances from one side of the epithelium to the other. Adherens junction anchor epithelial cells laterally into the cytoskeleton of adjacent cells. Desmosomes provide direct, spot-like, localised lateral junctions and anchoring sites for intermediate filaments between epithelial cells. The basement membrane is a specialised structure providing attachment sites for the overlying epithelial cells and underlying connective tissue. It contains collagens, proteoglycans and laminins, which help overlying epithelial cells to interact with extracellular matrix and also provides structural integrity to the epithelial cells. Hemidesmosomes anchor the intermediate filaments of the cytoskeleton into the basement membrane (Ross and Pawlina, 2006).

The human lung comes into direct contact with 10,000L of inhaled air every day and is exposed to diverse particulate matter including noxious gases, pollutants, common allergens and pathogens (Holgate, 2007; Holgate et al., 2000). The lung microenvironment and homeostasis is

maintained by the continuous release of TGFβ and IL-10. IL-10 is the main inhibitory cytokine released by CD4+ T cells, regulatory T cells (Treg) and epithelial cells which can repress multiple pro-inflammatory chemokines, cytokines and enzymes (Li et al., 2006a; Grünig et al., 1997). Indeed, IL-10-deficient mice are reported to exhibit increased airway inflammation after allergen challenge (Grünig et al., 1997). Also, a clinical study has reported a decrease in CD4+ and CD8+ T cells, a reduction in T cell proliferation and attenuation of TNFα and IL-1β expression, on administration of IL-10 in seventeen volunteers, in comparison to placebo (Chernoff et al., 1995). On the other hand, TGFβ is an effective regulatory cytokine with multiple roles. TGFβ differentially regulates haemopoietic cells and maintains immune tolerance by controlling the proliferation, differentiation and survival of lymphocytes (Li et al., 2006a). Additionally, TGFβ monitors and controls the initiation as well as resolution of inflammation by modulating the chemotaxis, proliferation and survival of dendritic cells, mast cells, lymphocytes and granulocytes. Taken together, TGFβ imparts tolerance towards self or innocuous antigens without suppressing the immune responses towards a pathogen (Li et al., 2006a, 2006b). A study reported that mice with deletion of the TGFβ receptor developed lethal inflammation associated with T cell activation and differentiation (Li et al., 2006b). Hence, TGFβ and IL-10 are incontrovertibly essential for maintaining normal physiological responses and the dysfunction, inhibition or loss of either can cause severe autoimmune diseases or allergic responses.

Epithelial cells also express the co-stimulatory molecule CD200, which is reported to inhibit alveolar macrophage activation by binding to its receptor, CD200R. CD200R is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily and is present on most leukocytes, specifically on cells of the myeloid lineage (Hussell and Bell, 2014). CD200 expression has been identified in the airways of mice and rats, where it is expressed on bronchial

epithelial cells and on type II alveolar epithelial cells, but not on type I alveolar epithelial cells (Hussell and Bell, 2014). The CD200-CD200R interaction blocks the activity of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and c-jun *N*-terminal kinase (JNK) leading to the inhibition of pro-inflammatory cytokine generation. It also plays an important role in maintaining homeostasis (Hussell and Bell, 2014).

Under normal physiological conditions the airway epithelium is impermeable to environmental insults due to intact tight junctions. Thus, structural integrity of the pulmonary epithelium is paramount in protection against airway pathogens. Airway remodelling is the result of epithelial structural changes due to faulty reparative processes and epithelial to mesenchymal transition (Figure 1.3). In addition, airway remodelling includes an increase in goblet cells, thickening of the smooth muscle layer due to smooth muscle hypertrophy and hyperplasia, an increase in connective tissue deposition and angiogenesis. In persistent asthma, the airway epithelium is unable to form tight junctions and is more susceptible to damage by reactive oxygen species present in cigarette smoke or in polluted air (Nelson et al., 2003). In addition, the airway epithelium expresses high levels of the epidermal growth factor receptor (EGFR) and releases increased amounts of epidermal growth factor (EGF) as a result of structural damage (Holgate, 2007; Knight, 2002). Moreover, the injured epithelium also releases a myriad of chemokines, cytokines and growth factors, including RANTES, eotaxin, thymic stromal lymphopoietin (TSLP), IL-25, IL-33, TNFα, IL-1β, fibroblast growth factor, platelet-derived growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factors and insulin-like growth factor, that can amplify inflammatory responses several fold (Holgate, 2007; Knight, 2002; Knight et al., 2004). Notably, the aforementioned growth factors can interact with fibroblasts present in the underlying mesenchyme, causing their differentiation and proliferation into

myofibroblasts (Figure 1.3). Remarkably, the inflamed epithelium itself can transdifferentiate into myofibroblasts (Holgate, 2007). Thus, the damaged epithelium exhibits chronic wound-like characteristics, with incomplete repair due to enhanced secretions of growth factors, chemokines and cytokines, that can lead to airway remodelling (Holgate et al., 2000).

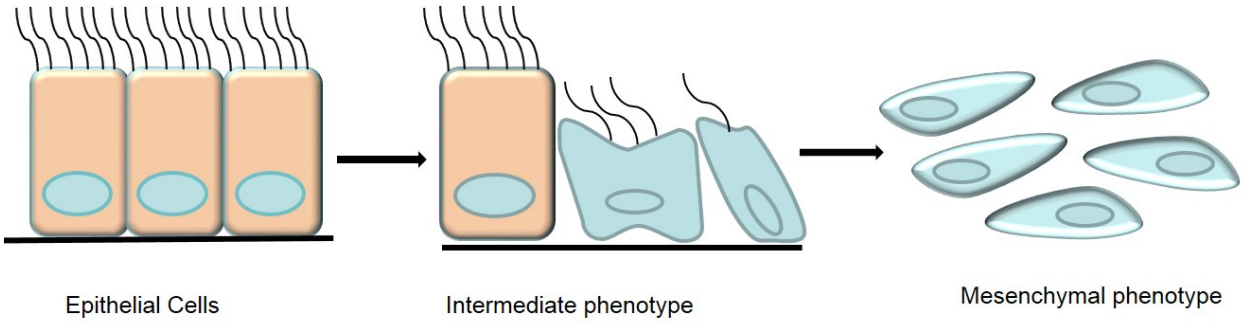


Figure 1.3 Epithelial to mesenchymal transition.
As epithelial cells transition to a mesenchymal phenotype, they lose their surface markers ZO-1, laminin-1, E-cadherin, cytokeratin, syndecan and desmoplakin, and start acquiring mesenchymal surface markers such as vimentin, α-SMA, fibronectin and β- catenin.

Cytokines released by damaged epithelial cells activate and recruit several immune cells to the asthmatic lung. For example, TSLP, IL-25 and IL-33 that are secreted by epithelial cells, activate dendritic cells. These, in turn, prime T_H2 cells to produce T_H2 type cytokines including IL-5, which causes eosinophilia in allergic asthma. Notably, epithelial cells express FcεRII (the CD23 receptor) which can bind and move IgE-antigen complexes or IgE present in the airway lumen, across the epithelium by transcytosis towards underlying mast cells, macrophages or dendritic cells, which become activated.

1.2.2 *Airway Smooth Muscle*

In addition to chronic inflammation, ASM hyperplasia and hypertrophy are now widely accepted as important patho-physiological features of asthma (Johnson et al., 2001). Although, the pathological role of ASM is well established in asthma, the exact mechanism behind the increase in ASM mass is still unclear. Additionally, ASM is also involved in multiple, normal physiological processes, including branching of the respiratory tree during lung embryogenesis, coordinating the distribution of ventilation within the airways, mucus propulsion and exhalation (Bara et al., 2010; Bentley and Hershenson, 2008; Gerthoffer, 2008). In response to various external as well as cellular stimuli, ASM is the main effector of bronchial contraction. Moreover, ASM has also been considered an inflammatory cell because it is capable of releasing multiple chemokines and cytokines, which can contribute to mast cell activation and survival (Bara et al., 2010). Some of the inflammatory mediators which are synthesized by asthmatic ASM are COX2, PGE_2, IL-8, IL-6, GM-CSF, eotaxin, RANTES, IFNγ and monocyte chemoattractant proteins (MCP) 1, 2 and 3; which can consequently recruit multiple immune cells (Busse and Lemanske, 2001; Busse et al., 2000; Chung, 2000). Further, allergen-dependent mast cell activation and degranulation results in the release of multiple inflammatory mediators, which can facilitate ASM phenotypic conversion from a contractile to a mesenchymal proliferative phenotype (Busse and Lemanske, 2001). A growing body of literature suggests that asthmatic ASM has an increased ability to deposit extracellular matrix (ECM) including fibronectin, elastin fibers, laminin and collagen (Bara et al., 2010; Bentley and Hershenson, 2008; Dekkers et al., 2009). These ECM proteins can enhance ASM proliferation and phenotype switching in response to mitogens released by epithelial cells, mast cells, neutrophils and eosinophils (Bara et al., 2010). Furthermore, differences in contractile

responses exist between ASM cells derived from asthmatics and healthy subjects, which could explain hyper-contractility in asthma (Nelson et al., 2003). Thus, asthmatic ASM cells contract with greater velocity compared to healthy ASM, which may be attributed to increases in the expression of smooth muscle myosin light chain kinase, transgelin and myosin heavy chain (Dekkers et al., 2009).

1.2.3 *Mast Cells*

Mast cells are the first effector cells against allergic or antigenic insults. They are abundant in the skin, connective tissue and mucosal tissues including the respiratory tract and are enriched in secretory granules (Wernersson and Pejler, 2014). Mast cell granules contain a vast array of enzymes, broncho-constrictors, lipid mediators, and growth factors (for details see Owen et al., 2013; Ross and Pawlina, 2006). In addition, on activation, mast cells also release many cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-13, GM-CSF, IFNγ, RANTES, MIP-1α, eotaxin and TNFα, that potentiate the priming and recruitment of eosinophils, neutrophils, T cells and dendritic cells (Busse and Lemanske, 2001).

In asthma and other allergic conditions, the concentration of IgE antibodies secreted by biased plasma cells in the lung mucosa increases drastically (Galli and Tsai, 2012; Gould and Sutton, 2008) . Biased plasma cells are terminally differentiated, tissue-resident, antigen-specific IgE-secreting B cells. Therefore, in addition to the bone marrow, the lung mucosal population of B cells and plasma cells are fully equipped to generate high affinity, antigen-specific IgEs by class switching and clonal expansion (Gould and Sutton, 2008). Thus, IgEs bind to the Fc epsilon receptor I (FcεRI), present in high numbers on mast cells, and sensitizes them for degranulation.

This process, that is known as ‘piecemeal degranulation’ takes place as a result of IgE binding, which subsequently lead to release of granular content from mast cells piece-by-piece in a controlled and specific way without lysis or exocytosis (Rosenberg et al., 2013).

1.2.4 *Eosinophils*

Eosinophilic asthma or allergic asthma is the most reported and studied phenotype. Eosinophils are generated from pluripotent stem cells in the bone marrow, which are released into the peripheral blood circulation and are recruited to specific tissues in response to chemoattractants including eotaxin and IL-5 (Rosenberg et al., 2013). Like mast cells, eosinophils contain specific granules that store highly toxic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN), which can be released in response to inflammatory stimuli and further perpetuate adaptive immune responses during airway inflammation (for details see Owen et al., 2013; Ross and Pawlina, 2006). The release of these highly toxic mediators can also induce the shedding of the surface epithelium and can damage the underlying bronchi (Rosenberg et al., 2013). Moreover, eosinophils play important role in airways remodeling, by releasing growth factors, elastase and metalloproteases, which stimulate fibroblasts causing pulmonary fibrosis. Eosinophil granules also contain several cytokines, enzymes and growth factors as well as Charcot-Leyden crystals, or crystalloid bodies, whose presence in the airways is one of the diagnostic features of airways inflammation (Rosenberg et al., 2013). Eosinophils have numerous surface receptors for chemokines, growth factors, adhesion molecules, chemotaxins, degranulation and intercellular communications. Specifically, eosinophils express IL-5R that responds to the T_H2-cell-derived cytokine, IL-5,

which causes their differentiation, proliferation and survival of eosinophils. Eosinophils also express FcεRI, which bind IgE and, thereby sensitizes them to release co-stimulatory factors or granular contents, leading to an amplification of the inflammatory response (Gould and Sutton, 2008). Finally, eosinophils also contain lipid bodies which are storage as well as synthesis sites of leukotrienes, prostaglandins and thromboxanes (Khoury et al., 2014).

1.2.5 *Dendritic Cells*

Dendritic cells are bone marrow-derived, most efficient antigen-presenting cells, with respect to other professional antigen-presenting cells such as macrophages and B-cells. Multiple subsets of dendritic cells have been identified on the basis of expression of a diverse array of cell surface markers. These subtypes have both distinct and overlapping functions (Lambrecht and Hammad, 2015). Interestingly, pulmonary dendritic cells are equipped with the periscopic functions associated with their extending pseudopods or processes, which are stretched out between epithelial cells into the airway lumen and can continuously sample the luminal contents (Hammad and Lambrecht, 2008). Recent evidence suggests that dendritic cells play a pivotal role in inducing adaptive immune responses against pathogenic foreign antigens and anergy or tolerance towards benign common antigens by expressing distinct membrane glycoproteins involving major histocompatibility complex (MHC) class I, MHC class II and co-stimulatory proteins CD80/CD86 (Lambrecht and Hammad, 2003; Satpathy et al., 2012). Immature airway mucosal dendritic cells are mainly phagocytic and are strategically found above as well as under the basement membrane of pulmonary epithelial cells, which is the most efficient location for airway surveillance. Upon antigenic stimulation, the dendritic cells mature by reducing their

phagocytic activity, increasing antigen processing and increasing the surface expression of antigen presenting proteins (Lambrecht and Hammad, 2003; Satpathy et al., 2012). Moreover, dendritic cells continuously migrate from the lungs to the draining lymph nodes to prime $CD4^+$ and $CD8^+$ naïve T cells against bacterial or viral antigens by processing and presenting them on either MHC I or MHC II, along with the co-stimulatory molecules CD80/CD86 and CD40 (for details see Owen et al., 2013; Ross and Pawlina, 2006). Additionally, dendritic cells in thymus can also assist in the negative selection of double positive naïve $CD4^+CD8^+$ T cells exhibiting higher affinity towards self-antigens (Owen et al., 2013). Notably, dendritic cells can also secrete IL10, which activates regulatory T cells (Treg) (Lambrecht and Hammad, 2015). Tregs, in turn, suppress T_H2- or T_H1-mediated inflammatory processes and maintain homeostasis in the absence of any pathogenic breach of the airway epithelium (Satpathy et al., 2012).

1.2.6 *Lymphocytes*

Imbalance and dysregulation of T_H1 versus T_H2 responses are one of the underlying causes of allergic inflammation in asthma. T_H1 cells release IFNγ and IL-2 to maintain cellular defence against viral infections while T_H2 cells secrete GM-CSF, IL-4, IL-5, IL-6, IL-9 and IL-13 that promote and perpetuate allergic inflammation (Busse and Lemanske, 2001). In particular, IL-4 regulates T_H2 survival; IL-4 and IL-13 regulate IgE synthesis by B cell class switching; ILs-3, -9 and -13 are involved in mast cell differentiation as well as maturation, and IL-3, IL-5 and GM-CSF are responsible for the maturation and recruitment of eosinophils along with basophils (Holgate, 2011, 2012, 2013; Lambrecht and Hammad, 2015). However, recent evidence suggests roles of other T lymphocyte subsets including T_H17, Treg and T_H9 cells (for details see Owen et

al., 2013). It has also been suggested that T cells remain plastic, instead of committing to a particular subset, and are affected by the surrounding microenvironment (Lloyd and Hessel, 2010). T_H17 cells are a distinct lineage of CD4+ T cells and are so named because they produce IL-17. TGFβ and the pro-inflammatory cytokines IL-6, IL-21 and IL-23 are reported to be important for differentiation of T_H17 cell in mice, although it is not clear which cytokines are required for T_H17 development in humans (Lloyd and Hessel, 2010). It has been reported that IL-17 is enhanced in the lungs of patients with severe, refractory asthma and that the T_H17 subset is associated with pulmonary neutrophilia and glucocorticoid resistance (Al-Ramli et al., 2009).

T_H9 cells are another distinct subset of CD4+ T cells, which secret IL-9. T_H9 cells require IL-25 and TGFβ for their proliferation as well as development (Lloyd and Hessel, 2010; Luster and Tager, 2004). IL-9 is responsible for allergic inflammation in the lungs by inducing IgE class switching, mast cell recruitment and mucus production. IL-9 is also produced by mast cells and eosinophils, although immunohistochemistry studies with BAL fluid from asthma patients have detected IL-9 co-localized with the CD4+ T cell subset (Erpenbeck et al., 2003).

In contrast to the above mentioned CD4+ subtypes, Treg and γδT cells (see below) are involved in lung homeostasis and downregulation of inflammatory responses in the lung (Lloyd and Hessel, 2010; Luster and Tager, 2004). The most common Treg cell subset is forkhead box P3 $(FOXP3)^+CD4^+CD25^+$, which needs constant, chronic local antigen exposure for maintenance and survival (Hartl et al., 2007). In addition to releasing IL-10 and TGFβ, Tregs can downregulate MHC class II, along with the co-stimulatory molecules, which are expressed in high numbers on antigen presenting cells (Lloyd and Hessel, 2010). If Tregs do not get continuous antigen exposures, their suppressive effect diminishes and airway responses to innocuous allergens return. A study reported that in the airways of children with asthma, the percentage of

$(FOXP3)^+CD4^+CD25^+$ Treg cells is lower than in healthy children and interestingly the number of these cells can be increased by glucocorticoid treatment (Hartl et al., 2007).

γδ T cells are an innate T cell subtype expressing the γδ T cell receptor (TCR) instead of the more prevalent αβ TCR. Generally, in 95% of T cells, the TCR consists of an αβ heterodimer chain complexed with CD3 as a co-receptor molecule, whereas on the remaining 5% of T cells the TCR consists of a γδ heterodimer plus the CD3 chain (Carding and Egan, 2002; Owen et al., 2013). In contrast to αβ T cells, γδ T cells are $CD4^-CD8^-$ and mainly perform immune-surveillance functions by residing close to the pulmonary epithelium (Carding and Egan, 2002; Owen et al., 2013). Thus, γδ T cells form the first line of defence by imparting remarkable resilience to the lung against diverse environmental injuries. These cells respond to stress signals as well as tissue damage. γδ T cells mostly release T_H1 type cytokines rather than T_H2 type cytokines. Moreover, γδ T cells can respond to both protein as well as non-protein antigens and hence are not MHC restricted (Carding and Egan, 2002). Plasticity of T cell subsets and flexibility of cytokine secretion allows the lung T cell population to efficiently respond to a variety of insults (Islam and Luster, 2012).

1.3 Asthma Therapy

Asthmatics have been classified into four categories: intermittent, mild persistent, moderate and severe; on the basis of the disease severity, symptoms and spirometry (Global initiative for asthma (GINA), 2015; Rabe and Schmidt, 2001). Drug treatment of asthma relies on relievers and controllers. Relievers are bronchodilators, which are taken on an as-needed basis to relieve symptoms while controllers are anti-inflammatory agents. GINA has proposed a stepped

plan (Table 1.1) for asthma control and treatment based on disease severity with step 1 and step 5 referring to the treatment of mild disease and severe disease respectively (Global initiative for asthma, 2015). Step 1 advises the use of short-acting β_2-adrenoceptor agonists (SABAs), on an as-needed basis, as bronchodilators to provide symptom relief. Step 2 involves the addition of an inhaled glucocorticoid (GC; more typically referred to as an inhaled corticosteroid [ICS]), as an anti-inflammatory medication to control the disease, and is given on a regular basis. Step 3 recommends a combination of a long-acting β_2-adrenoceptor agonist (LABA) and an ICS, rather than simply increasing the dose of ICS. Step 4 includes increasing the dose of ICS/LABA combination, while step 5 is for refractory asthma control and recommends the use of alternative, add-on treatments such as oral GCs, leukotriene receptor antagonists and/or anti-IgE therapy. ICSs and LABAs are the mainstay of asthma therapy (Global initiative for asthma, 2015). ICS/LABA combination therapy is considered the most effective, easily accessible, easy to administer and economic option in controlling moderate-to severe allergic, T_H2-mediated, eosinophilic asthma (Barnes, 2006a, 2006b; Global initiative for asthma, 2015; Rabe and Schmidt, 2001). However, in neutrophilic asthma, virus-induced asthma exacerbations and refractory asthma (such as that seen in cigarette smokers), combination therapy is less effective. Consequently, alternative treatment options have been proposed and several of them are undergoing clinical trials. However, so far, it appears that some of these alternative options (e.g. certain anti-cytokine therapies) have severe side-effects and/or are very expensive (anti-IgE therapy) and will not be easily accessible in developing countries.

TREATMENT STEPS				
STEP 1	STEP 2	STEP 3	STEP 4	STEP 5
asthma education				
environmental control				
as needed rapid-acting ß2-agonist	as needed rapid-acting ß2-agonist			
CONTROLLER OPTIONS	SELECT ONE	SELECT ONE	ADD ONE OR MORE	ADD ONE OR BOTH
	low-dose ICS	low-dose ICS *plus* long-acting ß2-agonist	medium- *or* high-dose ICS *plus* long-acting ß2-agonist	oral glucocorticosteroid (lowest dose)
	leukotriene modifier	medium- *or* high-dose ICS	leukotriene modifier	anti-IgE treatment
		low-dose ICS *plus* leukotriene modifier	sustained-release theophylline	
		low-dose ICS *plus* sustained-release theophylline		

Table 1.1 Stepwise asthma treatment strategy recommended by GINA.
(Global initiative for asthma, 2015).

1.3.1 *Conventional Therapies*

1.3.1.1 Glucocorticoids (GCs)

Endogenous GCs are involved in the regulation of amino acid, fatty acid and carbohydrate metabolism and are synthesized from a common cholesterol precursor, cholestane. The synthesis of endogenous GCs and their release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Barnes, 1996; Newton, 2000).

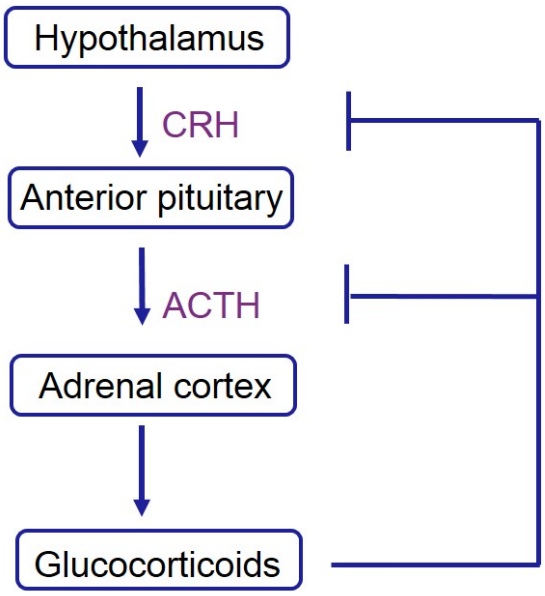
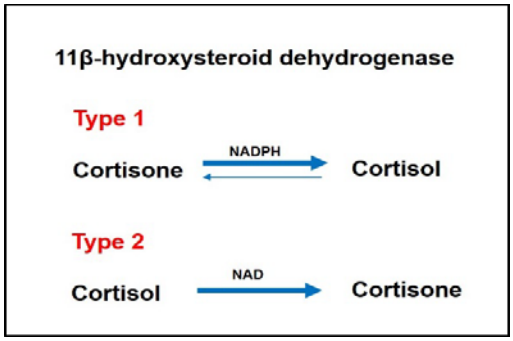


Figure 1.4 Schematic diagram demonstrating regulation of GC release by the hypothalamic pituitary adrenal (HPA) axis and negative feedback regulation by the released GCs.
Key: CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone.

The hypothalamus releases corticotrophin-releasing hormone (CRH), which induces the anterior pituitary to enhance the synthesis and secretion of adrenocorticotropic hormone (ACTH) (Newton, 2000; Taves et al., 2011). ACTH then induces the synthesis of the endogenous GC, cortisol (*aka* hydrocortisone) (Figure 1.4). The metabolism of cortisol is complex being tissue-dependent and controlled by multiple factors. Historically, the liver was regarded as the principal site of cortisol metabolism. However, it has been recognised over the years that many tissues within the body are capable of metabolizing cortisol (Tomlinson and Stewart, 2001). Cortisol can be converted to an inactive GC, cortisone, by the action of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which subsequently controls the concentration of active GC available to elicit physiological responses.

There are two isoforms of 11β-HSD: the type 1 enzyme functions as a reductase generating cortisol from inactive cortisone, whereas the type 2 enzyme functions as a dehydrogenase inactivating cortisol to tetrahydrocortisone (THE), 5β-tetrahydrocortisol (THF) and 5α-

tetrahydrocortisol (allo-THF) (Feinstein and Schleimer, 1999; Monder et al., 1989; Tomlinson and Stewart, 2001). In addition to the liver, kidney and GI tract, which are major metabolic organs, airway epithelial cells and ASM cells also express high levels of 11β-HSD (Tomlinson and Stewart, 2001). Notably, inflammatory stimuli such as TNF-α, IL-4, IL-5, IL-6 and IFNγ have been reported to induce Type 1 11β-HSD, which suggests there is localised GC generation under acute inflammatory conditions (Tomlinson and Stewart, 2001).



According to the current literature, cortisol is involved in regulating many functions such as development, growth, behaviour, apoptosis and suppression of inflammation (Zhou and Cidlowski, 2005). Corticosteroid-binding globulin (CBG) is the major blood transport protein for cortisol in humans, while, in contrast, synthetic GCs are reported to bind to plasma albumin in the circulation (Fernandez-Real et al., 2002). CBG is the dynamic regulator of GC bioavailability and is a member of the SERPIN (serine protease inhibitors) family. Interestingly, CBG provides a pool of circulating cortisol, available to be released both systemically and at a tissue level, on interaction with proteinases, such as neutrophil elastase, which is expressed by activated neutrophils during inflammation (Fernandez-Real et al., 2002). Synthetic GCs mimic the effects of cortisol and thus, act as effective anti-inflammatory controller medications in asthma. As shown in figure 1.4, GCs also act as negative feedback agents in the HPA axis and inhibit the release of CRH and ACTH (Newton, 2000; Taves et al., 2011). Moreover, GCs, due to their anti-inflammatory activity, inhibit

the expression of many pro-inflammatory mediators, which results in a reduction of asthma symptoms and exacerbations, an improvement in quality of life and lung function, and a reduction in AHR (Necela and Cidlowski, 2004; Zhou and Cidlowski, 2005). Some of the commonly used GCs for asthma treatment include fluticasone propionate, budesonide, beclomethasone dipropionate, ciclesonide and mometasone furoate which are administered either alone or in combination with a LABA (Rabe and Schmidt, 2001) [further discussed in sections 1.3.1.2 & 1.3.2].

The glucocorticoid receptor (GR, gene name: *NR3C1*), which is present in the cytoplasm of different cell types (further discussed in sections 1.4.3 and 1.4.5), is the cognate receptor for GCs (Zhou and Cidlowski, 2005). In the cytoplasm, unliganded GR exists in a complex with different chaperone proteins and immunophilins, which masks its nuclear localization signal. Some of the major chaperone proteins and immunophilins which are associated with GR are: heat shock protein 90 (Hsp90), protein phosphatase 5 (PP5) and FK506-binding proteins (FKBP51/FKBP52) (Vandevyver et al., 2012). GCs rapidly diffuse through the cell membrane and bind to GR, which undergoes a conformational change, resulting in FKBP51 to FKBP52 exchange, exposure of a nuclear localization signal and the association of importins and dynein-dynactin motor proteins. The latter promote the rapid translocation of the GC-GR-chaperone complex to the nucleus (Vandevyver et al., 2012). Once inside the nucleus the chaperone machinery and importins dissociate from the GC-GR complex and the free liganded GR can then interact with accessible glucocorticoid response elements (GREs) on target genes (see sections 1.4.2 to 1.4.5), to up-regulate or down-regulate gene transcription (Figure 1.5). Alternatively, it can bind to and prevent the activity of pro-inflammatory transcription factors (as discussed below).

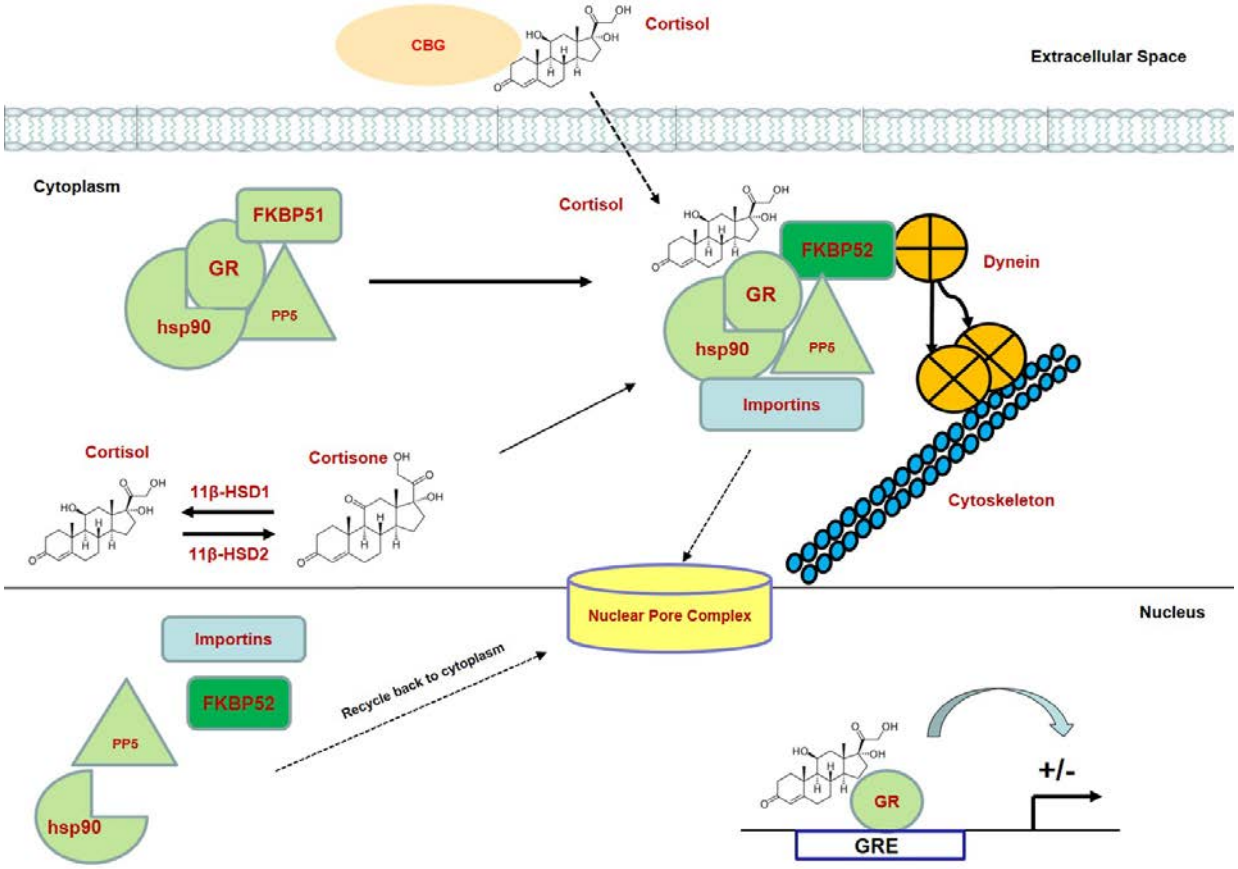


Figure 1.5 Chaperone-mediated translocation of GR.
When in the circulation, cortisol is normally bound to CBG, while synthetic GCs bind to albumin, to resist degradation. On receiving stress signals, proteases or elastases cleave CBG freeing cortisol, which can then enter the cytoplasm where it binds GR. In the cytoplasm, GR is present in a dormant state bound to multiple chaperones as well as immunophilins. However, on binding with GC, GR is translocated to nucleus by interacting with importins and dynein proteins. Once in nucleus the chaperone proteins and immunophilins dissociate from the GC-GR complex, which can then modulate gene expression. Cortisol and synthetic GCs can also be metabolised in the cytoplasm by a family of 11β-HSD enzymes, thereby reducing their biological activity (Adapted from Vandevyver et al., 2012).

Two related, interdependent, anti-inflammatory mechanisms of action of GCs have been reported, which are referred as 'transactivation' and 'transrepression'. Transrepression is a process whereby the ligand-bound GR directly inhibits the transcription of pro-inflammatory genes, whereas repression via transactivation mechanism involves the induction of anti-inflammatory

genes, which then switch-off a variety of pro-inflammatory responses (King et al., 2013; Newton, 2000; Newton and Holden, 2007; Newton et al., 2010). Some of the genes transactivated by the GC-GR complex exhibit therapeutic potential in asthma and include cyclin-dependent kinase inhibitor 1C (*CDKN1C/p57^{kip2})*, glucocorticoid-induced leucine zipper (*GILZ*), dual specificity phosphatase 1 (*DUSP1/MKP1*), IL-1 receptor antagonist and regulator of G-protein signaling 2 (*RGS2*) (King et al., 2013; Newton, 2000; Newton and Holden, 2007; Newton et al., 2010). For example, *RGS2* is bronchoprotective, as it inhibits signaling induced by agonists that act via G-protein-coupled receptors (GPCR), specifically those that work through the heterotrimeric G-protein, Gq; *RGS2* may also have anti-inflammatory activity (Holden et al., 2014). *p57^{kip2}* and *DUSP1* attenuate the activity of inflammatory kinases such as ERK and JNK (Owens and Keyse, 2007; Chang et al., 2003), whereas *GILZ* inhibits the transcriptional activity of both nuclear factor kappa-light-chain-enhancer of B cells (NF-κB) and activator protein 1 (AP-1) (Ayroldi and Riccardi, 2009). Conversely, some of the adverse effect proteins which are transrepressed by the GC-GR complex include osteocalcin, collagenase, IL-1β, AP-1 and NFκB (King et al., 2013; Newton, 2000; Newton and Holden, 2007; Newton et al., 2010).

However, not all genes induced by GCs are beneficial. Some of them, including metabolic genes, can result in serious side-effects. For this reason, transactivation was not always considered a desirable feature of GCs. Accordingly, to avoid negative effects of GC-induced gene transactivation, the pharmaceutical industry has used several approaches in attempts to reduce the unwanted actions of GCs. One of these is the development of 'dissociated' GR ligands or SEGRAs (SElective GR Agonists), which supposedly cause fewer side-effects than classical GCs, through their ability to promote transrepression but not transactivation (Newton, 2000; Newton and Holden, 2007). Examples of apparently dissociated ligands include RU 24858 (Belvisi et al., 2001;

Chivers et al., 2006; Vayssière et al., 1997) and GW870086X (GW; Leaker et al., 2015; Uings et al., 2013), which was tested in this study and is further discussed in the results sections. In addition to dissociated steroidal GR ligands, non-steroidal agonists such as GSK 9027 (GSK) have also been examined in an attempt to reduce GC related side-effects (Yates et al., 2010). We included GSK 9027 in our studies (see chapter 4) to compare its transactivation potential with other GCs. Furthermore, pro-drugs, or soft-steroids, is another approach used to limit side-effects. Such molecules demonstrate lung-specific activation and hence produce less systemic exposure (Barnes, 2004). Examples of soft steroids include des-isobutyryl ciclesonide (further discussed in Chapters 3 to 5) and beclomethasone dipropionate. Both of these GCs are only activated in the lung due to the presence of specific pulmonary esterases (Barnes, 2004). This mechanism of action requires the presence of a hydroxyl group in the steroid at position 21 that is attacked by the esterase. Accordingly, these GCs remain inactive until the hydroxyl group is cleaved. Finally, fluticasone furoate, budesonide and fluticasone propionate were synthesized for rapid hepatic clearance to reduce systemic exposure and, therefore, side-effects (Barnes, 2004).

Despite all efforts to reduce GC-related side-effects, it has not been possible to separate transactivation from transrepression (Figure 1.6). Moreover, this thinking was probably naïve given that both transactivation and transrepression are now believed to mediate the anti-inflammatory actions of GCs (King et al., 2013; Newton, 2000; Newton and Holden, 2007; Newton et al., 2010). Moreover, published data clearly demonstrate that the transcription inhibitor, actinomycin D and the protein translation inhibitor, cycloheximide, attenuate GC-induced suppression of pro-inflammatory genes including IL8, IL6, CXCL1, CXCL2, GM-CSF and COX-2 suggesting a role for GC-GR transactivation (Chivers et al., 2006; King et al., 2013). In addition, zinc finger protein 36 (ZFP36), an mRNA destabilizer, is induced by GCs and inhibits pro-

inflammatory proteins by destabilizing their mRNAs (King et al., 2009; Smoak and Cidlowski, 2006). Collectively, these data suggest that gene transcription and synthesis of new proteins (i.e. transactivation), along with transrepression is necessary for the anti-inflammatory actions of GCs (Figure 1.6). Consistent with this claim is the recent report that many of the aforementioned, GC-induced anti-inflammatory genes are induced in lung biopsies taken from asthma patients given budesonide by inhalation (Kelly et al., 2012).

Nonetheless, transactivation as a mechanism of action of GCs is still relatively unexplored when compared to transrepression. For this reason, the present study was conducted to investigate the pharmacology of GC-induced, gene transactivation with an emphasis on the pharmacodynamics of the GC-GR interaction. Furthermore, the present research explored the extent to which gene transactivation is affected by a panel of different, clinically-relevant GR ligands and how this process is modulated by the LABA, indacaterol (see below).

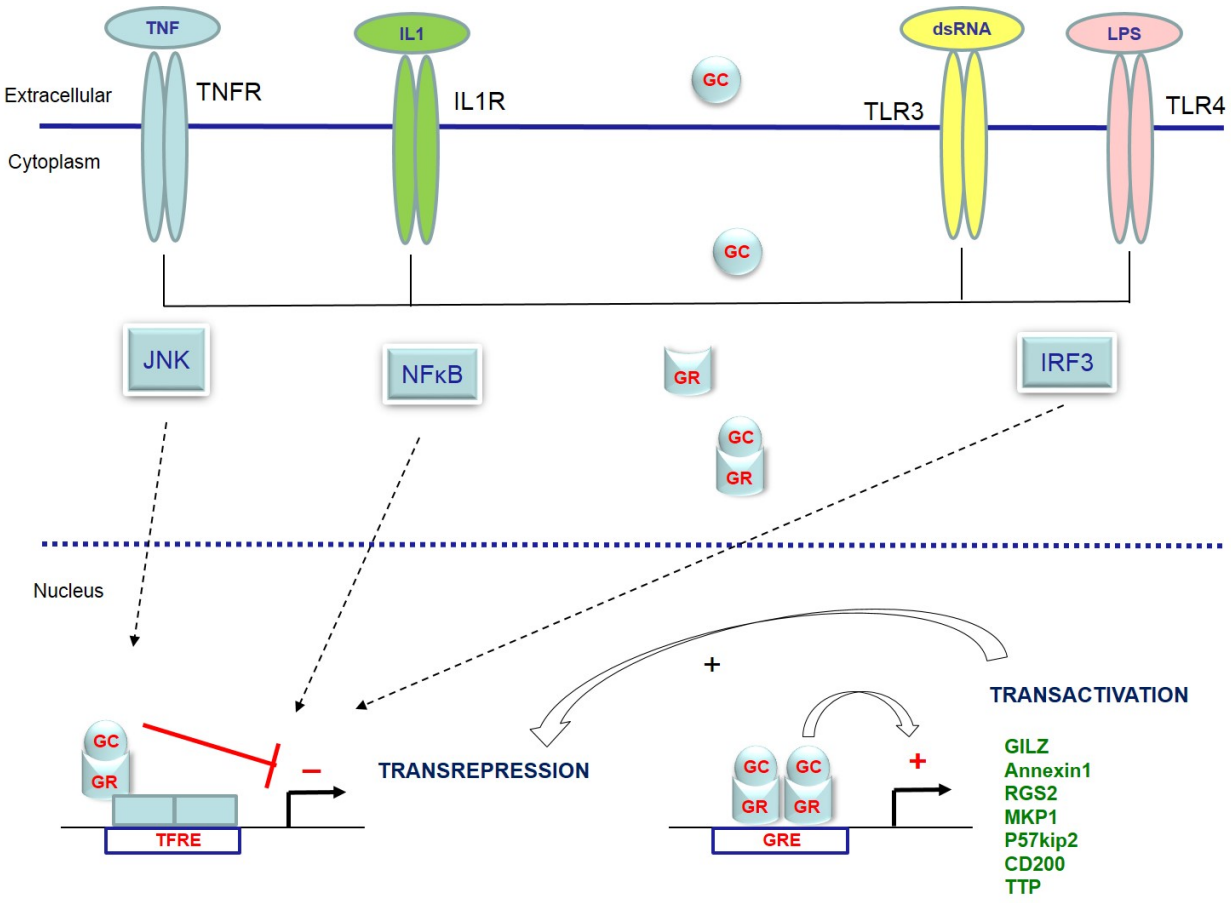


Figure 1.6 Transactivation and transrepression by GCs.
Simplified figure showing that in the presence of a pathogenic antigen, structural and immune cells release multiple pro-inflammatory mediators, which on binding to their respective receptors, activate downstream pro-inflammatory modulators such as IRFs, JNK and MAPK. In the absence of GCs these pro-inflammatory regulators activate NFκB, which translocate to the nucleus and lead to transcription of multiple cytokines and chemokines, consequently further amplifying inflammatory processes. In the presence of a GC, the GC-GR complex translocates to the nucleus and either (i) interacts as a dimer with GREs in the DNA sequence of anti-inflammatory genes (i.e. transactivation), which in turn represses pro-inflammatory gene expression, or (ii) tether physically to the NFκB, subsequently interfering in up-regulation of pro-inflammatory genes (i.e. transrepression) (Adapted from Beck et al., 2009).

1.3.1.2 Short- and long-acting β_2-adrenoceptor agonists (SABAs and LABAs)

β_2-adrenoceptor (β_2-AR) agonists are categorized into three groups based on their duration of action, namely: SABAs, effective for up to 4h; LABAs effective for 12h and ultra LABAs

effective for 24h (Cazzola et al., 2010). SABAs, such as terbutaline and salbutamol, are used as rapid relievers for asthma control (Holgate and Polosa, 2008; Rabe and Schmidt, 2001). β_2-AR agonists binds to the β_2-ARs expressed on airway smooth muscle to effect relaxation. However, they also probably activate β_2-ARs on epithelial cells, eosinophils, neutrophils macrophages and T-cells where they may exert additional therapeutic benefit especially in combination with an ICS (Johnson, 2006).

Adrenoceptors are GPCRs and have been classified in to two main types: α and β, which are further divided into six α-subtypes (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}) and three β-subtypes (β_1, β_2, β_3). On ligand binding, α-adrenoceptors, which can couple to the heterotrimeric G-proteins, G_q or G_i, can mediate an increase in Ca^{2+} levels and the inhibition of adenylyl cyclase (AC) respectively; in smooth muscle these effects typically cause smooth muscle contraction (Johnson, 2006). Many studies have reported the presence of both α and β adrenoceptors in the lung (Johnson, 2006; Johnson et al., 2001; Roth et al., 2002). However, β_2-ARs are primarily responsible for regulating ASM tone and on agonist binding induce smooth muscle relaxation by activating a G_s/adenylyl cyclase (AC)/cAMP-signalling cascade (Giembycz and Newton, 2006; Johnson, 2006).

When SABAs or LABAs bind to the β_2-ARs it undergoes a conformational change, causing the dissociation of $G_{\alpha s}$ from the βγ subunits, allowing $G_{\alpha s}$ to activate AC (Johnson, 2006). Activation of AC results in the production of cyclic adenosine 3'5'-monophosphate (cAMP) from adenosine 5'-triphosphate (ATP) (Figure 1.7). cAMP activates protein kinase A (PKA), which is a tetrameric enzyme containing two regulatory and two catalytic subunits (Johnson, 2006). The catalytic subunits dissociate from the regulatory subunits due to the conformational change that results following cAMP binding to the regulatory subunits (Billington et al., 2013). The catalytic subunits can then phosphorylate multiple cellular proteins, including the

inositol(1,4,5)trisphosphate receptor (IP_3R), heat shock protein 20KDa (Hsp20), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP) and the high conductance, calcium-activated, potassium channel (BK_{Ca}), which ultimately causes ASM relaxation and hence, bronchodilation (Figure 1.7) (Beall et al., 1999; Conti and Adelstein, 1981; Kume et al., 1989; Tertyshnikova and Fein, 1998; Velasco et al., 2002). In addition to PKA, an increase in cAMP may also affect bronchodilation by activating other effector pathways involving cGMP-dependent protein kinase (PKG) and exchange proteins directly activated by cAMP (Epac) (Figure 1.7) (Billington et al., 2013; Hamad et al., 2003; Roscioni et al., 2011). Of relevance to this thesis is that PKA catalytic subunits can also phosphorylate the transcription factor cAMP-response element-binding protein (CREB) at Ser^{133} leading to gene transcription (Mayr and Montminy, 2001).

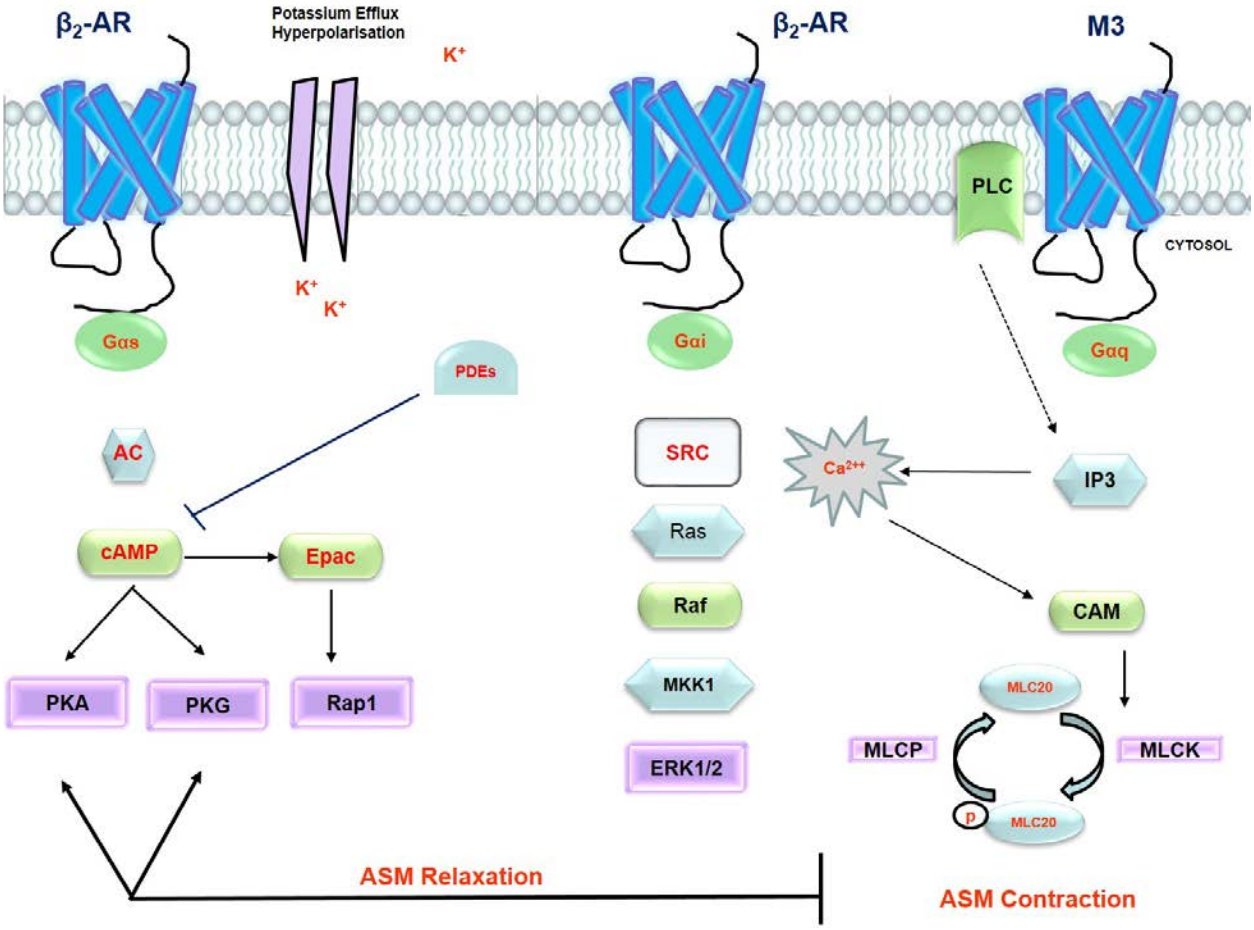


Figure 1.7 Regulation of multiple effector pathways by β2-ARs and mechanisms of smooth muscle relaxation.

Contractile agonists such as acetylcholine bind to Gq-coupled M_3-muscarinic receptors causing PLC activation and the generation of IP_3. IP_3 releases calcium stored in the endoplasmic reticulum into the cytosol. Calcium-calmodulin and MLCK form a complex leading to the phosphorylation of myosin light chain 20 (MLC20) and ASM contraction. ASM contraction is inhibited by PKA and PKG by reversing MLC20 phosphorylation. Key: AC, adenylyl cyclase; PDEs, Phosphodiesterases; PKA, protein kinase A; MKK1, mitogen activated protein kinase; Epac, exchange proteins activated by cAMP; MLCK, myosin light chain kinase; CAM, calmodulin; PLC, phospholipase C. (Adapted from Giembycz and Newton, 2006).

1.3.2 *Combination Therapy*

In asthma, the use of SABAs and LABAs as a monotherapy given on a regular basis is not recommended because they do not reduce airway inflammation and may even worsen the disease (Global initiative for asthma, 2015). However, LABAs are used in combination with ICSs in asthmatic subjects that are not well controlled with an ICS alone. Some examples of LABAs (generally used in combination with ICS) used for asthma control include formoterol and salmeterol (Cazzola and Matera, 2008; Newton et al., 2010). The combination of fluticasone propionate with salmeterol, and budesonide with formoterol show greater efficacy in asthma management than the ICS alone, which has resulted in the development of inhaler devices containing fixed combinations of the aforementioned drugs (Giembycz and Newton, 2006; Greening et al., 1994; Masoli et al., 2005; Newton et al., 2010; O'Byrne et al., 2001; O'Byrne et al., 2005; Rabe and Schmidt, 2001). Presently, four such drug combinations are available in the single inhaler devices, which are marketed as: Symbicort® (formoterol fumarate dihydrate plus budesonide), Seretide®/Advair® (salmeterol xinafoate plus fluticasone propionate), Foster®/Fostair® (formoterol fumarate dihydrate plus beclomethasone dipropionate) and, more recently, Zenhale®/Dulera® (formoterol fumarate dihydrate plus mometasone furoate). These combination therapies improve asthmatic symptoms, decrease exacerbations, improve lung function and decrease the risk of asthma-related hospitalizations (Greening et al., 1994; O'Byrne et al., 2001; O'Byrne et al., 2005).

With the tremendous success of Symbicort and Seretide/Advair in asthma management, second-generation, ICS/LABA combination therapy has been developed. An example is Breo®/Relvar® [vilanterol trifenatate plus fluticasone furoate (FF)], which has the advantage of

once-a-day dosing (Cazzola et al., 2011) and was recently granted marketing authorization by the European Commission and FDA. Additional, so-called, ultra-LABAs are likely to become available for asthma treatment within the next few years including indacaterol and carmoterol, which will probably be combined with long acting GCs such as mometasone furoate (Tamm et al., 2012).

1.3.2.1 Potential mechanisms explaining clinical superiority of combination therapy

Despite the many studies showing superiority of ICS/LABA combination therapy over ICS alone, the mechanistic basis of their efficacy remains to be elucidated. Several possibilities have been suggested that require some form of interaction between the two components of the combination therapy that are not mutually exclusive. The classical interaction of a SABA or a LABA with the β_2-AR results in cAMP accumulation and PKA activation. This may then lead to bronchodilation and gene transcription (Figures 1.8 & 1.9). It has been shown that ICSs potentiate β_2-AR-mediated signaling, reverse agonist-induced β_2-AR desensitization, increase β_2-AR number (Chong et al., 1997; Collins et al., 1988), and enhance both Gsα expression and coupling to adenylyl cyclase (Kalavantavanich and Schramm, 2000). Intriguingly, due to the presence of a GRE site in the promoter region of β_2-AR gene (*ADRB2*), ICSs can potentially augment the rate of transcription of *ADRB2* gene and hence, may counteract the agonist-induced desensitization of β_2-AR (Baraniuk et al., 1997; Mak et al., 1995).

Conversely, β_2-AR agonists and other stimuli that elevate cAMP, have been reported to upregulate GR number in the airways (Dong et al., 1989). There is also evidence that β_2-AR agonists can promote ligand-independent activation and translocation of GR to the nucleus in a cAMP/PKA-dependent manner (Eickelberg et al., 1999). Additionally, β_2-AR agonists can

enhance GR phosphorylation and GR sensitivity to activating ligands that is mediated by the direct or indirect activation of multiple kinases (PKA/PKG/MAPKs) (Chen et al., 2008; Galliher-Beckley and Cidlowski, 2009; Ismaili and Garabedian, 2004). Finally, PKA has been reported to regulate the recruitment of transcription factors at GREs in hepatoma cells (Espinás et al., 1995) and to enhance GR binding to GREs (Rangarajan et al., 1992).

Data obtained from several primary cells and cell lines show that LABAs and GCs can interact in either an additive manner, where the functional responses they induce simply summate, or in a synergistic manner, where the functional responses produced by the two drugs in combination is greater than the sum of their individual responses (Newton et al., 2010).

In this respect, ASM hypertrophy and hyperplasia are believed to be crucial in causing AHR and lung function decline in asthma (Dekkers et al., 2012). Notably, ICS/LABA combination therapy is reported to synergistically inhibit human bronchial smooth muscle proliferation by activating CCAAT-enhancer binding protein-α (C/EBP-α) and cyclin dependent kinase inhibitor p21 (Roth et al., 2002). Similarly, Dekkers *et al*., (2012) have reported that ICS/LABA combination therapy synergistically inhibits the release of platelet-derived growth factor, fibroblast growth factor and collagen I (which are involved in ASM proliferation and phenotypic conversion), in bovine tracheal smooth muscle by enhancing the expression of the cell cycle inhibitors $p57^{kip2}$ and *p21*. Another study reported that the combination of formoterol and budesonide synergistically inhibited the expression of adhesion molecules (ICAM-1, VCAM-1) on, and GM-CSF release from, human lung fibroblasts, which suggests the potential clinical superiority of the combination in controlling asthmatic inflammation (Spoelstra et al., 2002).

To account for these data, two possible interactions can model the superior efficacy of ICS/LABA combination therapy: (a) a LABA and a GC induce mechanistically-different signaling

pathways that collectively generate additivity or synergy, or (b) a LABA augments the functional response of a GC through a common mechanism resulting in synergy (Figures 1.8 & 1.9).

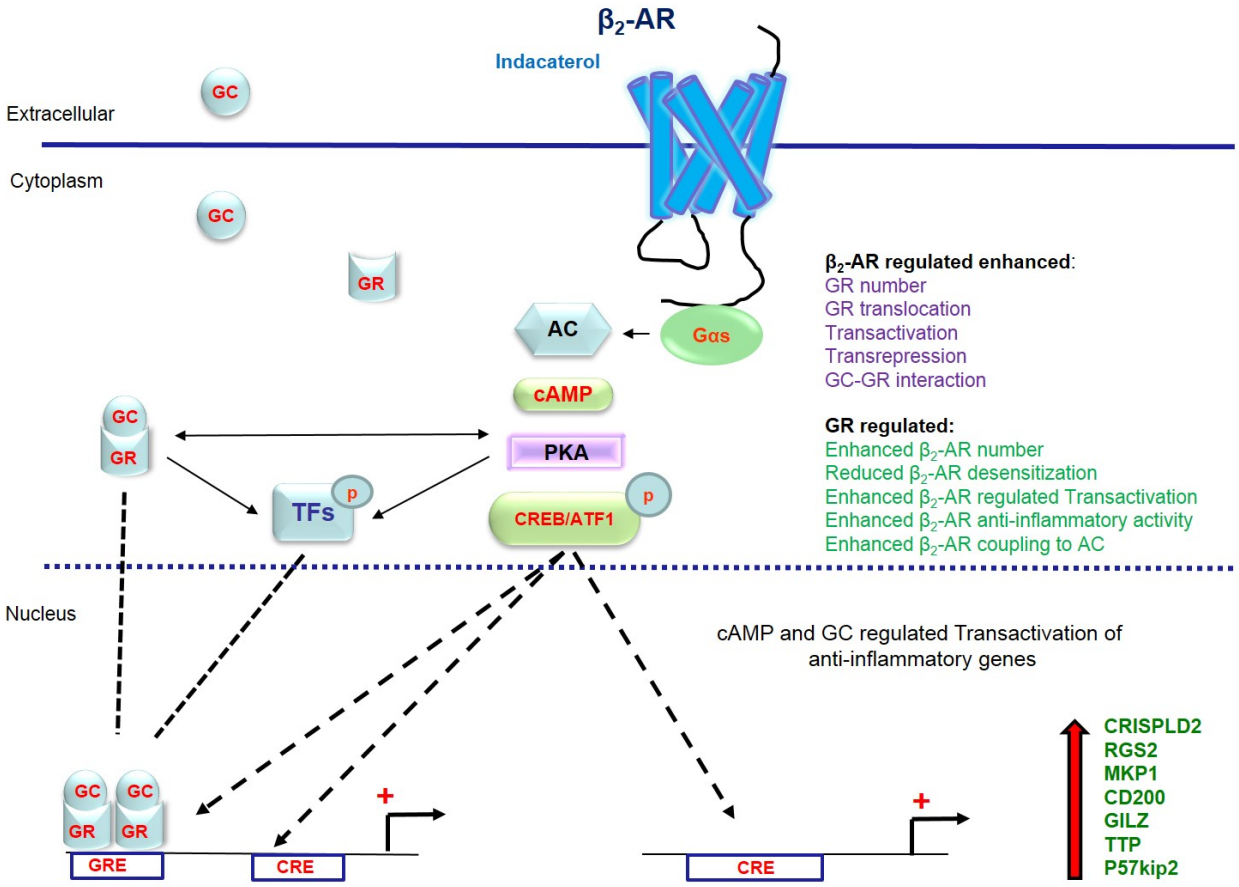


Figure 1.8 A simplified schematic showing mechanisms that could explain the enhanced clinical benefit of ICS/LABA combination therapy.
The figure shows that the GC-GR complex translocates to the nucleus and interacts with GRE sites in DNA leading to transactivation of beneficial genes. In the presence of a LABA, this effect is enhanced due, for example, to GR phosphorylation by PKA, or to the activation of cAMP dependent transcription factors (TFs). In addition, genes can be upregulated independently of the GR by PKA-dependent activation of additional transcription factors such as cAMP response element binding protein (CREB) and activating transcription factor-1 (ATF-1). Key: cAMP response element (CRE); glucocorticoid response element (GRE) (Giembycz and Newton, 2006, 2015; Giembycz et al., 2008).

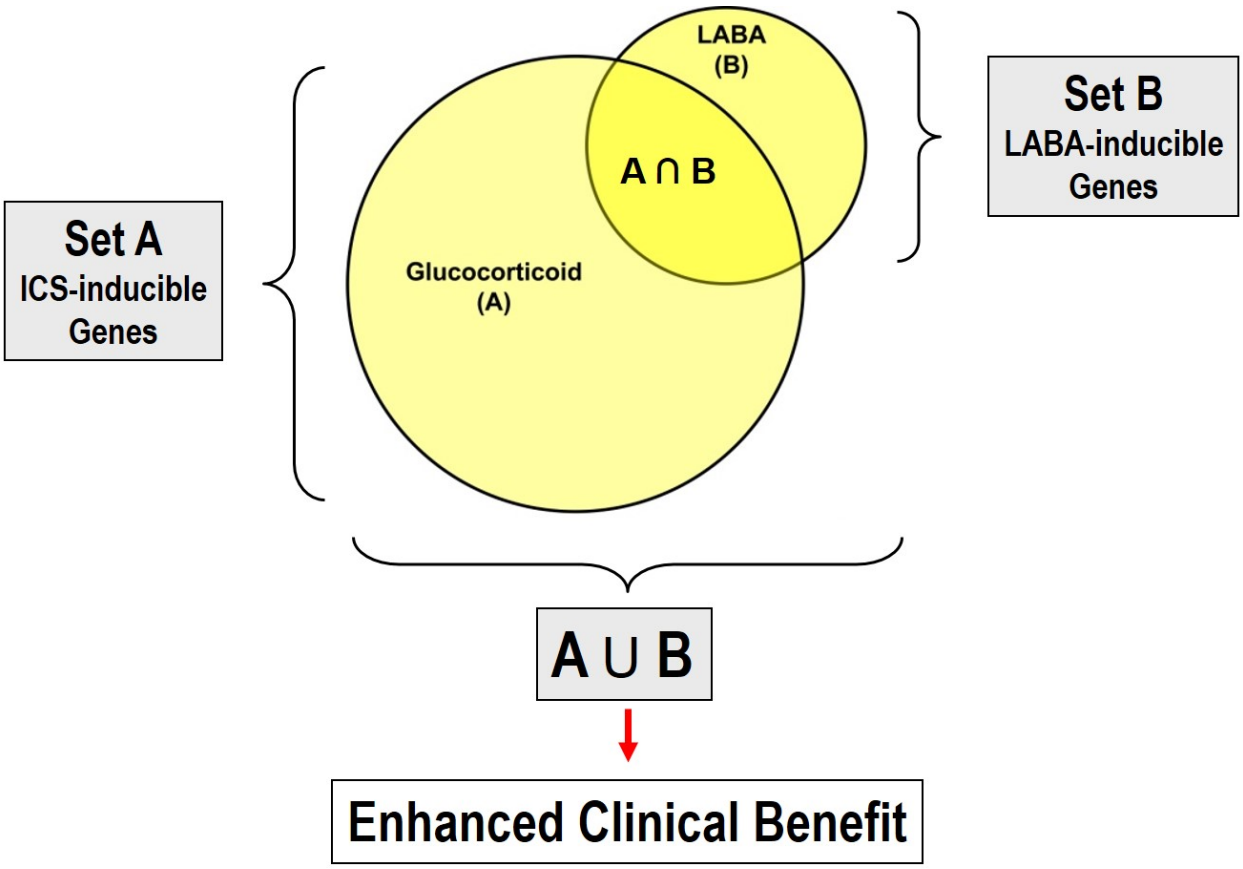


Figure 1.9 A model to explain the enhanced clinical benefit of an ICS/LABA combination therapy.
A LABA and an ICS each induce a set of genes. These sets overlap and genes in the intersection are induced in an additive or synergistic manner. Thus, there are certain genes which are only regulated by GCs (set A), and other genes which are only induced by LABAs (set B). The model indicates that the benefit of ICS/LABA combination therapy in asthma reflects the union (i.e. A U B) of all gene expression changes, which is dependent on those genes which are additively or synergistically co-induced by both GC and LABA (A ∩ B). Examples of such genes are discussed further in chapter 5 (Joshi et al., 2015).

1.3.3 Alternative Therapies: Muscarinic Receptor Antagonists, Oral GCs and Leukotriene Receptor Antagonists

Muscarinic receptor antagonists (e.g. tiotropium bromide), oral GCs (e.g. prednisone) and leukotriene receptor antagonists (e.g. Zileuton: trade name Zyflo®) are recommended add-on therapies for severe or difficult to treat asthma. Muscarinic receptor antagonists or anti-cholinergics, inhibit binding of acetylcholine to muscarinic receptor (Figure 1.7). Five muscarinic receptor subtypes, M_1-M_5, are recognised, which belong to the GPCR superfamily. M_1, M_2 and M_3 are localised to human airways (Moulton and Fryer, 2011). In severe, chronic asthma, the release of neuronal and non-neuronal acetylcholine from nerve cells, immune cells and structural cells increases, which may activate M_3 receptors on airway smooth muscle and glands to promote bronchoconstriction and mucus secretion respectively (Moulton and Fryer, 2011, Figure 1.7). Additionally, acetylcholine has been implicated in the enhanced secretion of cytokines and chemokines, which subsequently lead to the recruitment of multiple immune cells and pulmonary inflammation (Wessler et al., 1998). Thus, anticholinergics such as tiotropium bromide may be beneficial in these patients by alleviating bronchoconstriction and airway inflammation (Barnes, 2004).

Oral GCs such as prednisone are prescribed to patients with difficult to control, severe asthma (Global initiative for asthma, 2015). Prednisone serves as the most effective treatment for severe asthma attacks, when other treatments fail to provide relief. Prednisone is prescribed only for short durations. A short course may be as brief as 3-4 days or as long as 2-3 weeks (Chapman et al., 1991). In addition to duration, dose also varies on the basis of asthma severity and a patient's sensitivity to the medication. For rapid relief during a severe asthma exacerbation, a high dose is given, which is gradually reduced, until the oral GC is stopped; this process is referred to as a

"steroid taper" (Chapman et al., 1991; Scarfone et al., 1993). Prolonged and continuous treatment with oral GCs is generally avoided because of the undesirable side effects that often develop with this dosing regimen (Chapman et al., 1991; Scarfone et al., 1993).

In some patients with asthma, leukotrienes (LT) play a major role in compromising lung function. In these sensitive individuals, targeting CysLTR1 with selective antagonists can be beneficial because they often suppress bronchoconstriction, plasma exudation and mucus secretion (Barnes, 2004). Zileuton is an example of a leukotriene receptor antagonist used for asthma maintenance; it is also an inhibitor of 5-lipoxygenase, which is the rate-limiting enzyme involved in the biosynthesis of LTB_4, LTC_4, LTD_4, and LTE_4 (Barnes, 2004).

1.3.4 Protective Tolerance and Allergen Avoidance

Atopic individuals can be protected against sensitization to some of the common innocuous aeroallergens by administration of specific immunotherapies. Studies have reported that exposure to allergen in early infancy instead of later years can be beneficial at inducing tolerance towards an allergen such as peanut and animal dander (Holgate and Polosa, 2008). However, in adults, specific immunotherapy (SIT) is an option of inducing tolerance against common aeroallergens. After repeated experimental low dose exposures of allergens, SIT induces immunological tolerance by boosting IgG antibodies and reducing allergen specific IgE subtypes (Holgate and Polosa, 2008; Holt and Sly, 2012). Uniquely, SIT decreases the recruitment and activation of mast cells, basophils, neutrophils and eosinophils. SIT can be administered in two ways: a) by subcutaneous immunotherapy (SCIT), which involves regular subcutaneous injections of increased dose of allergen, and b) by sublingual immunotherapy (SLIT), which comprises the oral administration of allergen to induce tolerance (Holgate and Polosa, 2008). These two SIT therapies

augment Treg proliferation, which release IL10 and TGFβ to attenuate allergen-induced immune responses. SCIT and SLIT have few mild side-effects and are suitable for children (Holgate and Polosa, 2008).

1.3.4.1 Anti IgE monoclonal antibody

Xolair® (*aka* omalizumab) is administered to allergic asthmatics, especially those with moderate-to-severe, inadequately controlled disease despite receiving daily, high doses of ICS/LABA combination therapy. Omalizumab is a monoclonal, humanised, IgE-specific, IgG1 antibody that binds to the C3 domain of IgE and, hence, blocks IgE-FcεR1 interaction on the surface of mast cells, eosinophils, dendritic cells and basophils (Holgate and Polosa, 2008). Additionally, omalizumab reduces surface bound IgE on FcεRI-bearing cells, limiting the degree of release of allergic mediators. It also reduces the number of FcεRI receptors on basophils in atopic patients (Pelaia et al., 2012). Moreover, by inhibiting the binding of IgE to FcεRI expressed on dendritic cells, omalizumab can reduce the efficiency of antigen presentation to T lymphocytes and lowers free serum levels of IgE by 96–99% (Pelaia et al., 2012). TGFβ and endothelin-1 are involved in eliciting structural changes in the airways, such as sub-epithelial fibrosis and proliferation of bronchial smooth muscle cells. Omalizumab suppresses the production of TGFβ in a cellular model of allergic asthma and decreases endothelin-1 in the exhaled breath condensate of patients with severe, persistent, allergic asthma (Zietkowski et al., 2010). A recent study reported that omalizumab reduces airway wall thickness, mucous gland metaplasia and fibrosis in mouse models of allergic asthma (Hoshino and Ohtawa, 2012). Notably, using computed tomography imaging data obtained from asthmatics, omalizumab was reported to reduce airway wall thickness and increase bronchial luminal area (Riccio et al., 2012).

1.3.4.2 Anti-cytokine therapies

Interleukin-4 is responsible for class switching of B cells and IgE generation. Hence IL-4-targeted monoclonal antibodies have been developed and are effective in decreasing IgE synthesis and AHR in a murine model of asthma (Zhou et al., 1997). Moreover, the humanized, IL-4-targeted, monoclonal antibody, pascolizumab, effectively neutralized IL-4 responses *in vitro* and in a randomized, placebo controlled, Phase I trial carried out in patients with mild-to-moderate asthma, was well tolerated (Pelaia et al., 2012). However, pascolizumab failed subsequent phase II clinical trials due to its inability to provide clinical improvements in asthma symptoms. Similarly, an inhaled, non-immunogenic human recombinant IL-4 receptor for asthma treatment failed in clinical trials due to lack of efficacy and bioavailability (Holgate and Polosa, 2008).

Interleukin-5 induces eosinophilia by enhancing the differentiation of eosinophil progenitors and survival. Mepolizumab (*aka* Nucala®) is an anti-IL-5 antibody recently approved by the FDA that was found to be efficacious in patients with severe asthma characterized by persistent, ICS-resistant eosinophilia (Nair et al., 2009). Mepolizumab decreases the number of eosinophils and frequency of exacerbations, as well as ICS consumption (Pelaia et al., 2012).

Interleukin-13 is responsible for IgE production, recruits eosinophils, macrophages, T cells into the airways and promotes ASM hyperplasia (Barnes, 2004, 2006b; Holgate and Polosa, 2008). Thus, antagonising IL-13 is a potential treatment option. An anti-IL-13 humanised antibody has been developed and after successful phase I trial has entered phase II evaluation (Pelaia et al., 2012). Similarly an anti-IL-9 antibody has been reported to reduce airway inflammation and AHR in human asthma and is currently in phase II clinical development (Parker et al., 2011). The results of these two efficacy trials will be of considerable interest.

Initial studies in experimental animal models might provide the optimistic view that targeting cytokines can be the ultimate solution for treating asthma. However, the translation of these findings to asthmatic patients is not evident and has not yet fulfilled expectations. Some of the reasons that account for the poor success of anti-cytokine therapy could be biological redundancy between cytokines (e.g. between IL-4 and IL-13), which could be addressed by targeting them together instead of separately. Also, the reported discrepancies between studies in animal models and humans with asthma could be due to several reasons. In particular, mice, which are often used for such preclinical studies, do not spontaneously develop asthma, unlike humans, and do not exhibit heterogeneity of the asthma phenotype.

In concluding this section, it is clear that many of the more novel, or alternative, therapies are often associated with severe side-effect profiles, lack of efficacy, clinical outcome discrepancies and problems in securing approval from the US FDA; thus, in 2016 conventional therapies still remain the most effective treatment options for asthma management.

1.4 Regulation of GR- and β_2-AR-Mediated Gene Transcription

1.4.1 *Response Elements and Transcription Factors*

Proteins that are involved in chromatin rearrangement and transcription of a gene are known as transcription factors (TFs). TFs are directly or indirectly involved in the modulation of the transcriptional machinery and hence can regulate the expression of a gene. Usually, a TF has two distinct domains: one for DNA binding (DBD) and the other for activation of transcription (an activation function [AF]) (Todeschini et al., 2014). TFs such as GR, which also acts as a receptor, possess a third domain for a ligand to bind (LBD) (Clark and Belvisi, 2012). Furthermore, TFs can

interact with other sets of proteins, so-called co-activators or co-repressors, which tether to TFs and modulate their activity (Gertz et al., 2013). Thus, TFs modulate transcription by interacting with co-activators or co-repressors, which influences the binding of RNA polymerase II to the promoter of target genes and, thereby, the efficiency of transcription (Gertz et al., 2013). TFs recognize and bind to specific target sequences in the eukaryotic genome; such binding sites are referred to as response elements (REs) (Khorasanizadeh and Rastinejad, 2001). Interestingly, according to recent studies only 0.01% of the genome is bound by TFs despite the presence of a multitude of REs (Gertz et al., 2013). Several REs are found associated with a gene promoter mostly at an upstream location, which suggests the significant involvement of REs in transcriptional regulation. Incidentally, a gene may contain multiple copies of a specific RE upstream of its promoter sequence (Todeschini et al., 2014). Additionally, REs contain short consensus sequences, which can be similar in different genes but are not identical. Often, REs consists of two half sites which can be direct repeats or palindromes, separated by 0-4 base pairs (Khorasanizadeh and Rastinejad, 2001). Consequently, RE recognition depends upon the orientation of half sites and spacing. Notably, the co-occurrence of two distinct half sites together (generally referred to as composite sites) results in context-dependent transcriptional control and is the basis of spatiotemporal gene regulation (Matzinger and Kamala, 2011).

1.4.2 *Cell Type- and Gene-Specific Transcriptional Regulation*

Strict gene regulation is mandatory for a targeted, cellular response against an environmental cue. Accordingly, differential recruitment of TFs to respective binding sites is a well-orchestrated regulatory affair. Many genome wide studies have identified numerous response

elements in the human genome, although further analysis is required to understand the subtle differences of RE occupancy under *in vivo* and *in vitro* conditions (Brewster et al., 2014). For example, in a particular cell type, TF binding can be insignificant and transient but interpreted as significant when *in vitro* experimental procedures are used. Chromatin accessibility varies with different cell types due to differences in chromatin modifications such as methylation, acetylation, phosphorylation, ubiquitinylation and sumoylation. Moreover, arrangement of REs in the chromatin also varies because of the differences in orientation, distance, number and transcriptional modifications (Gertz et al., 2013; Matzinger and Kamala, 2011; Todeschini et al., 2014). Composite REs (Figure 1.10D) play a central role in mediating cell- and gene-specific transcription. This is because co-occurrence of two TFs in a composite RE may correspond to synergism or inhibition of the bound TFs, through their ability to interact with co-activators or co-repressors, resulting in enhancement or suppression of gene transcription (Kassel and Herrlich, 2007). Co-occurrence as well as the composition of two TF binding sites varies from gene to gene and tissue to tissue. For example, there can be concomitant GRE and NFκB or GRE and AP1 binding sites, whose number and availability will vary among genes and tissues (Pan and Nussinov, 2011). Additionally, co-occurrence of two TFREs can also potentiate the binding of enhancers causing gene-dependent augmentation of expression (Pan and Nussinov, 2011). Likewise, overlapping REs (Figure 1.10C) can modulate transcription of a gene depending on what TF is bound (e.g. an activator or a repressor) (Kassel and Herrlich, 2007). Interestingly, sharing of TFs by multiple genes, competition among TFs for diverse REs, TF affinity differences for binding sites and the presence of non-functional binding sites, may all be responsible for tissue-specific regulation of gene expression (Gertz et al., 2013; John et al., 2011). It has been assumed that TFs are present in excess in a cell but, on the contrary, the concentration of the TF can be limiting

during transcription of a gene of interest (Todeschini et al., 2014). Some of the factors which can lead to a decrease in TF concentration for the gene of interest include the simultaneous transcription of multiple genes, the presence of numerous identical functional REs during transcription (which can cause scarcity of a specific TF), the presence of several, non-functional and accessible REs (to which the TFs bind instead of those that are functionally competent) and high copy number of another housekeeping or constitutively expressed gene undergoing transcription (Todeschini et al., 2014).

1.4.3 *The Glucocorticoid Response Element (GRE)*

In its most simplistic form, the work presented in this thesis is focussed on gene regulation by two response elements: the GRE and the CRE. A GRE can be located in a gene promoter as well as in an enhancer (Watson et al., 2013). A gene promoter is a region in a DNA sequence that initiates transcription of a particular gene and contains many TFREs. Promoters are located upstream of the transcription start sites (i.e. towards the 5' region) of genes and can be about 100–1000 base pairs long. In contrast, an enhancer is located far from the transcription start site of a gene (e.g. 1Mbp distance from promoter); it can contain multiple REs that maybe downstream or upstream of a start site. Enhancers generally potentiate the transcription of a gene by promoting multiple interactions between coactivators and a TF bound to its RE, to the transcriptional machinery bound with the promoter as well as start site of a gene. It is proposed that DNA loops and folds in such a way that an enhancer located thousands of bases away from the transcription start site of a gene, can still positively interact with the transcriptional machinery of that gene. Simple GREs (Figure 1.10A) have the palindromic consensus sequence: 5’-GGTACA NNN TGTTCT-3’ (where "N" can be any nucleotide acting as a spacer) and are classified as canonical,

classical GREs (Clark and Belvisi, 2012; Newton, 2000). When a GR binds to a GRE to activate transcription of a gene, the site is also known as a positive GRE. Conversely, when the GR downregulates transcription of a gene the site is referred to as a negative GRE (Surjit et al., 2011; Figure 1.10A). Other mechanisms that regulate gene transcription by GR involve the tethering to other TFs that are already pre-bound to REs (Figure 1.10B) and the co-occupation of a composite GRE with another TF (Figure 1.10D). Interestingly, negative composite GREs correspond to the binding of GR to DNA, which promotes the dissociation of the co-occupant TF at the neighbouring composite binding site and coincident transrepression of that gene (Kassel and Herrlich, 2007; Figure 1.10D). Several composite sites have been identified for AP-1 and GR as well as CREB and GR, which can be positively or negatively regulated depending upon the external cellular stimuli and the promoter context. Overlapping GREs (Figure 1.10C) have also been reported, which are simply GRE sites that overlap with another TF binding site. Thus, the binding of GR sterically inhibits the binding of the other TF causing gene-specific regulation (Kassel and Herrlich, 2007; Figure 1.10). Similarly, GR can inhibit a DNA bound TF by tethering to it; this can lead to gene transrepression by either inhibiting the binding of co-activators, recruiting co-repressors or both (Busillo and Cidlowski, 2013).

1.4.4 *The cAMP Response Element (CRE)*

Cyclic adenosine 3',5'-monophosphate (cAMP) directly regulates a myriad of cellular pathways and "cross talks" with other signalling cascades that it does not affect directly. The cAMP level in a cell is maintained by adenylyl cyclases and phosphodiesterases (Fimia and Sassone-Corsi, 2001). A major target of cAMP is the enzyme PKA, for which multiple protein

substrates have been identified and are known to be regulated by phosphorylation. PKA can directly phosphorylate several TFs, including CREB, activating transcription factor 1 (ATF1) and cAMP response element modulator (CREM) (Zhang et al., 2005). Phosphorylation is one of the major events in activation of TFs. PKA targets serine residues, specifically Ser^{133} in CREB, Ser^{117} in CREM and Ser^{63} in ATF1 (Mayr and Montminy, 2001). Phosphorylation of Ser^{133} in CREB accelerates its binding to a CRE (Figure 1.8) and it further recruits the CREB-binding protein, CBP/p300, which successfully results in cAMP-dependent gene induction (Mayr and Montminy, 2001; Zhang et al., 2005). CREs exist as a full palindromic site 5'-TGACGTCA-3' or as a half site 5'-TGACG/CGTCA-3'(Montminy et al., 1986). Differences in CRE sequence and placement within 200bp of promoter results in differential regulation of cAMP dependent gene expression. CREs which are situated more than 300bp upstream of the promoter are less effective in binding CREB (Zhang et al., 2005). Interestingly, methylated CREs cannot bind CREB and hence this post-translational modification is one of the factors that results in gene silencing (Zhang et al., 2005).

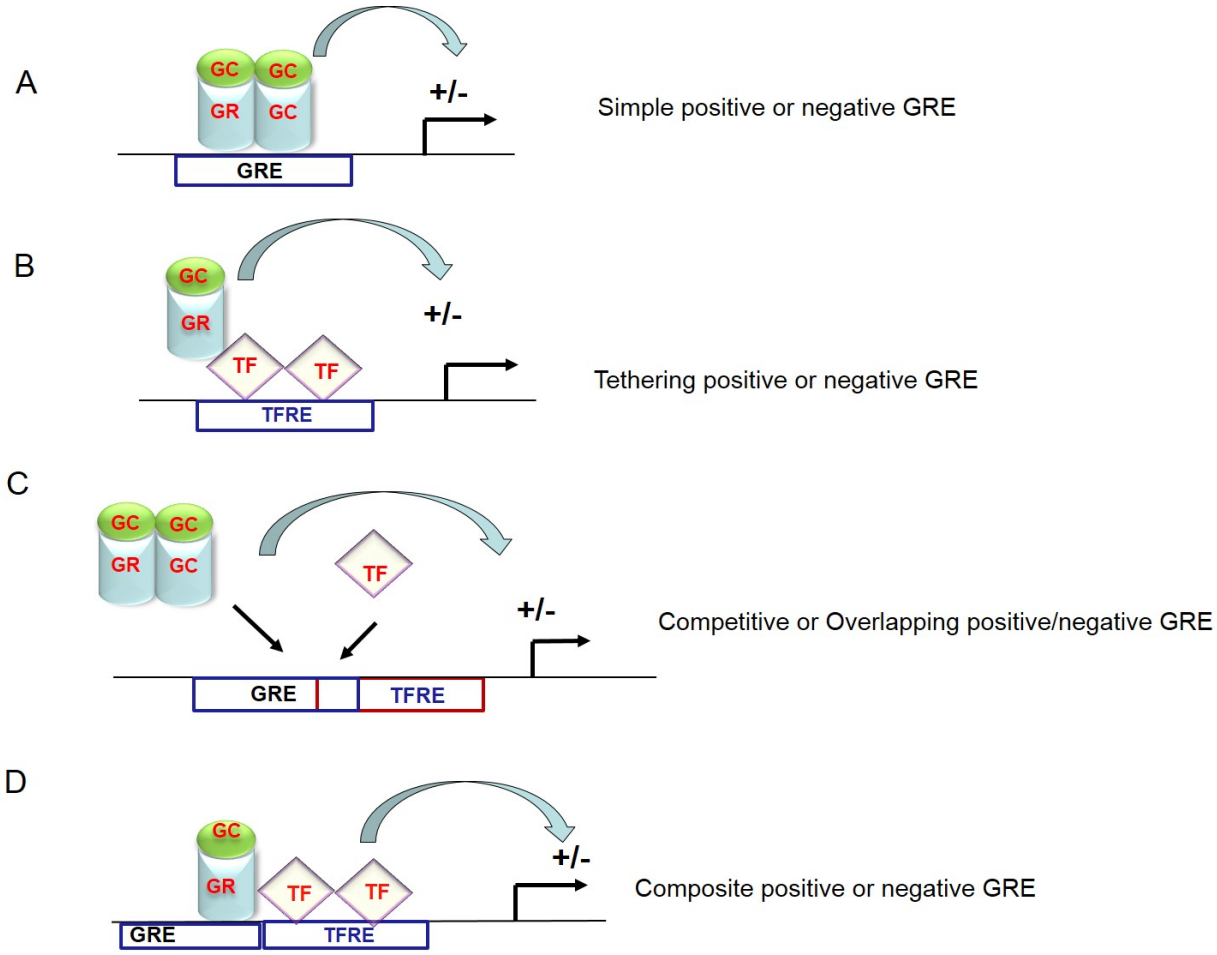


Figure 1.10 Schematic diagram explaining different models of interactions between GRE sites and other transcription factors (TFs) as well as transcription factor response elements (TFREs).

Panel A represents simple positive or negative GRE, which is recognised and bound by GC-GR complex in the nucleus. If a GRE is positive that means it will transactivate a gene while if the GRE is negative, it will inhibit the gene transcription or will transrepress a gene. Panel B shows tethering of GC-GR complex to TFs bound to their respective REs. Tethering of GC-GR can induce or suppress transcription. Panel C shows simplified competitive GRE containing overlapping binding sites for GR as well as another TF, again such sites can induce or suppress the transcription of a specific gene depending on which TF will bind. Panel D represents composite or allied GRE which contains GR and another TF binding sites side by side and can be responsible for the additive or synergistic transactivation or transrepression of a specific gene (Newton, 2000).

1.4.5 *GR as a Transcription Factor*

GR is a nuclear hormone receptor and a ligand-activated TF. GR is encoded by the *NR3C1* gene located on chromosome 5 and is composed of nine exons (Clark and Belvisi, 2012; Newton, 2000). Multiple splice variants of GR have been reported namely: GRα, GRβ and GRγ, but GRα is the predominant form, which has 777 amino acids and a molecular weight of ~97kDa (Clark and Belvisi, 2012). These isoforms exist due to alternative splicing and differences in translation initiation. In addition, these major isoforms have several of their own variants (Clark and Belvisi, 2012).

The activity of GR as a TF is differentially regulated by post-translational modifications, in particular phosphorylation. All GR modifications rely on its binding by GC which changes its conformation, making it more or less susceptible to different cellular kinases (Busillo and Cidlowski, 2013; Chen et al., 2008). Notably, GR can be phosphorylated by PKA, JNK, glycogen synthase kinase 3 (GSK3), cyclin dependent kinases (CDK) and ERK (Busillo and Cidlowski, 2013; Chen et al., 2008). It is believed that phosphorylation of GR by one or more of these kinases generates differential patterns of GR-dependent gene regulation. Phosphorylation is a major GR modification which can make GR more or less efficient in inducing gene transcription. For example, GR phosphorylated at Ser^{203} fails to bind GREs in a gene promoter (Galliher-Beckley and Cidlowski, 2009; Rhen and Cidlowski, 2005). Transcriptionally favourable phosphorylation sites in human GR are Ser^{211} and Ser^{226} (Galliher-Beckley and Cidlowski, 2009; Rhen and Cidlowski, 2005). GR has also been reported to interact with phosphatases such as pp1, pp5 and pp2A, which can also modulate GR interaction with co-activators and the transcriptional machinery (Hinds and Sánchez, 2008; Zhang et al., 2009).

Chromatin immunoprecipitation assays (Chip) followed by sequencing (Chip-seq) has identified numerous composite or overlapping GREs in conjunction with pro-inflammatory transcription factor REs (Busillo and Cidlowski, 2013). This suggests that inflammation can be potentiated by GCs and resolution can be promoted by inflammatory TFs such as NFκB. Thus, the repression or resolution of inflammation by GCs is complicated as it depends upon the context of gene transcription, which is modulated by the concentration of TFs (e.g. GR *vs*. NFκB), the accessibility of binding sites, the strength of cellular as well as environmental stimuli, the physiological state (i.e. healthy *vs.* disease), genetic factors, diet and other co-occupant TFs. For example, studies in HeLa cells demonstrated that when GC and an inflammatory stimulus such as TNFα were co-administered, in addition to the conserved sites, a significant proportion of 'allied' GRE and NFκB sites were identified (Rao et al., 2011). Interestingly, the study showed that GR down-regulated some of the TNFα-induced genes while, on the other hand, synergistically enhanced another subset of TNFα-regulated genes. Whole genome microarray analysis has identified approximately 900 genes, which are co-regulated by GR and TNFα (Lannan et al., 2012).

A common co-occupant of GREs is AP1, which can control the pro-inflammatory or anti-inflammatory actions of GC-GR complexes (Biddie et al., 2011). Interestingly, it is reported that AP1 can induce GR binding and the absence of AP1 can reduce GR occupancy, causing attenuation of GR-mediated responses (Biddie et al., 2011; Kassel and Herrlich, 2007). However, the exact *modus operandi* of GC and AP1 interdependence is unknown. In contrast to AP1, co-occurrence of CREs with GREs can give rise to synergistic, anti-inflammatory effects by recruiting CREB and CBP/p300 (Busillo and Cidlowski, 2013; Kassel and Herrlich, 2007).

1.5 Pharmacology of Drug Receptor Interactions

Receptor theory underpins drug discovery. As described ahead, the quantification of drug action is based on the *Law of Mass Action*, which was initially described to explain simple elementary chemical reactions. To date, receptor theory has, almost exclusively, been developed from the study of GPCRs and ion channels. Whether such theory extends to other receptor systems such as nuclear hormone receptors that often act as ligand-dependent TFs is largely unexplored. In this thesis, current receptor theory has been applied to study GR-mediated gene expression and to interrogate if GCs used to treat asthma are therapeutically equivalent. The remainder of this thesis should be read in conjunction with Appendix A, which provides a glossary of pharmacodynamics terms.

1.5.1 *Historical Perspective*

In the 4th century, pharmacologia or pharmacum were the Latin terms used to describe a drug or a medicine. Today we use the Greek word, pharmacology, to denote a broad discipline dealing with the study of drug action to treat a disease (Norton, 2005). A drug can be defined as any known chemical excluding a nutrient or dietary constituent, which when administered produces a biological effect (Rang et al., 2007). A drug can be a synthetic chemical, a chemical extracted from a plant or an animal and a genetically engineered substance. A cellular protein, which on interacting with a ligand or drug, mediates a (patho)physiological response is known as a receptor (Rang et al., 2007).

Pharmacological test systems have changed drastically in the last 20 years due to advances in the field of molecular biology (Kenakin, 2009). Traditionally, animal tissues were used as the surrogate for human tissues for the testing of new drug candidates, ignoring potential genotypic

and phenotypic differences. The idea was that a given receptor in an animal faithfully represented the human ortholog in all, or most, pharmacological aspects. However, a significant leap of faith was needed to believe that the receptor systems in the two species were equally sensitive and coupled in the same way to second messengers or other cellular pathways (Kenakin, 2009). With the advent of recombinant systems expressing human receptors came the ability to test drugs directly on human cellular receptors. However, there are still differences between recombinant human receptor expressing drug testing systems and the real physiological as well as pathological conditions in a human body. Nevertheless, transfected human cell lines expressing desired human cDNA are used in drug testing and drug discovery. These cell lines are robust, exhibit rapid growth rates, possess low background activity and are stable under experimental conditions. Human cell line test systems produce physiological responses in presence of a drug which can be measured by pharmacological experiments (Kenakin, 2009).

Pharmacological subdisciplines include pharmacodynamics and pharmacokinetics; the former is the study of a drug's effect in a biological system, while the latter is the study of the effects of biological system on a given drug in terms of absorption, distribution, metabolism and excretion (Norton, 2005). Basic understanding of the concentration-response relationship of a drug in a biological system is a prerequisite to study pharmacology. The biological effects of a drug when it binds to its receptor or a biological target, can be measured in terms of its efficacy (often termed tau (τ)), affinity (typically defined as $1/K_A$ or $1/K_B$ - see below) and potency (typically denoted as an effective concentration (EC) that produces a given level of response relative to the maximum). Efficacy is the measurement of drug's potential to produce a biological response while affinity is a drug's tenacity (or strength) to bind a specific receptor (Kenakin, 2009). Mathematically, affinity is defined as the reciprocal of the equilibrium dissociation constant

designated as K_A (for an agonist) and K_B (for an antagonist) (Rang et al., 2007; Kenakin, 2009). Stated differently, affinity is the concentration of a drug that occupies 50% of a given receptor type at equilibrium. Thus, at equilibrium 50% of the receptors are occupied and 50% of the receptors are free. It follows that the lower the value of K_A the more receptors are occupied by a given concentration of a ligand and higher is the affinity of that ligand for that particular receptor (Kenakin, 2009). Finally, potency is the drug concentration required to produce half (50%) of the maximum response and is usually described as an EC_{50}. Concentration-response curves in a given biological system are the representation of a graded response produced by an agonist as a function of its concentration. Generally, concentration-response curves are plotted in semi-log format of agonist concentration and yield a sigmoidal curve (Figure 1.11). An agonist's potency and maximum response can be estimated from its concentration-response curve. More often potency and affinity are expressed in negative log format, typically pEC_{50} and $pK_{A(B)}$ respectively.

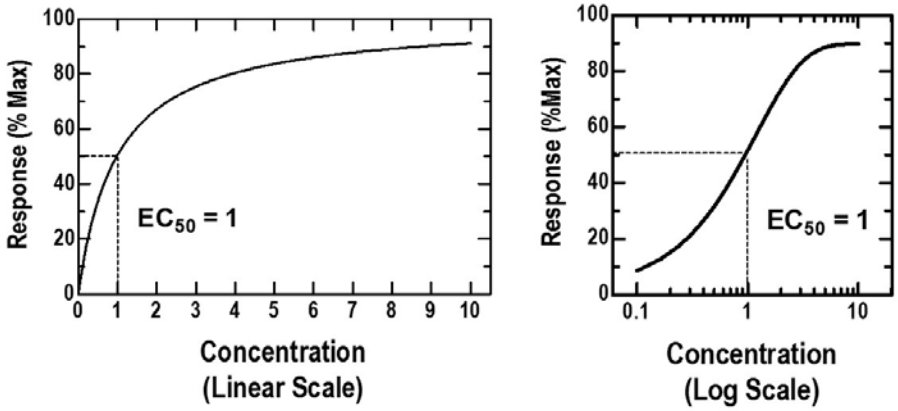


Figure 1.11 Hypothetical graphs representing the relationship between agonist concentration and response, on a linear (rectangular hyperbola) and log_{10} scale (sigmoidal curve).
(Kenakin, 2009).

1.5.2 *The Law of Mass Action*

The *Law of Mass Action*, which was first introduced by Guldberg and Wage in 1864 to describe elementary chemical reactions, is traditionally used for the quantitative study of drug receptor interactions (Ruffolo, 1982; Kenakin, 2009). For a simple bimolecular reaction, the *Law of Mass Action* assumes that the rate of reaction is dependent upon the concentration of each individual species involved in that reaction. For a bimolecular reaction involving the reversible binding of a drug (D) and receptor (R), the result of their interaction is the product, D.R, as follows,

$$\mathbf{D+R} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathbf{D.R}$$

where k_{on} and k_{off} are the association and dissociation rate constants respectively

At equilibrium, the rate of association of D to R is equal to rate of dissociation of the D.R complex. Thus,

$$\boldsymbol{k_{on}[D][R] = k_{off}[D.R]} \quad \textbf{(a)}$$

Rearranging (a) gives:

$$\frac{\mathbf{[D][R]}}{\mathbf{[D.R]}} = \frac{\boldsymbol{k_{off}}}{\boldsymbol{k_{on}}} \quad \textbf{(b)}$$

where, k_{off}/k_{on} is equivalent to the equilibrium dissociation constant (K_A), which is defined as the concentration of ligand that at equilibrium will occupy 50% of the available receptors. That is, at equilibrium [D] = K_A, which means half the receptors are occupied and the other half are free. In this case the above equation simplifies to:

$$\frac{K_A}{[D]} = \frac{[R]}{[D.R]} \quad \textbf{(c)}$$

or**,** because K_A and [D] are equivalent

$$\mathbf{1} = \frac{[R]}{[D.R]} \quad \textbf{(d)}$$

on rearranging

$$\mathbf{[R] = [D.R]} \quad \textbf{(e)}$$

Further rearrangement of equation (b) for the concentration of D.R complexes gives:

$$\mathbf{[D.R]} = \frac{\mathbf{[D][R]}}{K_A} \quad \textbf{(f)}$$

In the above equation, K_A is a constant, receptor number [R] is fixed in a specific cell at a given moment in time and only the drug concentration can vary. In addition, the *Law of Mass Action* also allows the calculation of fractional receptor occupancy at equilibrium. For calculating receptor number:

$$\mathbf{R_t = [R] + [DR]} \quad \textbf{(g)}$$

Where R_t total receptor number, [R] is number of free receptor and [DR] number of bound receptor. On rearranging the above equation:

$$\mathbf{[R] = [R_t] – [DR]} \quad \textbf{(h)}$$

Substituting this expression, which describes free receptors [R], into equation **(b)** and simplifying gives:

$$\frac{[DR]}{[R_t]} = \frac{[D]}{[D]+K_A} \quad \textbf{(i)}$$

which describes fractional receptor occupancy ($[DR]/R_t$) (Ruffolo, 1982; Stephenson, 1956).

1.5.3 Basic Concepts of Receptor Theory: The Origin of Pharmacology

John Newport Langley in 1878 introduced the concept of a receptor but he called it a "receptive substance" (Ruffolo, 1982; Kenakin, 2009). It was Paul Ehlrich in 1913 who coined the term 'receptor'. Langley described that on binding, hormone or any drug stimulates the receptive substance, which in turn passes on this stimulus to different organs (Kenakin, 2009; Ruffolo, 1982).

Alfred Joseph Clark in 1926 presented the first quantification of receptor theory and the foundation of receptor occupancy concept. Clark stated that pharmacological effect of a drug is directly proportional to the number of receptors it occupies (Kenakin, 2009; Ruffolo, 1982). Hence, according to Clark's receptor occupancy theory, which is based on the *Law of Mass Action* (see above), maximum response is produced by a drug when all the receptors are occupied and that binding ***and*** function are superimposable relations such that $EC_{50} = K_A$. Thus, the assumption of this theory is that the maximal response produced by a given drug is equal to the maximum tissue response (i.e. all agonists acting at the same receptor should produce the same maximal response). However, this assumption was subsequently shown to be untrue, which lead Ariens in 1954, to propose the concept of intrinsic activity (α). While affinity represented the attachment of a ligand to a receptor, the term intrinsic activity was introduced to refer to the ability of a drug to induce response after binding that was less than the maximal tissue response. The term α

represented a proportionality factor to scale responses produced by weak agonists. Thus α values of 0.45 and 0.22 refer to agonists that produce 45% and 22% of the maximal tissue response at full receptor occupancy (Figure 1.12). It followed that agonists were compounds that possess affinity and measurable, but often, variable intrinsic activity, whereas antagonists (i.e. drugs that bind to a receptor but do not activate it), demonstrate affinity but have zero intrinsic activity (Ruffolo, 1982).

Stephenson in 1956 described the concept of partial agonists. Partial agonists were those drugs that produced a lower or submaximal response (that is, they have lower intrinsic activity) when compared to other drugs acting at the same receptor that produced higher or a maximal response. Therefore, intrinsic activity of a full agonist is 1, intrinsic activity of a partial agonist is > 0 but < 1 and for an antagonist it is zero (Stephenson, 1956) (Figure 1.12).

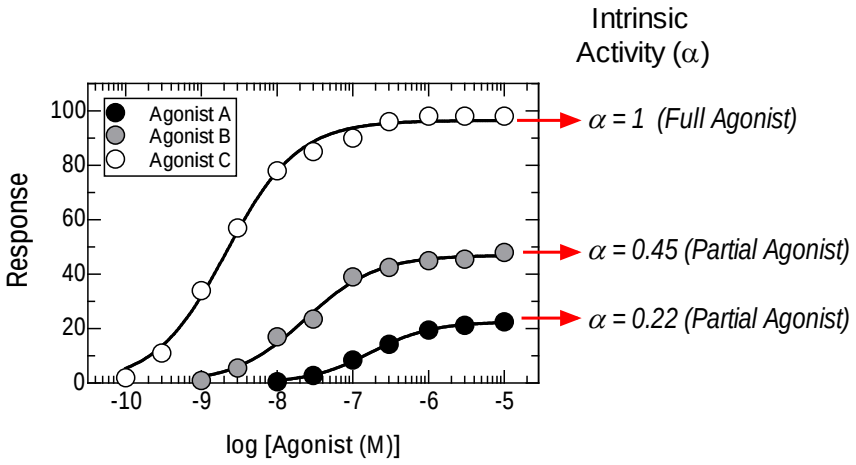


Figure 1.12 Graph depicting concentration response curves of three different hypothetical agonists A, B and C with different intrinsic activity (α) values.
Rank order of potency of the three agonists: EC_{50} C > EC_{50} B > EC_{50} A.

Clark and Ariens both assumed that maximum response is produced by a drug or a full agonist when all the receptors are occupied; in other words 50% receptor occupancy will generate 50% of the response. In 1955, Furchgott, Stephenson and Nickerson demonstrated that this

assumption is untrue in many biological systems. In contrast, they suggested that it is possible for a drug to induce maximum response by occupying only a small percentage of the total receptor population (Ruffolo, 1982; Stephenson, 1956). Notably, different drugs can have differing potential to induce a response and hence needed to occupy different proportions of receptors, when producing equal responses. Stephenson referred to this property of a drug as its efficacy. Although efficacy and intrinsic activity are often used interchangeably, they are not the same. Thus, two full agonists (i.e. drugs that generate the maximal tissue response) that have intrinsic activity values of 1 may have different efficacies. To demonstrate this concept, Furchgott and Nickerson using an irreversible receptor antagonist (at a concentration that inactivates a fraction of the total receptor population to which it binds) demonstrated that different agonists acting on the same receptor in the same tissue displayed different sensitivities to receptor inactivation under identical experimental conditions (Ruffolo, 1982; Stephenson, 1956). Thus, in a given tissue, inactivation of large number of receptors may be needed to inhibit a response produced by one agonist but not another. This finding gave birth to the concept of spare receptors or receptor reserve.

1.5.4 The Concept of Receptor Reserve and Spare Receptors

The concept of receptor reserve or spare receptor implies that a full agonist can produce a maximum response without needing to activate all of the available receptors to which it can bind (i.e. there are more receptors present in a tissue than are required to produce a maximum response to a particular agonist). Consequently, receptor occupancy is not directly proportional to the response elicited by a full agonist. Additionally, enormity of the receptor reserve depends on the efficacy of an agonist and the type of a biological system or a tissue (Ruffolo, 1982; Stephenson, 1956). Therefore, in a given tissue, for a high efficacy agonist, receptor reserve will be very large

in comparison to a partial agonist or low efficacy agonist, for which the receptor reserve can be zero. Clearly, a partial agonist (relatively low efficacy) may need to occupy all available receptors to produce the same response as a full agonist in a particular tissue. In this situation, response can be a linear function of receptor occupancy (Figure 1.13). For a very high efficacy, full agonist, response is a hyperbolic function of receptor occupancy while the curve gets flatter as the efficacy of the agonist decreases resulting in a linear relationship between response and receptor occupancy (Ruffolo, 1982; Stephenson, 1956). Notably, the potency and affinity for a high efficacy full agonist are not equal but for a low efficacy partial agonist as the response is a linear function of the receptor occupancy, affinity and potency are equal. In other words, when K_A=$EC_{50,}$ there is no receptor reserve, while when K_A >> $EC_{50,}$ a large receptor reserve exists (Figure 1.13). Thus, the K_A/EC_{50} ratio provides a measure of receptor reserve in a given tissue for a particular agonist, which is also a measure of agonist efficacy.

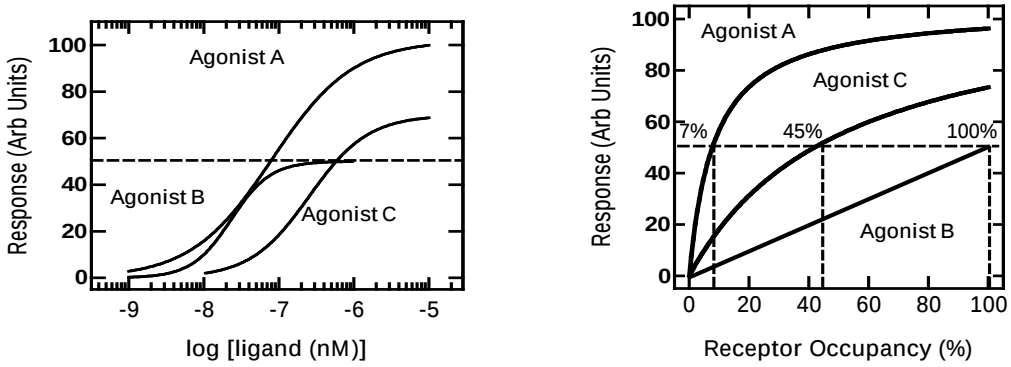


Figure 1.13 Graphs showing the relationship between response and receptor occupancy for three different agonists, acting on same receptor in the same tissue.
Agonist A is a high efficacy, full agonist and its response is a hyperbolic function of receptor occupancy; it generates 50% of the maximum response by occupying only 7% of the total receptor population. Agonist C, which is a partial agonist, requires 45% receptor occupancy to generate 50% of the maximum response produced by Agonist A, while Agonist B, a very low efficacy partial agonist, needs to occupy 100% of the receptors to generate a response that is 50% of the response produced by Agonist A. A large receptor reserve exists for Agonist A, while there is no receptor reserve for Agonist B. (adapted from Ruffolo, 1982).

1.6. Objectives and Rationale of PhD Research

1.6.1 Hypothesis

It is well established from the study of GPCRs, that agonists acting at the same receptor in the same tissue display differences in affinity, intrinsic activity and efficacy (see above) and that these pharmacodynamics parameters underpin the selection of drug candidates for development. Surprisingly, there is little literature comparing the pharmacodynamics of ligands that act as agonists at nuclear hormone receptors, such as GR. This is despite GCs being a mainstay therapy for asthma and other inflammatory conditions. In light of this gap in knowledge and using gene transactivation as a therapeutically-relevant, mechanism of action of GCs, the experiments reported in this thesis were designed to interrogate the pharmacodynamics of GC-GR interaction. The work described in this thesis was designed to test the **overriding hypothesis that GCs are not equal in their ability to promote gene transactivation and that, in a given tissue, GC-inducible gene expression is a distinct functional response that is described by a unique concentration-response relationship governed by GR number and agonist efficacy.** The hypothesis also incorporates, by extension, the idea that the ability of a LABA to enhance GR-mediated gene expression, will vary in an agonist-dependent manner.

Implicit in these ideas is that a given tissue will respond to a GC with a particular gene induction “fingerprint” that is governed by several tissue- and GC-dependent factors. These include the structure of gene promoters, the number of GR in a given tissue, the complement and abundance of various obligatory co-factors and the affinity as well as efficacy of a particular GC for GR. Two things follow from this concept: (i) GCs used in asthma management **may not be equal** with respect to their ability to transactivate gene promoters; and (ii) a change in the

concentration of one or more tissue-dependent factors (e.g. GR) will alter the concentration-effect relationship that describes the induction of a particular gene by a particular GC. This may be reflected by a change in the GC potency and/or the degree of agonism that can be produced in a given tissue.

Herein, the pharmacodynamics of a panel of seven GCs to promote gene expression in the BEAS-2B human airway epithelial cell line has been compared (see Fig 3.1). These include the clinically-relevant compounds FF, dexamethasone (Dex) and desisobutyryl-ciclesonide (DC) as well as GW, a novel ligand that displays partial agonism on a number of functional outputs (Uings et al., 2013), and a nonsteroidal GR agonist, GSK (Yates et al., 2010). For completeness, the naturally-occurring agonist, HC, was also included in the analysis and two, purported, GR antagonists, mifepristone (Mif; Gagne et al., 1985) and Org (Peeters et al., 2004). To test this hypothesis, a simple GRE luciferase reporter construct was used as a model system coupled with an analysis of a panel of GC-inducible genes encoding proteins with both anti-inflammatory and adverse-effect potential. While GCs affect many inflammatory and immune cells to produce therapeutic benefit, airway epithelial cells were used in this study because they are believed to play a profound pathogenic role in asthma and are a primary target for ICS and ICS/LABA combination therapy.

The primary objective of this research is to provide a pharmacodynamics framework of GC-induced gene expression in human bronchial epithelial cells. It is hoped that this work will generate impetus for comprehensive gene profiling studies across a variety of “target” and “off-target” tissues, and to understand what these genes do. It is proposed that the generation of GC-inducible, gene “fingerprints” based on the pharmacodynamics of the GC-GR interaction

will contribute important information that could assist the rational design of optimised ICS for asthma and allied inflammatory diseases.

1.6.2 Specific Objectives

Based on the hypothesis, four interrelated objectives were formulated:

1. To compare pharmacodynamically in the human airway epithelial cell line, BEAS-2B, the ability of a panel of clinically-relevant GCs to activate a GRE luciferase reporter by applying an analytical pharmacological approach.

2. To investigate the ability of the same panel of GCs to induce *real* genes that have anti-inflammatory potential including GC-induced leucine zipper (*GILZ*), kinase inhibitor protein 2 of 57kDa ($p57^{kip2}$) and cysteine-rich secretory protein LCCL domain-containing 2 (*CRISPLD2*). A potential adverse-effect gene, pyruvate dehydrogenase kinase 4 (*PDK4*), was also studied.

3. To estimate the fractional receptor occupancy and receptor reserve for the aforementioned GCs for induction of the luciferase reporter and of *GILZ*, $p57^{kip2}$, *CRISPLD2* and *PDK4*.

4. To compare, pharmacodynamically, the ability of a panel of clinically-relevant GCs in combination with Ind, to induce the luciferase reporter and of *GILZ*, $p57^{kip2}$, *CRISPLD2* and *PDK4*.

Chapter Two: **Materials and Methods**

2.1 Reagents and Suppliers

American type culture collection (ATCC; Rockville, MD, USA): BEAS-2B cells

Applied Biosystems (Burlington, Ontario, Canada): Fast SYBR mastermix, 96 well PCR plates, adhesive sealing film, StepOnePlus real-time PCR system

Biotium Inc. (Hayward, CA, USA): Luciferase reporter assay kit

Corning Inc. (NY, USA): Tissue culture flasks, 6/12/24/48 well plates

Eppendorf (NY, USA): Temperature controlled bench top centrifuges

GlaxoSmithKline (Stevenage, Hertfordshire, UK): Fluticasone furoate (GW 685698X; 6α,9α-difluoro-17α-[(2-furanyl carbonyl)oxy]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioic acid *S*-fluoromethyl ester), GW 870086X (6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(2,2,3,3-tetramethylcyclopropylcarbonyl)-oxoandrosta-1,4-diene-17β-carboxylic acid cyano-methylester)

Invitrogen (Burlington, Ontario, Canada): DMEM-F12, FBS, L-glutamine, trypsin-EDTA, penicillin-streptomycin antibiotics, HBSS, RNase/DNase free water

Nycomed (Konstanz, Germany): Des-isobutyrylciclesonide (2′(*R*)-cyclohexyl-11β,21-dihydroxy-16β-*H*-dioxolo [5′,4′:16, 17]pregna-1,4-diene-3,20-dione)

Organon Laboratories (Oss, The Netherlands): Org 34517 (11β-(1,3-benzodioxolo)-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one)

Promega (WI, USA): 20/20n luminometer, pGL3 basic and control luciferase reporter plasmids

Qiagen (ON, Canada): RNase free DNase set, RNeasy mini kit, QIAshredders

Quanta Biosciences (MD, USA): qScript cDNA synthesis kit

Sigma Aldrich (Ontario, Canada): DMSO, G418 disulphide salt

Steraloids (Newport, RI, USA): Mifepristone (RU 38486; 11β-[4-(dimethylamino) phenyl]-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one), Dexamethasone, Dexamethasone 21-mesylate

Thermo Fisher Scientific Inc. (Waltham, MA USA): NanoDrop 2000, CO_2 incubator, biosafety hood

Tocris (Bristol, UK): Carbenoxolone and hydrocortisone

VWR International (ON, Canada): Serological pipettes, microcentrifuge tubes, 25/50ml conical tubes, HP total RNA extraction kit, RNase/DNase free micro-pipette tips.

2.2 Dilutions and Cell Culture Preparations

All drugs were dissolved in dimethylsulphoxide (DMSO) and diluted to the desired working concentrations in culture medium. The highest concentration of DMSO used in experiments described in this thesis never exceeded 0.2% (v/v) and did not affect any output measured.

2.3 Model System

The human bronchial epithelial cell line, BEAS-2B, stably transfected with a luciferase reporter plasmid, was used throughout the studies discussed in this thesis. BEAS-2B cells were isolated from normal human bronchial epithelium obtained from autopsy of healthy individuals (Lechner and LaVeck, 1985). The cells were then infected with an adenovirus 12-SV40 (polyomavirus) virus hybrid (Ad12SV40) and cloned. BEAS-2B cells are non-tumorigenic,

immortalized (which can grow continually without senescence when cultured *in vitro* in a suitable growth medium) and adherent human epithelial cells (Reddel et al., 1989).

2.4 Generation of a GRE Reporter Cell Line

A previously developed and stably transfected human bronchial epithelial cell line BEAS-2B was utilised for the studies reported in this thesis (Catley et al., 2004; Chivers et al., 2004; Strähle et al., 1988). For the generation of the luciferase reporter plasmid construct, Promega's pGL3-basic luciferase reporter plasmid was chosen and modified by insertion of the neomycin gene expression cassette to confer resistance to geneticin, and a minimal β-globin promoter driving a luciferase gene which was positioned upstream of two copies of a consensus, simple GRE site (sense strand, 5'-TGT ACA GGA TGT TCT-3') resulting in the construct, pGL3.neo.TATA.2GRE (Catley et al., 2004; Strähle et al., 1988). To generate stable transfections of BEAS-2B cells with the pGL3.neo.TATA.2GRE plasmid, cells at ~70% confluence in T162 flasks were transfected with 8µg of plasmid DNA and 20µl of Tfx-50 (Promega, Madison, WI, USA), in a final volume of 10ml DMEM-F12. After 24h, geneticin (100µg/ml) was added until foci of stable transfectants appeared, which were harvested to create heterogeneous populations of cells in which the site of integration was randomized.

2.5 Culture of 2×GRE BEAS-2B Reporter Cells

2×GRE BEAS-2B cells were cultured for two days under a 5% CO_2/air atmosphere at 37ºC in 24-well tissue culture plates containing DMEM/F12, supplemented with 10% FBS, L-glutamine (2.5mM) and sodium bicarbonate (0.15% v/v). The cells were then growth-arrested for 24h in

serum-free medium (SFM). At this time, cultures were confluent and were processed for luciferase measurements or gene expression as described in the results section.

2.6 Treatment of 2×GRE BEAS-2B Cells and Measurement of Luciferase Activity

Confluent, 2×GRE BEAS-2B reporter cells in SFM were treated with FF, DC, Dex, GW, HC, Mif, GSK or Org alone or in combination with Ind or forskolin (Fsk) as indicated in the results sections and incubated at 37°C under a 5% CO_2 atmosphere. In some experiments, 2×GRE BEAS-2B reporter cells were incubated for 6h with Dex, GW or Dex and GW in combination as indicated. Alternatively, cells were pre-treated (30min) with Org (Peeters et al., 2004) or the alkylating agent, dexamethasone 21-mesylate (Dex-Mes; Simons and Thompson, 1981) at the concentration(s) indicated in the text. When Dex-Mes was used, cells were washed with SFM after the pre-incubation period, allowed to recover for 60min before being exposed to GC for 6h. Total RNA was extracted and the expression of a panel of GC-inducible genes was assessed (Table 2.1). Alternatively, 2×GRE BEAS-2B cells were lysed in 100µl 1× firefly luciferase lysis buffer and luciferase activity was measured using a 20/20^n Luminometer, according to the manufacturer's instructions. Data are expressed as fold induction of luciferase activity relative to unstimulated cells.

2.7 RNA Isolation, Reverse Transcription and Real-Time PCR

Total RNA was extracted from 2×GRE BEAS-2B reporter cells by using RNeasy Mini Kits. The purity and quantity of RNA was determined using nano-drop and was reverse transcribed using a qscript cDNA synthesis kit according to the manufacturer's instructions. Real-time PCR

analysis of cDNA was performed using the primer sequences shown in Table 2.1 (designed using Primer Express® software, ABI) that amplify glucocorticoid-induced leucine zipper (*GILZ*; HUGO gene name: transforming growth factor β-stimulated clone 22, domain family member 3 [*TSC22D3*]), kinase inhibitor protein 2 of 57 kDa ($p57^{kip2}$; HUGO gene name: cyclin-dependent kinase inhibitor 1C [*CDKN1C*]), cysteine-rich secretory protein LCCL (Limulus clotting factor C, Cochlin, Lgl1) domain-containing 2 (*CRISPLD2*) and pyruvate dehydrogenase kinase 4 (*PDK4*). These reactions were performed using an ABI StepOnePlus® instrument on 2.5µl of cDNA in 10µl reactions using Fast SYBR® Green chemistry according to the manufacturer's guidelines. Relative gene expression levels were determined from a cDNA standard curve that was analyzed simultaneously with the test samples and are presented as a ratio to *GAPDH*, whose expression was not affected by any of the GCs used in this study. Amplification conditions were: 95°C, 20s; followed by 40 cycles of: 95°C, 3s; 60°C, 30s. Dissociation (melt) curves (95°C, 15s; 60°C, 1min; 95°C, 15s) were constructed to confirm primer specificity.

Table 2.1 Primer pairs for real-time PCR.

Gene	Oligonucleotide	Accession Number(s)
PDK4		NM_002612.3
Forward	5'-GCT GTC CAT GAA GCA GCT ACT G-3'	
Reverse	5'-CGC AAA AAT GCA AAA GAA GTT CT-3'	
p57^{kip2} (CDKN1C)		NM_000076.2, NM_001122630.1 NM_001122631.1
Forward	5'-CTG TCC GGG CCT CTG ATC T-3'	
Reverse	5'-CAT CGC CCG ACG ACT TCT-3'	
GILZ (TSC22D3)		NM_198057.2, NM_004089.3, NM_001015881.1
Forward	5'- TGG CCA TAG ACA ACA AGA TCG A-3'	
Reverse	5'- CAC AGC ATA CAT CAG ATG ATT CTT CA-3'	
CRISPLD2		NM_031476.3
Forward	5'-CAA ACC TTC CAG CTC ATT CAT G-3'	
Reverse	5'-GGT CGT GTA GCA GTC CAA ATC C-3'	
GAPDH		NM_002046.4, NM_001256799.1
Forward	5'-ATG GAA ATC CCA TCA CCA TCT T-3'	
Reverse	5'-CAG CAT CGC CCC ACT TG-3'	

Forward and reverse primers for each gene are listed. Common genes symbols are shown and, where appropriate, official HUGO (human genome organisation) gene symbols are given in brackets. Generic primers were used for genes encoding multiple isoforms.

2.8 Fitting of Concentration-Response Curves by using Non-Linear Regression

Monophasic agonist concentration-response curves were fitted by least-squares, non-linear iterative regression to the following form of the Hill equation (Prism 4®, GraphPad Software Inc, San Diego, CA, USA) (Motulsky and Christopoulos, 2003):

$$E = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{(pEC_{50} - p[A])^n}} \qquad (1)$$

where E is the effect, E_{min} and E_{max} are the basal response and maximum agonist-induced response respectively, p[A] is the negative log molar concentration of agonist, pEC_{50} is a location parameter equal to the negative log molar concentration of agonist producing $(E_{max} - E_{min})/2$ and n is the gradient of the concentration-response curve at the pEC_{50} level.

2.9 Determination of Antagonist and Partial Agonist Equilibrium Dissociation Constants

Antagonist and partial agonist affinity values were determined by least-squares, non-linear regression using a modification of the Hill and Gaddum/Schild equations (Waud et al., 1978). Concentration-response curves were constructed to GC in cells pre-treated (30min) with vehicle or the GR antagonist, Org (Peeters et al., 2004) at concentrations of 10nM, 30nM and 100nM. Each “family” of concentration-response curves were then fitted simultaneously to equation 2.

Thus,

$$E = E_{min} + \left(\frac{(E_{max} - E_{min})}{1 + \left(\frac{10^{p[A]_{50}} \left[1 + \left([B] / 10^{-pA_2} \right)^S \right]}{[A]} \right)^n} \right) \quad (2)$$

where [A] and [B] are the molar concentration of full agonist and partial agonist/antagonist respectively, S is the Schild slope factor, which indicates the nature of antagonism, and pA_2 is the equilibrium dissociation constant of the partial agonist and antagonist when S = 1, which is equivalent to the pK_A or pK_B respectively. To determine whether S deviated significantly from unity, the entire family of concentration-response curves that made up an individual experiment was fitted globally to equation 2 under two conditions: one where S was constrained to a constant equal to 1 and the other where it was a shared value for all data sets. The *F*-test was applied to determine the equation that gave the best fit, which was used for the analysis.

2.10 Determination of Agonist Equilibrium Dissociation Constants by Controlled, Fractional GR Inactivation

The K_A of FF and Dex in 2×GRE BEAS-2B reporter cells was estimated by "irreversibly" inactivating a fraction of the total functional GR population with the alkylating agent, Dex-Mes (Simons and Thompson, 1981) according to Furchgott (1966). Agonist concentration-response curves were generated in cells treated (for 30min) with vehicle or Dex-Mes at the concentrations

indicated. Each set of concentration-response curves was then fitted simultaneously to the operational model of agonism (equation 3) and Hill equation (equation 1), which describes a theoretical relationship between pharmacological effect (E) and agonist concentration (Black and Leff, 1983). Algebraically,

$$E = \frac{E_m.\tau^n.[A]^n}{(K_A + [A])^n + \tau^n.[A]^n} \qquad (3)$$

where E_m is the theoretical maximum response of the tissue (which may or may not be the maximal agonist-induced response), [A] is the agonist concentration, n is the slope of the relationship between the concentration of agonist-receptor ([AR]) complexes and response (to account for concentration-response curves with gradients that are not equal to a value of 1) and τ is the operational efficacy of the agonist; this is the ratio of the total functional receptor concentration [R_t] to [AR] required to produce half-maximal effect (Leff et al., 1990). In these analyses a common value of E_m, K_A and n is assumed (Black and Leff, 1983, Leff et al., 1990). Only τ, which at sub-maximal responses decreases proportionally with the remaining fraction of non-inactivated receptors, was allowed to vary between individual concentration-response curves (Black and Leff, 1983, Leff et al., 1990). Thus, for each experiment a single estimate of E_m, n and K_A was calculated as well as the operational efficacy of the agonist, τ.

2.11 Determination of Partial Agonist Equilibrium Dissociation Constants by the Comparative Method

The K_A of partial GR agonists was also estimated by operational model fitting (Black and Leff, 1983) using the comparative method (Barlow et al., 1967) in which concentration-response curves of the partial agonist is compared against a reference full agonist. Each pair of concentration-response curves (i.e. those generated by the full agonist and partial agonist) were fitted simultaneously to equation 1 and equation 3 respectively (Motulsky and Christopoulos, 2003), which yielded an estimate of K_A and τ for the partial agonist, the E_m and n of the tissue and a pEC_{50} for each full agonist curve (Leff et al., 1990).

2.12 Determination of Receptor Reserve

Receptor occupancy-response curves were constructed to Dex, GW and FF using their K_As determined by Schild analysis, or receptor inactivation as indicated. At each concentration of agonist and, therefore, at each level of response, fractional GR occupancy (i.e. the ratio of agonist occupied GR (R_A) to R_t) in control cells was determined (Furchgott, 1966). Thus,

$$R_A/R_t = [A]^n/K_A{}^n + [A]^n \qquad (4)$$

2.13 Statistical Analysis

Data points, bars and values in the text and figure legends represent the mean ± s.e. mean of N independent determinations. Data were analyzed by Student's two-tailed t-test or repeated measures one-way ANOVA followed, when appropriate, by Tukey's multiple comparison test. In

experiments where GC-induced gene expression was examined, all statistical analyses were performed on untransformed data. The null hypothesis was rejected when $P < 0.05$.

Chapter Three: **Validation of a 2×GRE Luciferase Reporter Stably Transfected into BEAS-2B Cells**

Some of the data discussed in this chapter have been published as a peer reviewed manuscript:

Taruna Joshi, Malcolm Johnson Robert Newton & Mark A. Giembycz (2015). An analysis of glucocorticoid receptor-mediated transcription in human airway epithelial cells identifies unique, ligand-directed, gene expression fingerprints with implications for asthma therapeutics. *British Journal of Pharmacology*, **172** (5), 1360–1378.

3.1 Background Information and Rationale

Concepts of receptor theory have evolved since the 1900s predominantly from the study of GPCRs and ion channels. However, these concepts have not been extended to the study of nuclear hormone receptors such as GR, which is the target of anti-inflammatory GCs. Thus, to fill this void in knowledge, the present chapter is focussed on studying the pharmacodynamics of GR-mediated gene transactivation in BEAS-2B cells stably transfected with a reporter plasmid containing 2× consensus GRE sites upstream of a luciferase gene using agonists that are used clinically, or are in development, for the treatment of asthma.

The activity of a ligand at a given receptor depends upon two, interdependent parameters: affinity, which is defined as the reciprocal of its equilibrium *dissociation* constant (K_A) and efficacy, which is defined as the ability of a ligand to produce a response. Classically, affinity

(1/K_A or the equilibrium *association* constant) is an invariant parameter across tissues within an organism while efficacy will vary depending upon the receptor density in the target tissue, the function of interest and coupling efficiency between a receptor and its effector molecule. Hence, these are essential parameters that underpin the design of new drugs, including GCs for asthma.

Multiple GCs have been synthesised and introduced by the pharmaceutical industry to treat chronic inflammatory diseases such asthma. However, unlike the study of drugs that interact with GPCRs (e.g. LABAs), GCs have rarely been compared head-to-head in the same assay systems such as their ability to transactivate genes. Theoretically, as described in Chapter 1, transactivation of beneficial as well as adverse effect genes can vary depending upon the tissue of interest and the intrinsic efficacy of the GC. Thus, in this Chapter, we have compared, using a quantitative pharmacodynamics approach, the ability of a panel of seven GCs (Figure 3.1) to induce luciferase activity in 2×GRE BEAS-2B reporter cells.

3.2 Hypothesis

The overall hypothesis is that the GCs used in asthma management are not equal in their ability to induce gene expression because of differences in their intrinsic efficacy. In this chapter an analytical pharmacological approach was adopted to test this idea using 2× GRE BEAS-2B luciferase reporter cells as a surrogate of anti-inflammatory gene expression.

3.3 Results

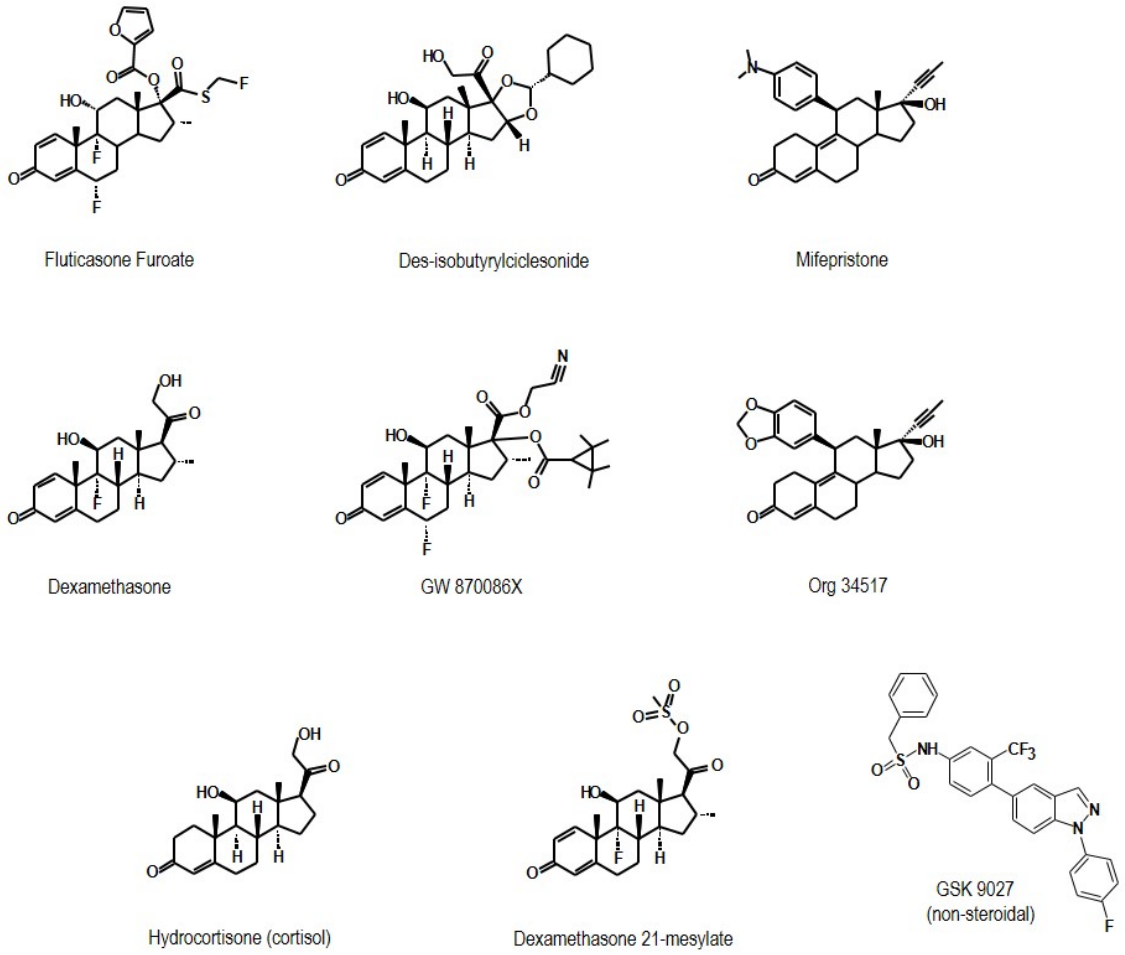


Figure 3.1 Chemical structures of the GR ligands examined in this study.

3.3.1 *Kinetics of GRE-Dependent Gene Transcription using Luciferase as a Surrogate Anti-Inflammatory Gene*

To validate the reporter plasmid system, initial experiments were conducted to determine the optimal time-point of luciferase gene induction. 2×GRE BEAS-2B reporter cells were treated with the maximum effective concentrations of four GR ligands (Dex-1μM, DC-100nM, FF-100nM and GW-1μM) and their ability to increase luciferase activity measured over a 24h time-frame. As figure 3.2A shows, each of these GR agonists produced a time-dependent enhancement of luciferase activity, which peaked at approximately 6h after stimulation. With the exception of GW, luciferase activity then declined over the next 18h. Although, the kinetics of the four GCs was similar, maximum luciferase activity at 6h was not equal (FF > Dex > DC > GW in decreasing order of activity, Figure 3.2A). Figure 3.2B and C shows that the maximum enhancement of luciferase activity was seen at ~6h, irrespective of the concentrations of FF and DC used.

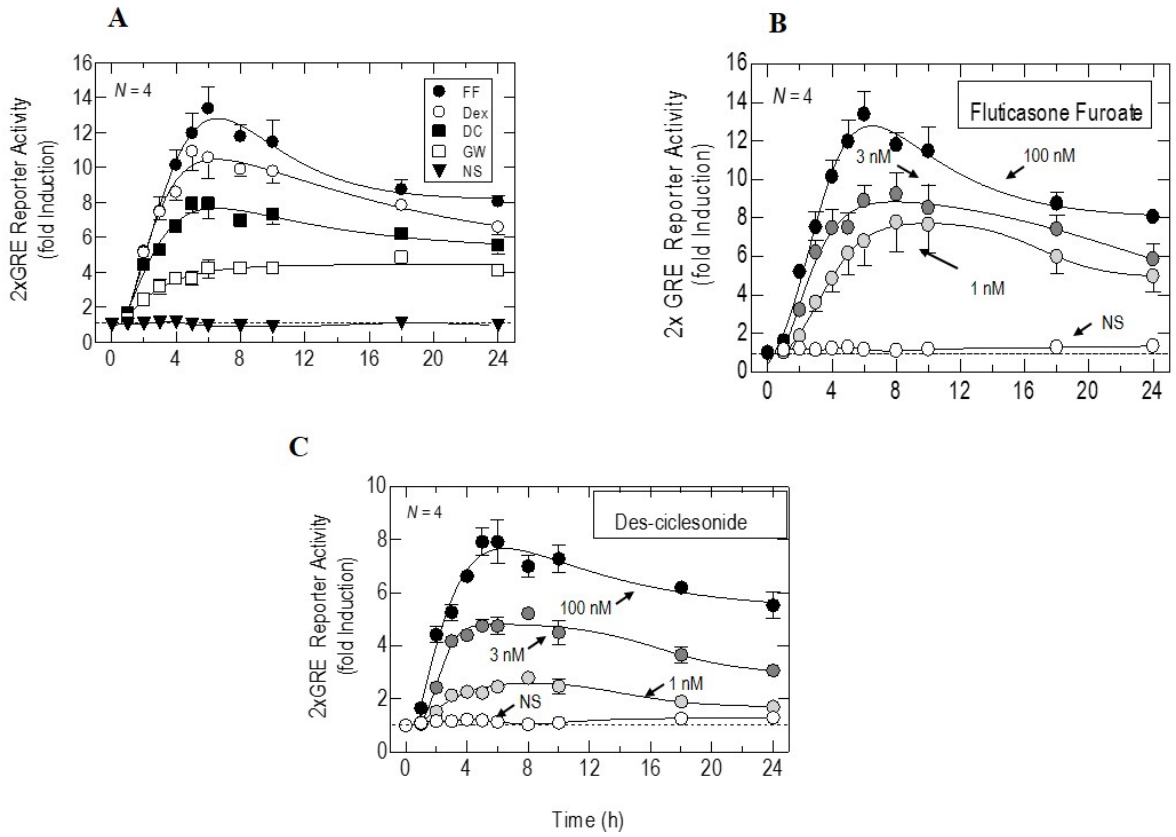


Figure 3.2 Kinetics of GRE-dependent transcription.
Panel A: 2×GRE BEAS-2B reporter cells were treated with fluticasone furoate (FF; 100nM), dexamethasone (Dex; 1μM), des-ciclesonide (DC; 100nM), GW 870086X (GW; 1μM) or vehicle (NS). Panels B and C: cells were treated with fluticasone furoate, des-ciclesonide (at 1nM, 3nM or 100nM) or vehicle (NS). At the times indicated, cells were harvested for the determination of luciferase activity. Data points represent the mean ± s.e. mean of *N* independent determinations. The dashed line in each panel defines baseline luciferase expression.

3.3.2 *Differences in GRE-Dependent Transcription by a Panel of GCs*

To validate the reporter plasmid system, a second set of experiment was conducted to compare the intrinsic activity (α) and potency (EC_{50}) of a panel of selected GCs in their ability to induce GRE-dependent luciferase activity. 2×GRE BEAS-2B reporter cells were treated with varying concentrations of the selected GR ligands for 6h, after which the cells were harvested for luciferase analysis. Concentration-response curves were constructed to six different GCs: Dex, FF, DC, GW, Org and Mif (Figure 3.3, Table 3.1). In addition to these synthetic GCs, an endogenous GR ligand, HC (Figures 3.1 & 3.3C, Table 3.1) and a non-steroidal ligand, GSK were also examined (further discussed in section 4.3.2).

Figure 3.3A and B, show that these GR ligands demonstrated agonism with the rank order of potency (EC_{50}) of FF > DC > Mif > Dex ≥ GW. Dex, a standard GC, was selected as a reference agonist to calculate the intrinsic activity values of the other GR ligands and was assigned a value of 1 (α = 1). Compared to Dex, FF was a stronger agonist (α = 1.21), which indicated that Dex, was in fact, a partial agonist on the reporter system (Table 3.1). In contrast, DC, GW and Mif showed decreasing intrinsic activity values consistent with increasing partial agonist behaviour. Org was inactive at all concentrations tested (Table 3.1).

A separate experiment was conducted to compare the ability of HC and Dex to transactivate the luciferase gene. As figure 3.3C demonstrates, HC induced luciferase activity in a concentration-dependent manner but acted as a partial agonist, by maximally inducing luciferase activity by only 8 fold (α=0.55) when compared to the reference agonist, Dex, which maximally induced luciferase activity by 13 fold (α=1). Thus, Dex and, by definition, FF can be referred to as super-agonists with respect to HC. A super-agonist is one that is able to induce a greater maximum response than an endogenous agonist at the same receptor.

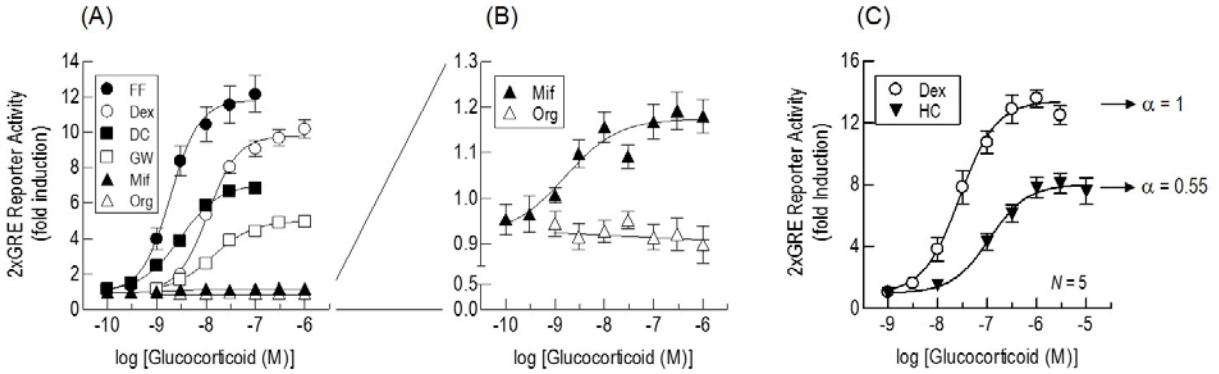


Figure 3.3 Comparative effects of a panel of GR ligands on GRE-dependent transcription. Panel A: 2×GRE BEAS-2B reporter cells were exposed to FF (100pM to 100nM), Dex (1nM to 1µM), DC (100pM to 100nM), GW (1nM to 1µM), Mif (100pM to 1µM) or Org (1nM to 1µM). In panel B, the *y* axis has been expanded to illustrate the very low intrinsic activity of Mif. Panel C: concentration-response curves were constructed to Dex (1nM to 1µM) and HC (1nM to 10µM). At 6h cells were harvested, luciferase activity was determined and concentration-response curves were constructed. Data points represent the mean ± s.e. mean of *N* independent determinations (see Table 3.1).

Table 3.1 Potency, E_{max} and intrinsic activity values of a panel of GR ligands for promoting GRE-dependent transcription.

Glucocorticoid	***N***	**pEC$_{50}$ (M)**[a]	***E*$_{max}$**[a] (fold induction)	**Intrinsic Activity**[a,b] (α)
Dexamethasone[c]	31	7.95 ± 0.03	9.9 ± 0.5	1.00
Fluticasone Furoate	13	8.72 ± 0.10	11.8 ± 1.0	1.21
Des-ciclesonide	25	8.61 ± 0.09	7.0 ± 0.3	0.70
Hydrocortisone	5	6.95 ± 0.08	6.4 ± 0.2	0.61
GW870086X	25	7.96 ± 0.07	4.94 ± 0.3	0.44
Mifepristone	7	8.04 ± 0.22	1.3 ± 0.1	0.03
Org 34517	7	inactive	0.9 ± 0.1	0.01

[a]Parameters calculated from the graphs shown in figure 3.3; [b]Intrinsic activity (α) = $E_{max}^{(\text{GR ligand - 1})}/E_{max}^{(\text{Dex - 1})}$; [c]Dex selected as a reference agonist (α = 1).

3.3.3 *Effect of an Inhibitor of GC Metabolism, Carbenoxolone, on GRE-Dependent Transcription*

11β-Hydroxysteroid dehydrogenase-2 (11β-HSD-2; Monder et al., 2003), is involved with the cellular metabolism of GCs. To determine if variable GC metabolism by 11β-HSD-2 was not responsible for the data shown in figure 3.3, 2×GRE BEAS-2B cells were treated with carbenoxolone (CBX) at a concentration reported to be selective for 11β-HSD-2 (Monder et al., 2003). To perform these experiments, cells were pre-incubated for 30min. with 1μM CBX and then treated with FF, Dex, DC and GW for a further 6h, after which cells were harvested and luciferase activity was measured.

Pre-treatment of 2×GRE BEAS-2B reporter cells with CBX failed to affect the concentration-response curves that described FF-, Dex-, HC-, DC- and GW-induced GRE-dependent transcription (Figure 3.4). These results agree with a previous report that BEAS-2B cells lack 11β-HSD-2 activity and that synthetic GCs are not susceptible to metabolism by 11β-HSD-2 (Feinstein and Schleimer, 1999). In addition, microarray analyses did not detect mRNA transcripts for 11β-HSD-2 in BEAS-2B cells (our unpublished data). Thus, this experiment confirmed that the data obtained with GR agonists shown in figure 3.3 are unlikely to be attributable to variable metabolic inactivation.

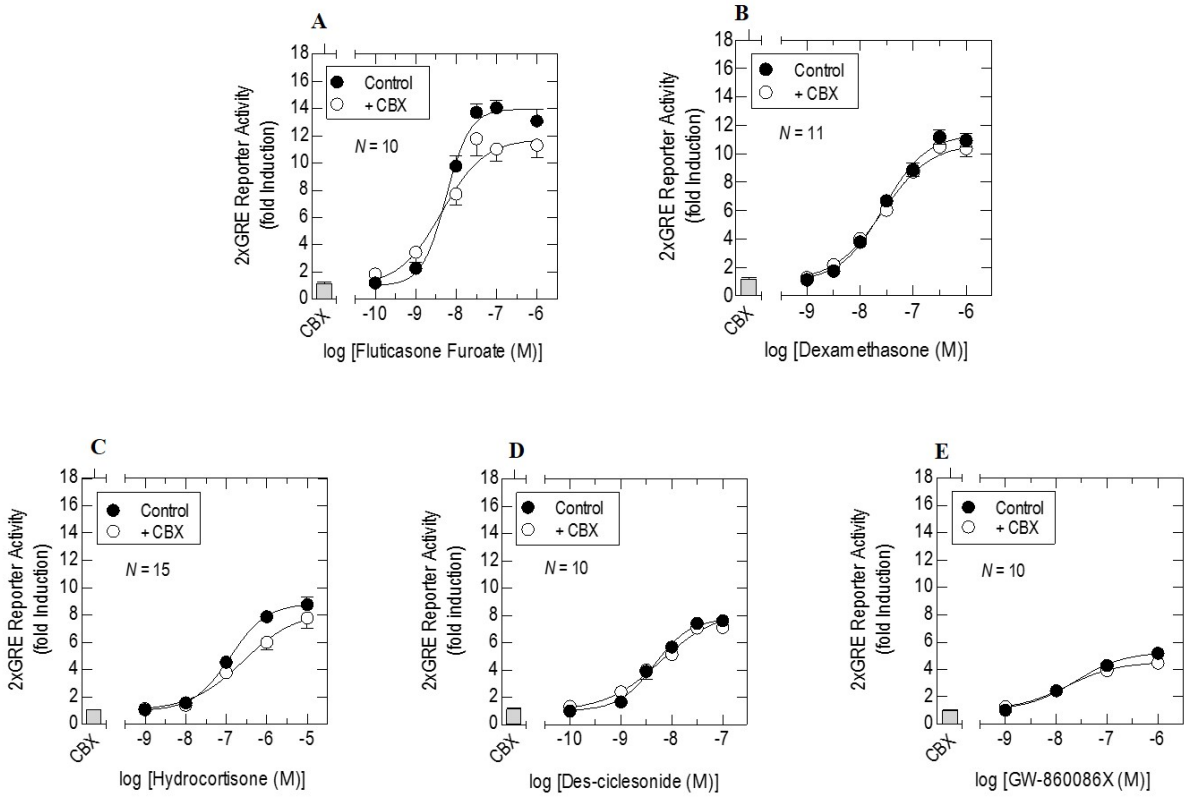


Figure 3.4 Effect of carbenoxolone on GRE-dependent transcription.
2×GRE BEAS-2B reporter cells were pre-treated with carbenoxolone (CBX; 1μM for 30min) or its vehicle and concentration-response curves were constructed to fluticasone furoate (A), dexamethasone (B), hydrocortisone (C), des-ciclesonide (D) and GW-870086X (E). After 6h, cells were harvested for the determination of luciferase activity. Data points represent the mean ± s.e. mean of *N* independent determinations.

3.3.4 *Effect of a GR Antagonist, Org 34517 (Org), on GRE-Dependent Transcription*

To determine that the GCs used in these studies acted through GR and did not cross-activate other nuclear hormone receptors, the affinity (K_B) of the GR antagonist, Org, was determined by Gaddum-Schild analysis. According to receptor theory, the affinity of Org for GR should be the same if all agonists used in the analysis mediate their effect through the same receptor.

2×GRE BEAS-2B cells were pre-incubated with vehicle or Org (10, 30 or 100nM) and concentration-response curves of GR agonists were then constructed to FF, Dex, DC and GW at 6h. As shown in figure 3.5, Org produced graded, dextral displacements of the concentration-response curves that described FF-, Dex-, DC- and GW-induced GRE-dependent transcription. For each GR agonist, the Schild slope factor, S, did not deviate significantly from unity. Thus, Org apparently behaved in a manner consistent with surmountable, competitive antagonism. Accordingly, S was constrained to a value of 1 from which mean pK_B values of 8.34, 8.38, 8.12 and 8.46 for Org were derived with FF, Dex, DC and GW respectively (Figure 3.5). These data provided strong evidence that the four GCs examined increased luciferase activity in 2×GRE BEAS-2B cells by interacting with GR.

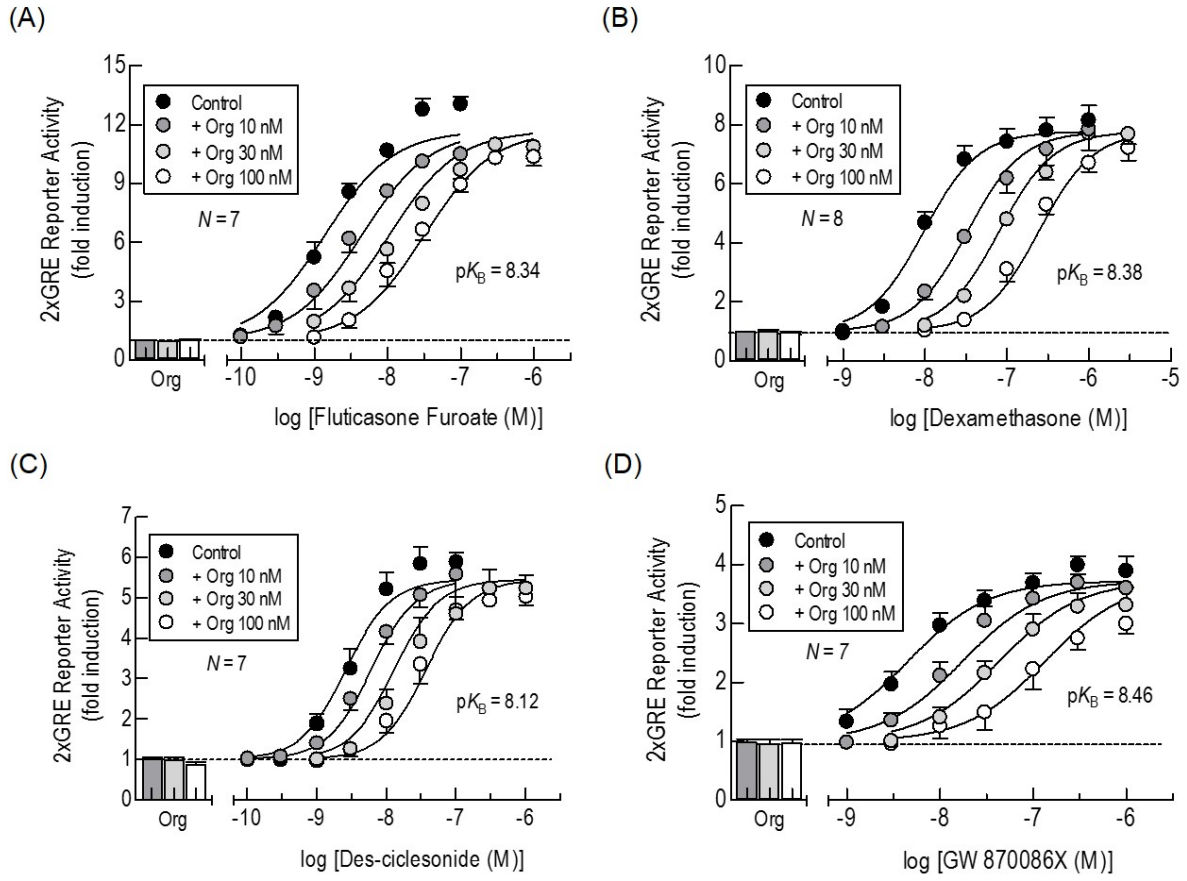


Figure 3.5 Gaddum-Schild analysis of the antagonism of GRE-dependent transcription by Org.

In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to FF (panel A), Dex (panel B), DC (panel C) and GW (panel D) alone and after pre-treatment (30min) with Org (10nM, 30nM and 100nM). Each family of concentration-response curves was then fitted simultaneously to equation 2 from which the affinity (pK_B) of Org was derived. The bars show the effect on luciferase activity of each concentration of Org alone; the horizontal dashed line in each panel defines baseline luciferase activity. Data points and bars represent the mean ± s.e. mean of *N* independent determinations.

3.3.5 *Competitive Antagonism by a Partial Agonist, GW 870086X (GW)*

In GPCR research, classical receptor theory predicts that a partial agonist should antagonise the effect of a full agonist. To determine whether this applies to agonists that act at nuclear hormone receptors, the effect of the partial agonist, GW, on Dex-induced, GRE reporter activation was determined. Cells were pre-incubated with vehicle or GW (100nM or 1μM) for 1h, and concentration-response curves were then generated for Dex in the continued presence of vehicle or GW. At concentrations of 100nM and 1μM, GW increased luciferase activity by 4.5 fold and 5.6 fold respectively. At the same concentrations, GW produced dextral, parallel displacements of Dex concentration-response curves. Analysing these data in the absence and presence of GW, by applying the Gaddum-Schild equation showed that GW behaved as a surmountable, competitive antagonist (S = 1) with a mean pK_B value of 8.08 (Figure 3.6A).

The affinity of GW was also determined by operational model-fitting using the comparative method with FF and Dex as reference agonists (Figures 3.6B & C). This experimental approach yielded pK_A values that were very similar to the affinity of GW derived by Gaddum-Schild analysis (Figure 3.6; Table 3.2). Furthermore, inspection of the data in figure 3.6D shows that the pK_A of GW was equivalent to its pEC_{50} for driving GRE-dependent transcription as would be expected for a low efficacy partial agonist where response approximates to a linear function of receptor occupancy (i.e. where K_A and EC_{50} are a similar value). In a similar manner, the comparative method was applied to calculate the affinity of another partial agonist DC, from which pK_A values of 8.52 and 8.59 were derived, when FF and Dex were used as reference agonists respectively (Figure 3.7; Table 3.2). Again, these values were very similar to the potency of DC (pEC_{50} = 8.61) for driving GRE-dependent transcription (Figure 3.7A & B*;* Table 3.2) and are

consistent with a lack of GR reserve for this GC in BEAS-2B reporter cells for enhancing luciferase activity.

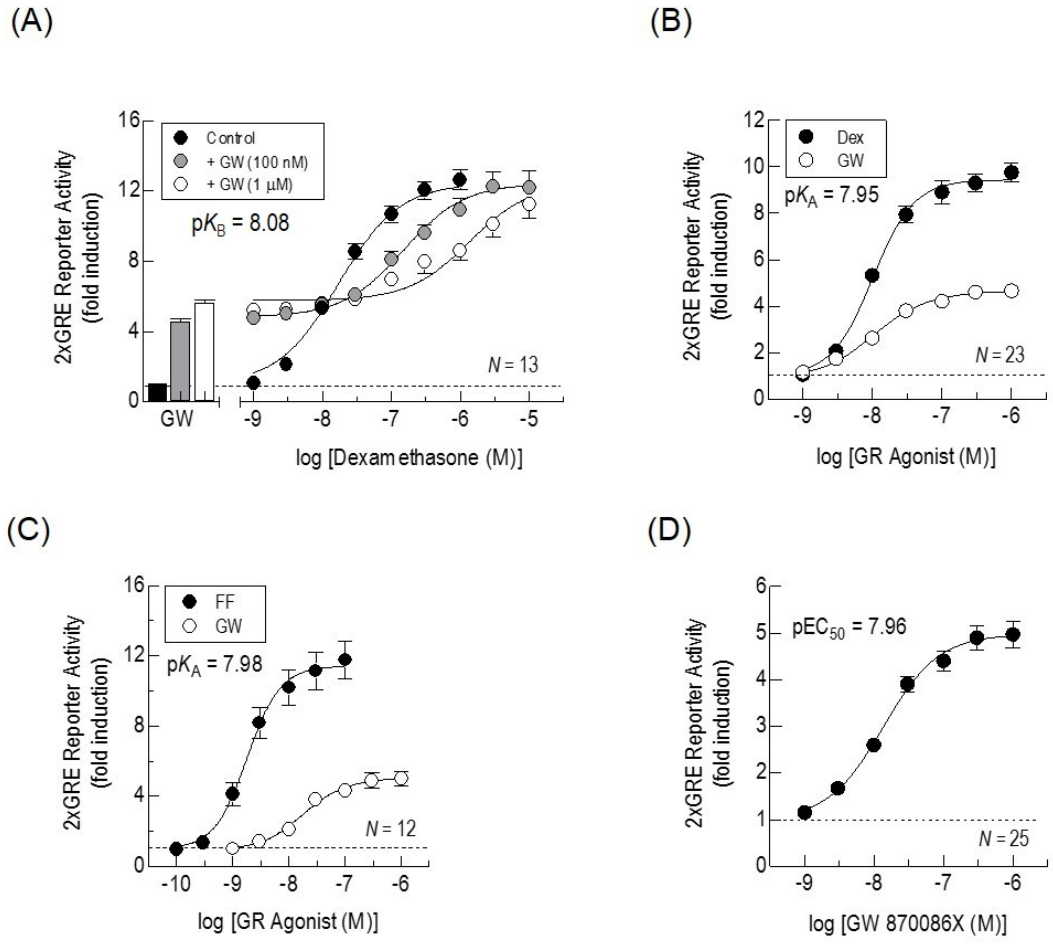


Figure 3.6 Determination of the affinity of GW by Gaddum-Schild analysis and the comparative method.

Panel A: In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to Dex alone and in the presence of GW (100nM and 1µM). The entire family of concentration-response curves were then fitted simultaneously to equation 2 from which a pK_A of 8.08 was derived. The bars in panel A show the effect of GW alone on luciferase activity. Panels B and C: concentration-response curves were constructed to GW and Dex, and GW and FF respectively and the resulting pairs of curves fitted simultaneously to equations 1 and 3 from which estimates of K_A, τ, n, E_m and pEC_{50} (of the reference agonist curve) were derived (see Table 3.2). Panel D shows the concentration-response relationship for GW-induced, GRE-dependent transcription. The horizontal dashed line in each panel defines baseline luciferase activity. Data points represent the mean ± s.e. mean of *N* independent determinations.

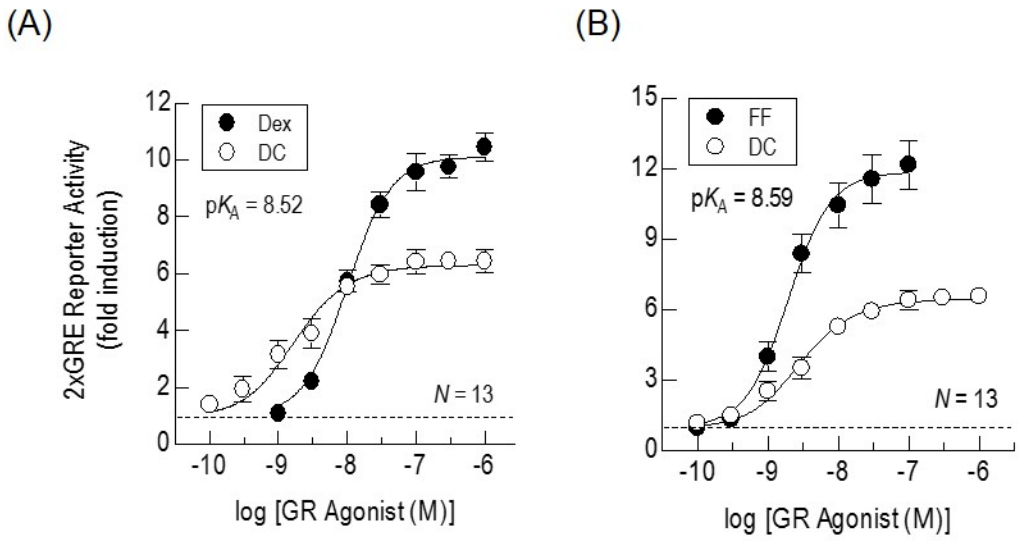


Figure 3.7 Determination of operational parameter estimates for DC-induced, GRE-dependent transcription by the comparative method.

In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to DC (100pM to 1μM) and compared against those constructed to Dex (1nM to 1μM; panel A) and FF (100pM to 100nM; panel B). The resulting pairs of curves were fitted simultaneously to equations 1 and 3 respectively from which estimates of K_A,τ, n, E_m and pEC_{50} (of the reference agonist curve) were derived (see Table 3.2). The horizontal dashed line in each panel indicates baseline luciferase activity. Data points represent the mean ± s.e. mean of *N* independent determinations.

Table 3.2 Operational parameter estimates that define the ability of a panel of GR agonists to promote GRE-dependent transcription.

	Parameter Estimates[a]				
Agonist	**Method**	**pK_A** (M)	**E_m** (fold)	***n***	**τ**
Dexamethasone	GR Inactivation	7.93 ± 0.27	21.7 ± 3.6	1.90 ± 0.58	1.07 ± 0.34
Fluticasone Furoate	GR Inactivation	8.69 ± 0.17	32.2 ± 4.3	1.56 ± 0.38	0.74 ± 0.12
Des-ciclesonide	Comparative^{Dex}	8.52 ± 0.21	*10.1 ± 0.5*[b]	1.53 ± 0.08	*1.83 ± 0.33*[b]
Des-ciclesonide	Comparative^{FF}	8.59 ± 0.17	*11.8 ± 1.0*[b]	1.99 ± 0.13	*1.18 ± 0.10*[b]
GW 870086X	Comparative^{Dex}	7.95 ± 0.10	*9.5 ± 0.4*[b]	1.53 ± 0.07	*1.09 ± 0.17*[b]
GW 870086X	Comparative^{FF}	7.98 ± 0.07	*11.4 ± 1.0*[b]	1.98 ± 0.13	*0.88 ± 0.09*[b]

Dex and FF concentration-response curves were constructed in the absence and presence of Dex-Mes (3nM, 10nM and 30nM; 30min pre-incubation followed by washout) and the resultant sets of curves were analysed simultaneously by operational model fitting from which estimates of K_A, τ, n and E_m were derived. These parameters were also derived for DC and GW by using the comparative method with FF and Dex as reference agonists.

[a]Parameters were derived from the curves shown in figures 3.6B & C, 3.7A & B and 3.9A & B.

[b]E_m values calculated by the comparative method are equivalent to the E_{max} values determined directly from the agonist concentration-response curve (see Table 3.1) by logistic curve fitting (equation 1). Accordingly, values of τ assume that Dex and FF are full agonists and generate a response that is equivalent to the E_m. However, GR inactivation indicates that this assumption is incorrect. Thus, in the comparative method, E_m and τ values are significantly under-estimated and over-estimated respectively and are italicized for that reason.

3.3.6 *Effect of GR Inactivation with Dexamethasone 21-mesylate (Dex-Mes) to Determine the Relationship between Luciferase Activity and GR Occupancy*

The relationship between GR occupancy and response was determined by constructing agonist concentration-response curves before and after controlled inactivation of a fraction of the total GR population with an irreversible, alkylating agent, Dex-Mes (Simons and Thompson, 1981), as described by Furchgott (1966). At concentrations up to 10μM, Dex-Mes failed to promote GRE-dependent transcription under conditions where Dex gave a robust response (pEC_{50}= 8.12 ± 0.12; maximum fold induction = 18.6 ± 1.0; Figure 3.8). Because Dex-Mes-induced receptor inactivation is time-dependent (i.e. it is a chemical reaction that covalently modifies GR) it was necessary to establish an exposure time that did not result in the inactivation of all GR. Preliminary experiments established that the optimal incubation time for Dex-Mes was 30min.

To conduct function *versus* occupancy experiments, 2×GRE-BEAS-2B reporter cells were pre-treated for 30min with varying concentrations of Dex-Mes. After 30min cells were washed free of Dex-Mes to prevent further GR inactivation and then incubated for 1h at 37^{o}C in Dex-Mes-free medium to allow the cells to recover. Concentration-response curves were then constructed to FF, Dex, DC and GW.

At the outset of the study, it was assumed that Dex and FF were high efficacy GR agonists in driving GRE-dependent transcription (i.e. where $EC_{50} < K_A$). However, in 2×GRE BEAS-2B reporter cells treated with Dex-Mes (3nM, 10nM and 30nM), the maximal asymptote of the concentration-response curves that described FF- and Dex-induced, GRE-dependent transcription was depressed in a graded fashion in the absence of any significant change in agonist potency (Fig.

3.9A & B; Table 3.3). Applying the operational model yielded pK_A values of 7.93 and 8.69 for Dex and FF respectively, which were very close to their EC_{50} values in the absence of Dex-Mes (i.e. K_A/EC_{50} ~1; Table 3.3). Accordingly, the relationships between GR occupancy and response were essentially linear and did not deviate appreciably from the line of identity (Fig. 3.9C & D). The linearity between the concentration of agonist-occupied receptors ([AR]) and response is unusual in pharmacology because it indicates a lack of a receptor reserve.

The operational model also provides estimates of τ (the efficacy of an agonist in a given tissue), E_m (the maximum response that a given tissue can produce) and n (the slope of the curve that describes the relationship between the formation of AR complexes and response). Analyses of the data shown in figure 3.9A and B, indicate that FF and Dex had very low efficacies (mean τ values = 0.74 and 1.07 respectively; Table 3.2). This finding was consistent with estimates of E_m, which were significantly greater (~3-fold) than the E_{max} values determined by logistic curve fitting. Stated differently, the maximum, measured responses produced by FF and Dex in BEAS-2B reporter cells in the absence of Dex-Mes (filled circles in Fig 3.9A & B) were significantly lower than the maximum tissue response predicted by the operational model (dashed lines labelled E_m in Fig. 3.9A & B). Since the E_m is, by definition, produced by a full agonist, these data suggest that FF and Dex were partial agonists in promoting GRE-dependent transcription.

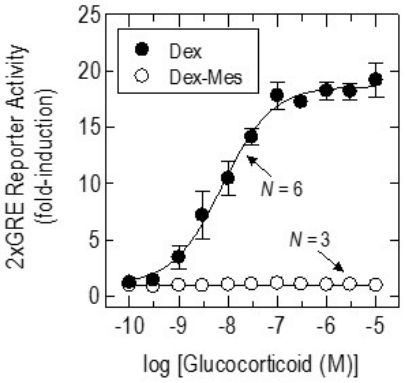


Figure 3.8 Effect of Dex and Dex-Mes on GRE-dependent transcription.
2×GRE BEAS-2B reporter cells were treated with Dex or Dex-Mes at the concentrations indicated. At 6h cells were harvested for the determination of luciferase activity. Data points represent the mean ± s.e. mean of *N* independent determinations.

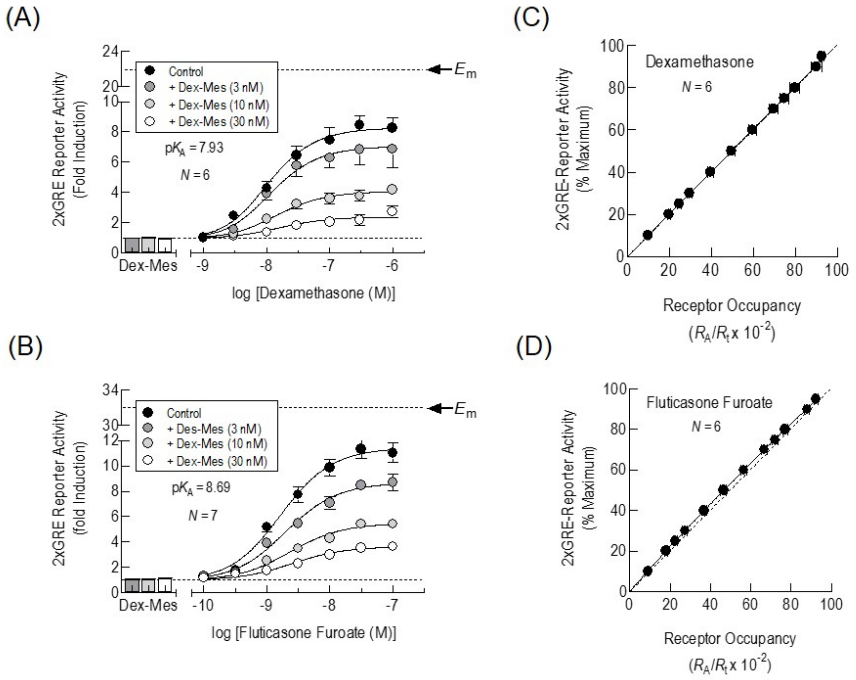


Figure 3.9 Relationship between GR occupancy and GRE-dependent transcription.
2×GRE BEAS-2B reporter cells were treated with Dex-Mes (3nM, 10nM or 30nM for 30min) or vehicle. The cells were washed in Dex-Mes-free medium and concentration-response curves constructed to Dex (panel A) or FF (panel B). The resulting sets of curves were analyzed by operational model fitting from which estimates of K_A, τ, n, E_m (indicated by the arrow) and pEC_{50} (of the control curve) were derived (Table 3.3). Data points represent the mean ± s.e. mean of N independent determinations. In panels C and D, the K_As of Dex and FF derived by GR inactivation were used to calculate the relationship between fractional GR occupancy (R_A/R_t) and GRE-dependent transcription. The horizontal dashed line in panels A and B toward the bottom of each graph indicates baseline luciferase activity. The dashed line in panels C & D is the line of identity where reporter activation is a linear function of GR occupancy.

Table 3.3 Relationship between the potency of FF and GW in promoting GRE-dependent transcription and their affinity for GR determined by fractional, irreversible GR inactivation with Dex-Mes

	Control	**+ Dex-Mes** (3 nM)	**+ Dex-Mes** (10 nM)	**+ Dex-Mes** (30 nM)
Fluticasone Furoate				
K_A (nM)[a]	2.04	2.04	2.04	2.04
EC_{50} (nM)[b]	1.81	2.33	3.14	3.24
K_A/EC_{50}	1.12	0.88	0.65	0.63
Dexamethasone				
K_A (nM)[a]	11.9	11.9	11.9	11.9
EC_{50} (nM)[b]	13.9	10.6	13.8	63.8
K_A/EC_{50}	0.86	1.12	0.86	0.19

See text and Table 3.2 for further details.
[a]Mean K_A values were calculated by the inactivation method (Fig. 3.9A & B; Table 3.2).
[b]Mean EC_{50} values were determined from the agonist concentration-response curves in the absence and presence of Dex-Mes (Fig. 3.9A & B).

3.4 Discussion

The results presented in this chapter demonstrate that the panel of GCs tested in this study did not behave equally in inducing luciferase activity in 2×GRE BEAS-2B reporter cells and thus, provide primary support for our overall hypothesis. Indeed, the intrinsic activity values of the GCs tested varied on the reporter with values consistent with full agonism, partial agonism, antagonism and even super agonism (where a synthetic agonist produces a greater maximum response than the natural ligand or ligands). Given the widespread distribution of GR, these findings are likely to apply to other cell types and could have a significant impact on the therapeutic activity of GCs in the treatment of asthma and other inflammatory disorders. For example, in a cell type where GR density is low, DC and GW may be weak in promoting the expression of anti-inflammatory genes because they have low intrinsic efficacy relative to Dex and FF.

As shown in figure 3.4, the panel of GCs tested on 2×GRE BEAS-2B reporter cells were unaffected by CBX, an inhibitor of 11β-HSD, confirming that the differences in GC intrinsic activity values was not due to variable metabolic inactivation of one GR agonist over another. Moreover, experiments involving Org confirmed that the increase in luciferase expression was due to the binding and activation of GR and not to cross-activation of other nuclear hormone receptors. Thus, Org pK_B values were ~8.3, irrespective of the GC tested, which is consistent with the affinity of Org for GR published previously by Peeters *et al.*, (2004).

According to classical receptor theory, which has developed principally from the study of GPCRs, a partial agonist will antagonise the effect of a full agonist acting at the same receptor. Our data involving the partial agonists GW and DC confirm that this also holds true for drugs that act at nuclear hormone receptors such as GR. These results are in agreement with a recent study by Uings et al., (2013), who showed that GW produced a competitive antagonism of Dex-induced,

GRE-dependent transcription in A549 (carcinomic lung alveolar epithelial cells) and MG63 osteosarcoma cell lines. The affinity of GW for GR, which has not been reported previously, was calculated using three different methods: Gaddum-Schild analysis, operational model fitting using the comparative method and operational model fitting using fractional GR inactivation (Figures 3.6 & 3.7, Table 3.2). All methods gave similar results ($pK_{A(B)} \sim 8$), which gives confidence that the derived affinity value is accurate.

Operational model fitting of the data in figure 3.9 showed that the pK_A values for Dex (7.93) and FF (8.69) were very close to their pEC_{50} values of 7.9 and 8.7 respectively (i.e. $K_A \approx EC_{50}$), which is unusual because it indicates the absence of a receptor reserve, even for the "super" agonist FF. Consistent with these data were the very low efficacy estimates of FF and Dex. Indeed, maximum luciferase activity produced by these agonists was ~3 times lower than the calculated system maximum (E_m). This is the maximum response that the tissue can produce and is typically achieved by a high efficacy, full agonist. Taken together, the data show that FF and Dex were partial agonists in promoting transcription in 2×GRE BEAS-2B reporter cells. However, one must be cautious in extrapolating these data to *bona fide* genes, as the unexpected pharmacodynamics parameters calculated here may simply represent an artifact of the artificial reporter system. This possibility is addressed in chapter 5.

Chapter Four: **Augmentation of GR-mediated, GRE Reporter Activity by Indacaterol: A Pharmacodynamics Study**

Some of the data discussed in this chapter have been published as a peer reviewed manuscript:

Taruna Joshi, Malcolm Johnson, Robert Newton & Mark A. Giembycz (2015). The long-acting β_2-adrenoceptor agonist, indacaterol, enhances glucocorticoid receptor-mediated transcription in human airway epithelial cells in a gene- and agonist-dependent manner. *British Journal of Pharmacology*, **172** (10), 2634–2653.

4.1 Rationale

Asthma is a heterogeneous, chronic inflammatory disease of the airways for which the mainstay treatments include anti-inflammatory agents, normally ICS, and bronchodilators such as β_2-AR agonists (SABAs or LABAs). Typically, SABAs and ICSs are administered as a monotherapy, while LABAs are always administered in combination with an ICS (Global initiative for asthma, 2015). β_2-AR agonists are administered as the standard reliever therapy for emergency hospital admissions and daily asthma management (Sears and Lötvall, 2005). However, due to their cardiopathic side-effects and that they mask the underlying inflammation that characterises asthma, β_2-AR agonists are not considered safe for daily, long-term use as monotherapy. For this reason, it is recommended that a LABA be combined with an ICS by all international asthma treatment guidelines (Bateman et al., 2008).

The β_2-AR is a GPCR and generally couples with the heterotrimeric GTP-binding protein, Gs. On ligand binding, cAMP is generated by the activation of adenylyl cyclase; cAMP then binds to the regulatory subunits of the PKA holoenzyme, which causes dissociation of the catalytic subunits from the regulatory subunit dimer and the phosphorylation of multiple proteins. In the case of airway calibre, this ultimately causes relaxation of the smooth muscle and bronchodilation (Giembycz et al., 2008).

In asthmatic subjects, Greening *et. al.,* in 1994 serendipitously discovered that combining the LABA, salmeterol, to a standard dose of beclomethasone dipropionate, was superior in gaining asthma control than was doubling the dose of the GC. Since that report, several studies have confirmed the clinical superiority of combination therapy in the treatment of moderate-to-severe asthma which cannot be controlled by GCs alone (Ducharme et al., 2010; Greening et al., 1994; O'Byrne et al., 2005; Sears, 2011). The molecular mechanism for the superiority of the combination therapy is still not clear. However, our research group has previously reported that β_2-AR agonists, acting via cAMP, synergistically enhance GC-induced gene expression, to a level that cannot be achieved by the GC alone (Kaur et al., 2008a). This enhancement of GRE-dependent gene expression by β_2-AR agonists was found to be either additive or synergistic depending on the gene of interest and the duration of drug exposure (Kaur et al., 2008a). Additivity of gene expression is when the effect produced by the two drugs in combination is equal to the sum of individual effects produced by the same drugs alone. In contrast, synergism is when the effect produced by two drugs in combination is greater than the sum of their individual effects.

In all previously reported studies, the effect of a LABA on the ability of a given GC to enhance gene expression (i.e. the strength of augmentation) was not considered. We propose that this is important to determine because if differences are apparent they could challenge the idea that

all inhaled GC/LABA combination therapies are therapeutically equivalent. To address this gap in knowledge, the transactivation potential of a panel of seven GCs to induce luciferase activity in 2×GRE BEAS-2B airway epithelial cells, alone and in combination with the ultra LABA, Ind was determined. We speculated, that the enhancement of GRE-dependent transcription by a β_2-AR agonist will be GR agonist- and gene-dependent, which will, in turn, depend upon GR number and GC efficacy. Thus, the primary aim of the work described in this chapter was to evaluate, pharmacodynamically, the effect of Ind on the potency, efficacy and affinity of seven GCs. Thus, these experiments extend the work reported in chapter 3, which was related to GCs as monotherapy, to the analysis of GC/LABA combination therapy.

Clinically relevant GCs, which were classified as full agonists, partial agonists and antagonists were analysed alone and in combination with Ind. As described in chapter 3, FF and Dex were classified as full agonists, DC and GW were partial agonist while Mif and Org were inactive in inducing luciferase activity. In addition, a novel, non-steroidal compound, GSK, synthesized by GlaxoSmithKline in an attempt to improve the therapeutic index of GCs in asthma, was also included in the analysis (Yates et al., 2010).

4.2 Hypothesis

We hypothesize that the combination of a GC and a LABA results in the enhancement of GRE-dependent reporter activation and that magnitude of enhancement by a given LABA depends upon the intrinsic efficacy of the GC.

4.3 Results

4.3.1 *Effect of Ind on GC-Induced, GRE-Dependent Transcription*

GR-mediated gene transcription in 2×GRE BEAS-2B reporter cells was determined in response to six GCs in the absence and presence of a maximally effective concentration of Ind (100nM) (Figure 4.1A-F). Five out of six GCs displayed varying degree of agonism as reported in chapter 3 and, in addition interacted synergistically with Ind at inducing luciferase activity. Surprisingly, Mif which was a very weak partial agonist in the absence of Ind, exhibited luciferase induction in concentration-dependent manner when combined with Ind with an enhancement of ~1.5 fold at the maximum concentration tested (Figure 4.1E). In addition, Org, which is reported to be a competitive, GR antagonist and, indeed, was inactive at inducing luciferase activity by itself, displayed extremely weak but detectable agonism in the presence of Ind (Figure 4.1F). The rank order of potency of the GCs tested was: FF > Dex = GW > DC > Mif and this was unchanged in the presence of Ind. Tables 4.1 and 4.2 show the maximum fold induction, potency and intrinsic activity values of each GC in the absence and presence of Ind, which were calculated from the data shown in figures 4.1 and 4.2. In comparison to FF, all GCs tested were partial agonists at inducing luciferase activity alone and in combination with Ind (Table 4.1), with the following rank order of intrinsic activity values (from high to low): FF > Dex > DC > GW > Mif > Org (Figure 4.1 & Table 4.1).

In a second set of experiments, concentration-response curves to Ind were constructed in presence of a maximally effective concentration of each GC. Figure 4.1G-L, shows the enhancement of luciferase activity by Ind for each GC studied. It is noteworthy, that despite the difference in intrinsic activity between each GC, Ind was equi-potent in potentiating luciferase

activity with an EC_{50} value of ~0.8nM. This also extended to Org, which was inactive by itself. Thus, these data show that when Ind was combined with a fixed concentration of a GC, or when a GC was combined with a fixed concentration of Ind, the potency of neither Ind nor the GC was changed (Table 4.2). In addition, the degrees of agonism and rank order of intrinsic activity values of the GCs studied were also unchanged in the presence of Ind (Tables 4.1 & 4.2; Figures 4.2A & B). Indeed, figure 4.2C shows that the relationship between the intrinsic activity values of each GR ligand in the absence and presence of Ind was linear with a Spearman's (ρ) rank order correlation value of 1. While it is possible that a LABA could have increased the potency of a GC, it is difficult to understand how this would lead to improved efficacy. Thus, the data conclusively show that the magnitude by which Ind enhances luciferase activity depended on the intrinsic activity of a GC; the higher the intrinsic activity, the greater was the enhancement. Therefore, the superiority of ICS/LABA combination therapy may be due to the ability of a LABA to enhance anti-inflammatory gene expression above the maximum level produced by a GC alone.

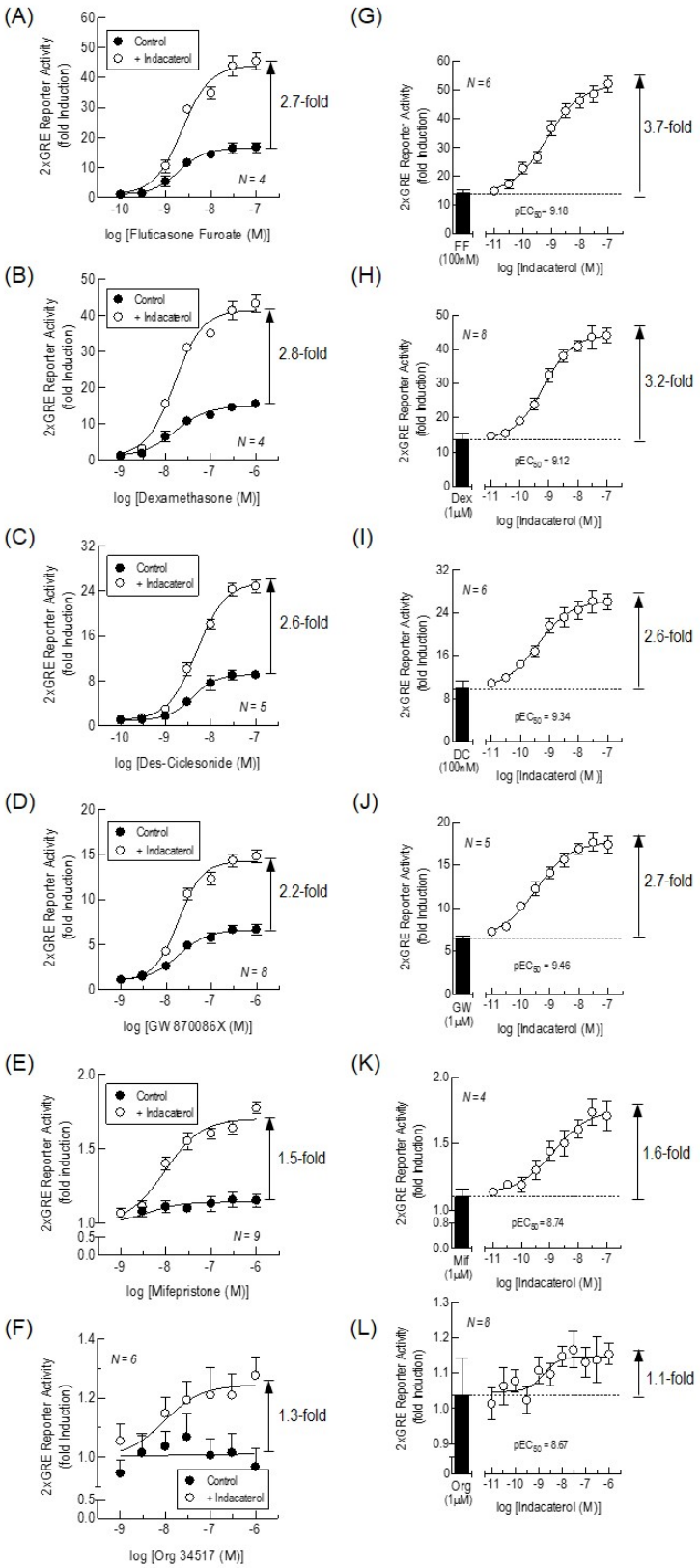


Figure 4.1 Effect of Ind on GRE-dependent transcription.
Cells were treated with fluticasone furoate (A), dexamethasone (B), des-ciclesonide (C), GW 870086X (D), mifepristone (E) or Org 34517 (F) at the concentrations indicated in the absence and presence of Ind (100nM), which was added concurrently. In panels G-H, cells were treated with Ind (10pM to 1μM) in the presence of a maximally-effective concentration of each GC that was determined from the data shown in panels A-F. At 6h cells were harvested, luciferase activity was determined and concentration-response curves were constructed. Data points represent the mean ± s.e. mean of *N* independent determinations. The vertical arrows in each panel indicate the maximal fold enhancement of GRE-dependent transcription produced by indacaterol (100nM). The horizontal dashed lines in panels *G-L* indicate luciferase activity produced by GC alone.

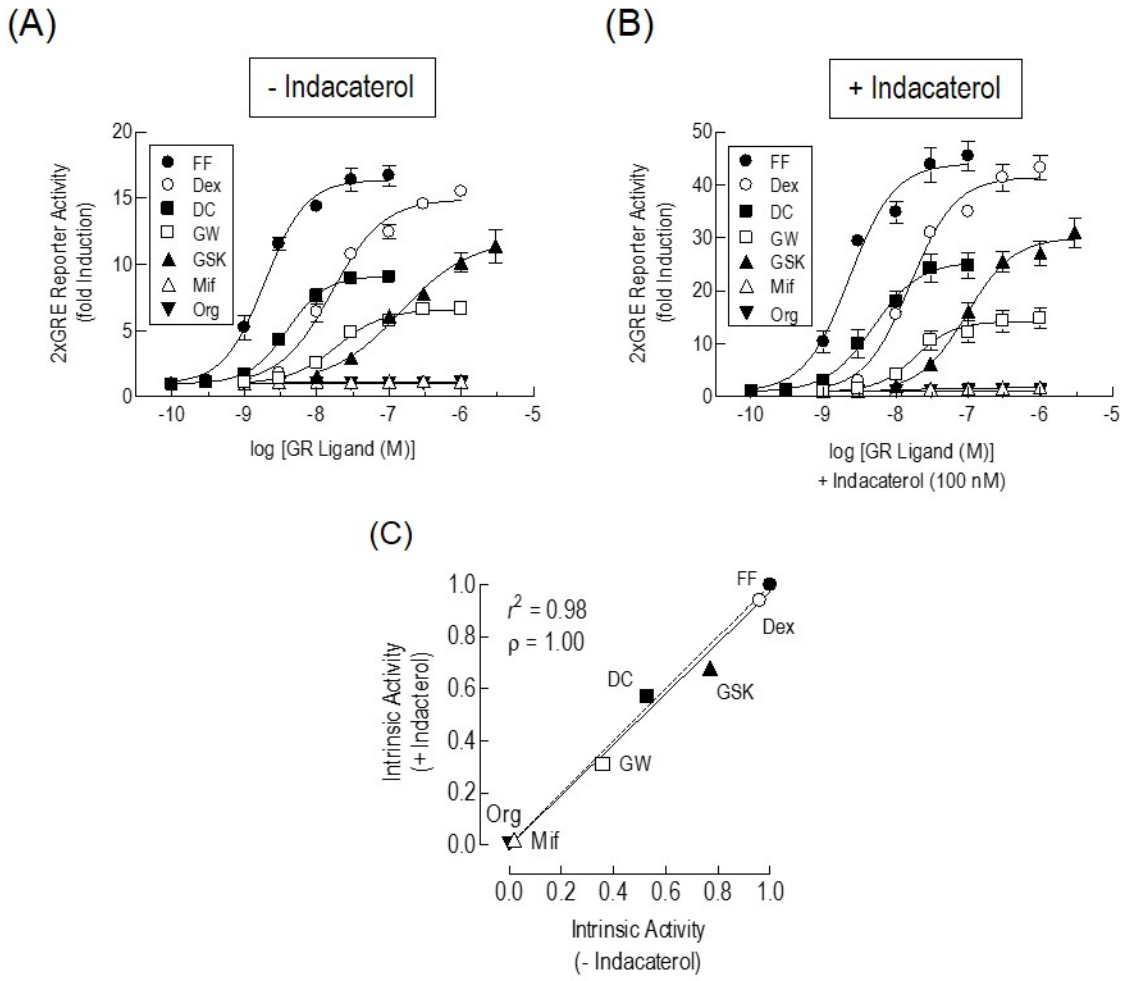


Figure 4.2 Effect of Ind on the intrinsic activity values of a panel of GR agonists in promoting GRE-dependent transcription.

Cells were treated with GR agonists at the concentrations indicated in the absence (panel A) and presence (panel B) of indacaterol (100nM) and concentration-response curves were then constructed. Panel C shows the relationship between the intrinsic activity values of each GR agonist in the absence and presence of indacaterol, with FF being assigned a value of 1. The dashed line indicates line of identity. Data points represent the mean ± s.e. mean of four to seven independent determinations (see Table 4.1). Key: FF, fluticasone furoate; Dex, dexamethasone; DC, des-ciclesonide; GW, GW 870086X; GSK, GSK 9027; Mif, mifepristone; Org, Org 34517.

Table 4.1 Effect of a maximally-effective concentration of Ind on the potency and intrinsic activity values of a panel of GR ligands to promote GRE-dependent transcription.

Glucocorticoid (N)	pEC_{50} -Ind	Fold Induction -Ind	α^a	pEC_{50} +Ind	Fold Induction +Ind	α^a
Fluticasone Furoate (4)	8.73 ± 0.05	16.4 ± 0.80	1	8.66 ± 0.08	44.1 ± 3.30	1.00
Dexamethasone (4)	7.79 ± 0.07	14.9 ± 0.46	0.96	7.80 ± 0.06	41.7 ± 2.54	0.94
GSK 9027 (5)	6.77 ± 0.17	12.8 ± 2.10	0.77	7.03 ± 0.05	30.1 ± 2.73	0.68
Des-ciclesonide (5)	8.39 ± 0.03	9.22 ± 0.24	0.53	8.32 ± 0.05	25.5 ± 1.15	0.57
GW 870086X (8)	7.72 ± 0.05	6.67 ± 0.22	0.36	7.72 ± 0.04	14.3 ± 0.67	0.31
Mifepristone (8)	7.67 ± 0.42	1.26 ± 0.09	0.02	7.75 ± 0.16	1.81 ± 0.06	0.02
Org 34517 (6)	inactive	0.99[b]	0	8.02[b]	1.24[b]	0.006

Values were calculated from the data shown in figures 4.1A-F and 4.6A using indacaterol at a concentration of 100nM.
[a]Intrinsic activity (fold induction by GR ligand – 1)/(fold induction by fluticasone furoate - 1).
[b]Values determined from pooled data.

Table 4.2 Concentration-dependent enhancement by Ind of GRE-dependent transcription.

Glucocorticoid	Concentration (μM)	*N*	2×GRE BEAS-2B Reporter Activity (fold induction)		Fold Enhancement by Indacaterol[a]	Indacaterol Potency (pEC_{50} (M))
			-Indacaterol	+Indacaterol		
Fluticasone Furoate	0.1	6	14.1 ± 0.58	51.7 ± 3.15	3.68 ± 0.19	9.18 ± 0.06
Dexamethasone	1	8	13.8 ± 0.71	45.1 ± 2.37	3.28 ± 0.13	9.12 ± 0.14
GSK 9027	3	5	8.50 ± 0.63	24.8 ± 1.03	2.94 ± 0.25	9.16 ± 0.04
Des-ciclesonide	0.1	6	9.85 ± 0.57	26.5 ± 1.53	2.72 ± 0.17	9.34 ± 0.18
GW 870086X	1	5	6.58 ± 0.18	17.4 ± 1.03	2.71 ± 0.14	9.46 ± 0.07
Mifepristone	1	4	1.10 ± 0.04	1.82 ± 0.15	1.61 ± 0.08	8.74 ± 0.13
Org 34517	1	8	1.04 ± 0.03	1.15 ± 0.03	1.11[b]	8.67[b]

Values calculated from the data shown in figures 4.1G-L and 4.4B.
[a]Maximum fold induction induced by GR ligand the presence of indacaterol/maximum fold induction by GR ligand alone.
[b]Values determined are the mean of eight experiments.

4.3.2 *Effect of ICI 118551 on the Augmentation of GRE-Dependent Transcription by Ind*

To confirm that the enhancement of Dex-induced, GRE-dependent transcription by Ind was a β_2-AR-mediated response, Schild analysis was performed with the highly selective β_2-AR antagonist, ICI 118551 (Figure 4.3). In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to Ind alone and after pre-treatment (1h) with ICI 118551 (ICI; 10nM) in presence of Dex (1μM). These curves were then fitted simultaneously to equation 2 from which a pA_2 of 9.49 was derived, which is consistent with a β_2-AR-mediated effect (Alexander et al., 2013). As expected, neither Ind nor ICI alone had any effect in 2×GRE BEAS-2B reporter cells.

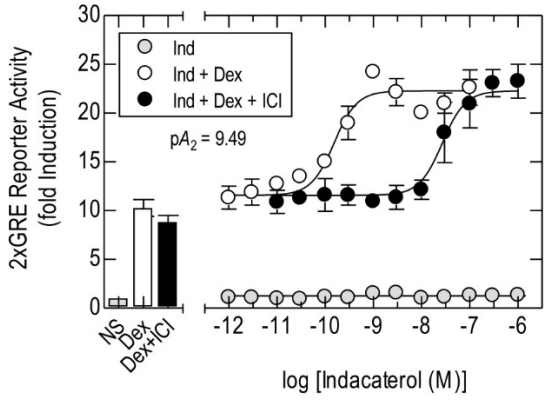


Figure 4.3 Effect of the selective and competitive β_2-AR antagonist, ICI 118551, on the enhancement of Dex-induced, GRE-dependent transcription by Ind.
In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to indacaterol (Ind) alone and after pre-treatment (60min) with ICI 118551 (ICI; 10nM) in presence of dexamethasone (Dex; 1μM). Each pair of concentration-response curves was then fitted simultaneously to equation 2 from which an affinity value of 9.49 for ICI was derived, which is consistent with a β_2-AR-mediated effect (Alexander et al., 2013). The effect of Ind in the absence of Dex is also shown. Bars and data points represent the mean ± s.e. mean of 5 independent determinations.

4.3.3 *Effect of Ind on GRE-Dependent Transcription Induced by a Non-Steroidal GR Agonist, GSK 9027 (GSK)*

The non-steroidal GR agonist, GSK, induced luciferase activity in concentration-dependent manner with an EC_{50} value of ~200nM. GSK was a partial agonist with intrinsic activity values of 0.77 (relative to FF) and 0.85 (relative to Dex) (Table 4.1). Similar to the other GCs tested, Ind (100nM) produced a synergistic enhancement (2.9 fold) of GSK-induced luciferase activity in the absence of any change in intrinsic activity or potency (Figures 4.2B, 4.2C & 4.4A; Table 4.1). In 2×GRE BEAS-2B reporter cells treated with a maximally-effective concentration of GSK (3μM), Ind enhanced luciferase activity in a concentration-dependent manner with an EC_{50} value of 0.78nM, which was similar to the potency of Ind for enhancing luciferase activity by the other GCs examined (Figure 4.4B; Table 4.1).

Pretreatment of 2×GRE BEAS-2B reporter cells with Org (1μM) antagonised (>90%) GSK-induced, GRE-dependent transcription in the absence and presence of Ind, suggesting that the non-steroidal ligand was acting through GR (Figure 4.5).

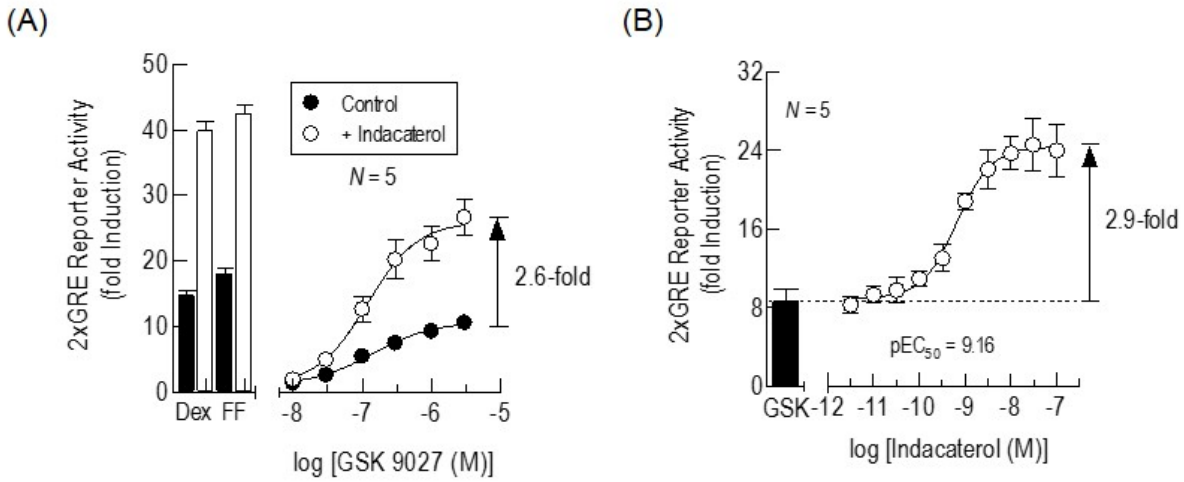


Figure 4.4 Effect of Ind on GSK-induced, GRE-dependent transcription.
In panel A, cells were treated with GSK 9027 at the concentrations indicated in the absence and presence of indacaterol (100nM), which was added concurrently. Dex, 1 μM and FF, 100nM, in the absence and presence of Ind, were included as comparators. In panel B, cells were treated with Ind (3pM to 1μM) in the presence of a maximally-effective concentration of GSK (3μM) determined from the data shown in panel A. At 6h cells were harvested, luciferase activity was determined and concentration-response curves were constructed. Data points represent the mean ± s.e. mean of *N* independent determinations. The vertical arrows in each panel indicate the maximal fold enhancement of GRE-dependent transcription produced by Ind. The horizontal dashed line in panel *B* indicates luciferase activity produced by GSK 9027 alone.

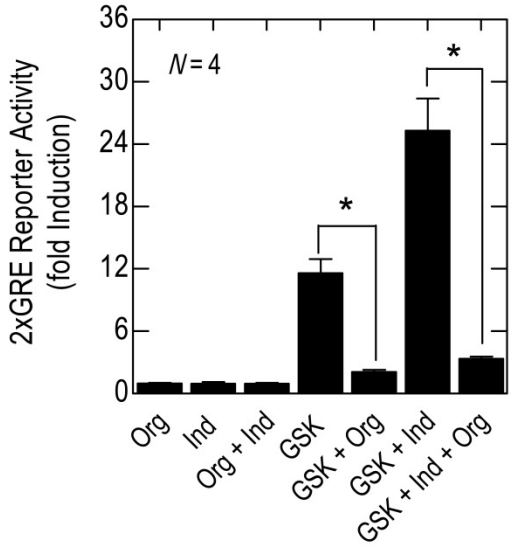


Figure 4.5 Effect of the GR antagonist, Org, on GSK-induced, GRE-dependent transcription. 2×GRE BEAS-2B reporter cells were pre-treated with Org 34517 (Org; 1μM; 30min) or its vehicle and then exposed to GSK 9027 (GSK; 3μM), or GSK 9027 and indacaterol (Ind; 100nM) in combination. At 6h cells were harvested and luciferase activity was determined. The effect of indacaterol and Org 34517 alone and in combination were also examined as controls. Bars represent the mean ± s.e. mean of *N* independent determinations. * $P < 0.05$, significant antagonism of GRE-dependent transcription by Org 34517.

4.3.4 *Relationship between GC Intrinsic Activity and the Augmentation of GRE-Dependent Transcription by Ind*

The relationship between the fold induction of luciferase activity by the seven GCs examined in the absence and presence of Ind was linear (Figure 4.6A). The stronger the agonism at GR, the greater was the fold induction produced by the LABA ($\rho = 1$). Replotting the *y*-axis as the fold enhancement of GRE-dependent transcription produced by Ind, for each GR ligand studied, indicated that the relationship was curvilinear and saturable (Figure 4.6B). Surprisingly, ligands with little (Mif) or no (Org) measurable agonist activity were rendered very partial agonists when combined with Ind.

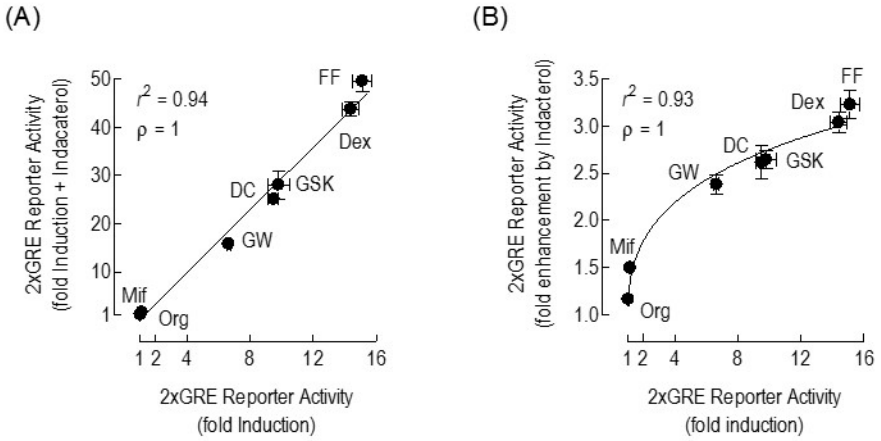


Figure 4.6 Influence of agonist intrinsic activity on the ability of Ind to enhance GRE-dependent transcription.
Panel A shows the relationship between GRE-dependent luciferase activity produced by seven GR agonists in the absence and presence of indacaterol (100nM). Panel B, shows that the fold enhancement by indacaterol of the maximal transcriptional response produced by each GR agonist was a saturable function of agonist intrinsic activity. Key: FF, fluticasone furoate; Dex, dexamethasone; DC, des-ciclesonide; GW, GW 870086X; GSK, GSK 9027; Mif, mifepristone; Org, Org 34517.

4.3.5 *Pharmacodynamics of GR-Mediated Reporter Activation in the Presence of Ind*

The pharmacodynamics of GC-GR interaction is poorly studied. Therefore, we applied the operational model of agonism to examine the effect of Ind on GR-mediated transcription. Concentration-response curves were constructed to Dex and FF in the absence and presence of Ind (100nM) before and after controlled, fractional GR inactivation with Dex-Mes. 2×GRE BEAS-2B reporter cells were treated with a concentration of Dex-Mes (10nM for 30min followed by washout) that suppressed the upper asymptote of the control agonist concentration-response curve. As shown in figure 4.7A and B, Dex-Mes treatment reduced the upper asymptotes of the Dex and FF concentration-response curves by 61% and 53%, respectively, without changing agonist

potency. Likewise, in the presence of Ind (100nM), Dex-Mes treatment reduced the upper asymptote of the Dex and FF concentration-response curves by 73% and 60% respectively but, again, there was no significant change in the potency of Dex and FF (Figures 4.7C & D).

Operational model fitting showed that Ind had no effect on the affinity (K_A) or operational efficacy (τ) of FF or Dex for GR (Table 4.3). Thus, K_A/EC_{50} ratios were unchanged and approached values of 1 indicating a lack of GR reserve where response is a linear function of occupancy. However, model estimates of the tissue maximum response parameter, E_m (dashed lines in figure 4.7), were considerably greater than the respective upper asymptotes of the Dex and FF concentration-response curves. Therefore, despite the significant increase in the transcriptional competency of ligand-bound GR when combined with Ind, there was still extra capacity in the system suggesting that these GCs behaved as partial agonists in driving GRE-dependent transcription.

Operational model fitting was also used to determine if the enhancement of GRE-dependent transcription by Ind was associated with a change in the affinity of partial agonists for GR. By applying the comparative method and using FF as a reference agonist, the pK_A values of GW, GSK and DC for GR were not changed by Ind (Figure 4.8; Table 4.3).

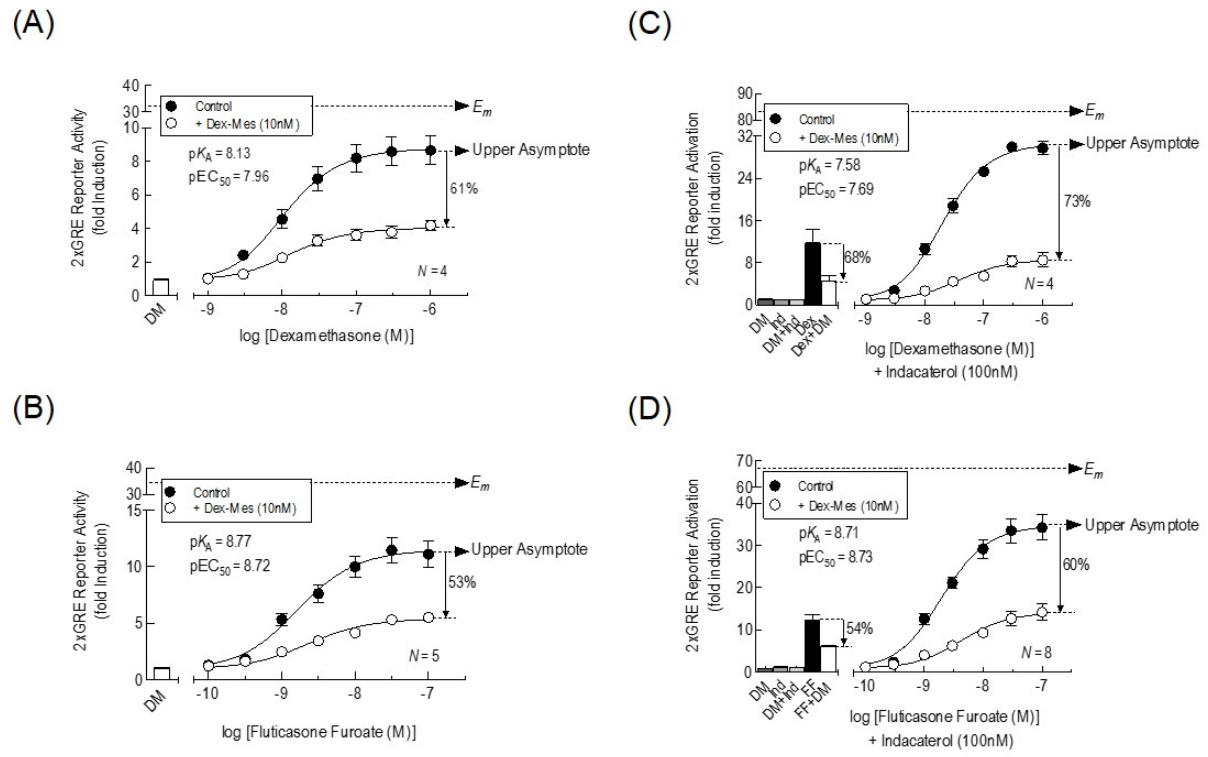


Figure 4.7 Analysis of Dex and FF concentration-response curve data in the absence and presence of Dex-Mes by operational model-fitting.

2×GRE BEAS-2B reporter cells were treated with dexamethasone 21-mesylate (Dex-Mes [DM]; 10nM or 30min) or vehicle. The cells were washed in Dex-Mes-free medium and concentration-response curves constructed to Dex (panels A & B) or FF (panels C & D) in the absence and presence of Ind (100nM). The model parameter estimates for Dex and FF are provided in Table 4.3. The bars in each panel show the effect of Dex-Mes, Ind, Dex-Mes + Ind, GR agonist and GR agonist after treatment of cells with Dex-Mes. The downward vertical arrows in each panel reflect the percentage inhibition of luciferase activity produced by Dex-Mes. The horizontal dashed arrow in each panel indicates the maximum tissue response (E*m*) predicted by the operational model. Data points represent the mean ± s.e. mean of *N* independent determinations. Key: DM, Dex-Mes; Ind, indacaterol; Dex, dexamethasone.

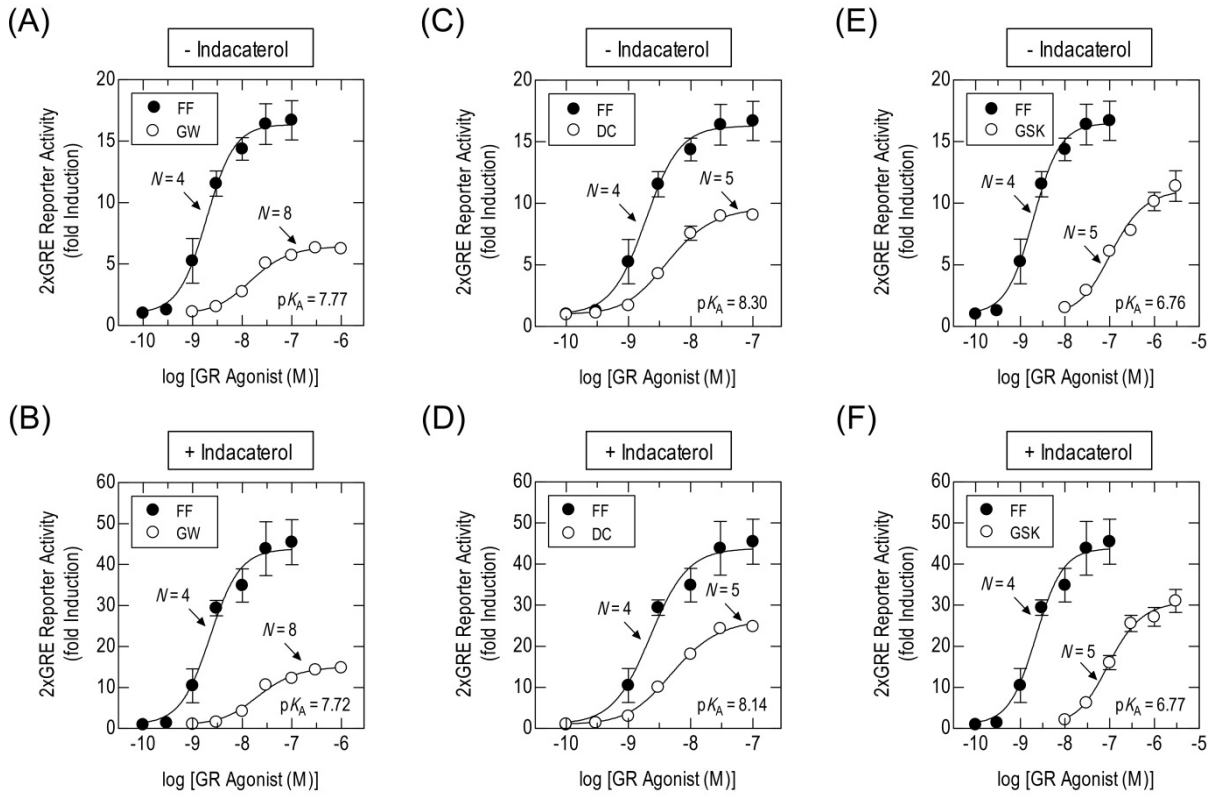


Figure 4.8. Determination of the affinity of GW, DC and GSK for GR.
In 2×GRE BEAS-2B reporter cells, concentration-response curves were constructed to fluticasone furoate (FF) and GW 870086X (GW; panels A & B), FF and des-ciclesonide (DC; panels C & D) and FF and GSK 9027 (GSK; panels E & F) in the absence and presence of indacaterol (100nM). The resulting pairs of curves were fitted simultaneously to equations 1 and 3 from which estimates of K_A were derived (see Table 4.3). Data points represent the mean ± s.e. mean of N independent determinations.

Table 4.3 Effect of Ind on pharmacodynamic parameters that define GRE-dependent transcription derived by operational model fitting.

GR Agonist	pK_A Indacaterol	pK_A +Indacaterol	τ -Indacaterol	τ +Indacaterol	Em^a -Indacaterol	Em^a +Indacaterol	*n* -Indacaterol	*n* +Indacaterol
Fluticasone Furoate	8.77 ± 0.44	8.71 ± 0.22	1.00 ± 0.05	1.73 ± 0.34	32.2 ± 7.9	67.9 ± 14.9	5.20 ± 4.00	2.29 ± 0.46
Dexamethasone	8.13 ± 0.23	7.58 ± 0.18	0.93 ± 0.35	1.49 ± 0.64	34.6 ± 9.8	83.1 ± 24.6	2.09 ± 0.61	1.53 ± 0.21
GSK 9027[b]	6.76	6.77	*1.55*	*1.80*	*16.5*	*43.9*	1.43	1.43
Des-ciclesonide[b]	8.30	8.14	*1.19*	*1.32*	*16.3*	*43.9*	1.59	1.45
GW 870086X[b]	7.77	7.72	*0.72*	*0.62*	*16.4*	*43.8*	1.54	1.45

[a]E_m values for GSK 9027, des-ciclesonide and GW 870086X were derived by the comparative method using fluticasone furoate as a reference full agonist and, by definition, are equivalent to the E_{max} values determined directly from the fluticasone furoate concentration-response curve by logistic curve fitting. Accordingly, values of τ assume that fluticasone furoate generates a response that is equivalent to the E_m. However, GR inactivation studies (Figure 4.7) indicate that this assumption is incorrect. Thus, in the comparative method, E_m and τ values are significantly under-estimated and over-estimated respectively and are italicized for that reason.
[b]Values determined from the mean data. Operational parameters were derived from the data shown in figures 4.7 and 4.8. Indacaterol was used at a concentration of 100nM.

4.3.6 *Effect of Ind and Forskolin (Fsk) on the Affinity of the GR Antagonist, Org, for GR*

In this set of experiments the effect of Ind on GRE-dependent transcription was compared with a direct activator of adenylyl cyclase, forskolin (Fsk). The aim here was to determine whether (i) the LABA-mediated augmentation of GC responses was strictly β_2-AR dependent or if could be reproduced by another cAMP elevator or mimetic, and (ii) if an increase in cAMP affects the affinity of antagonist binding to GR.

To interrogate these questions, 2×GRE BEAS-2B reporter cells were pretreated with Org (10nM and 100nM) or its vehicle for 1h. Concentration-response curves to FF, Dex, DC and GW were then constructed with and without either Ind (100nM) or Fsk (10μM), in the continued presence of antagonist or vehicle as indicated.

As shown in figure 4.9, Org, produced graded, parallel, dextral displacements of the Dex concentration-response curves. Analyses of these data using the Hill and Gaddum/Schild equations indicated that Org behaved as a surmountable, competitive antagonist (S=1) with a pK_B value (~8.3) that was unaffected by Ind (Figure 4.9A & B). The affinity of Org was also unchanged when Fsk (10μM) was substituted for Ind, using a larger panel of GCs namely FF, Dex, DC and GW (Figure 4.9C-J & Table 4.4).

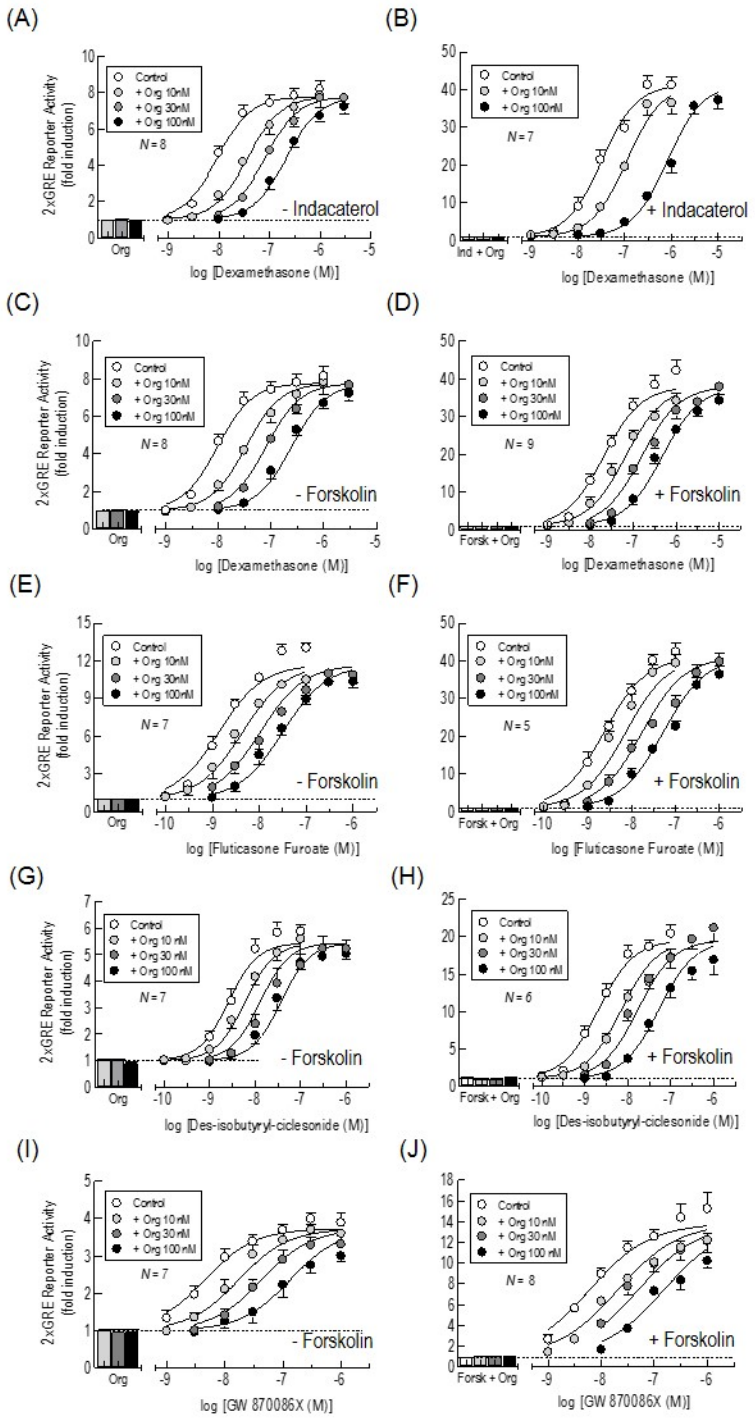


Figure 4.9 Gaddum-Schild analysis of the antagonism of GRE-dependent transcription by Org.

In 2× GRE BEAS-2B reporter cells, concentration-response curves were constructed to dexamethasone (panels A–D), fluticasone furoate (panels E and F), des-isobutyrylciclesonide (panels G and H) and GW 870086X (panels I and J) alone and after pre-treatment (60 min) with Org 34517 (Org) in the absence and presence of forskolin (Fsk; 10 μM) or indacaterol (Ind; 100 nM) as indicated. Each family of concentration-response curves was fitted simultaneously to equation 2 from which the pK_B of Org 34517 was derived. The bars in each panel show the effect on luciferase activity of each concentration of Org 34517 alone; the horizontal dashed lines define baseline activity. Data points and bars represent the mean ± SEM of *N* independent determinations. Panels A, C, E, G and I are reproduced from figure 3.5.

Table 4.4 Effect of Ind or Fsk on the affinity of Org for GR.

GR Agonist	**p*K*B**	**p*K*B** +Forskolin	**p*K*B** +Indacaterol
Fluticasone Furoate	8.34[a]	8.36	ND
Dexamethasone	8.38[a]	8.35	8.38
Des-ciclesonide	8.12[a]	8.39	ND
GW 870086X	8.46[a]	8.38	ND

[a]Data taken from Figure 3.5; ND, Not determined. The affinity of the GR antagonist, Org 34517, was calculated by Schild analysis from the data shown in figures 3.5 and 4.9. Indacaterol and forskolin were used at concentrations of 100nM and 10μM respectively.

4.4 Discussion

Asthma is a complex disease, displaying a variety of phenotypes. GCs are the first-line therapy for controlling airway inflammation in asthmatics. However, not all asthma phenotypes are adequately controlled by GC monotherapy regardless of dose. In such cases, asthma guidelines recommend combining a LABA with an inhaled GC concurrently in the form of a combination therapy. Combination therapy is the most effective asthma management option and is clinically superior at improving lung function and quality of life than available GC monotherapies (Giembycz and Newton, 2006; Greening et al., 1994; Masoli et al., 2005; Newton et al., 2010; O'Byrne et al., 2001; O'Byrne et al., 2005; Rabe and Schmidt, 2001). The molecular mechanism behind the superiority of combination therapy is vague and understudied (see below). However, the finding that Ind augmented GRE-dependent reporter activation by a mechanism that was antagonised by ICI 118551 with a K_B value of 9.49, is consistent with β_2-AR agonism. Moreover, the ability of forskolin to augment GRE-dependent transcription supports the idea that cAMP is central to the mode of action of LABAs.

As shown in figure 4.1, the GR ligands examined were not equivalent in their ability to promote GRE-dependent transcription. The intrinsic activity of these ligands varied from 1 for FF (super agonist) to a value of 0 for Org with the remaining ligands displaying partial agonist behaviour. In the presence of a maximum effective concentration of Ind, GR-mediated reporter activation was augmented in a synergistic manner and the magnitude of this effect was related to the intrinsic activity of the GR agonist. Thus, the rank order of GC intrinsic activity values was unchanged in the presence of Ind and a plot of α values in the absence and presence of Ind was

linear with a rank order correlation value of 1, indicating that the degree of augmentation was proportional to the intrinsic efficacy of the agonist.

Ind also enhanced GRE-dependent transcription produced by the non-steroidal, GR agonist, GSK. This is a significant observation because non-steroidal ligands such as AZD 5423 (Gauvreau et al., 2015), a structural analogue of GSK that is in phase II clinical development for asthma, may bind GR in a manner that is distinct from classical GCs and could form part of a novel combination therapy. Indeed, it is known that nuclear hormone receptors including GR, can adopt different conformations depending on the ligand to which it is bound (Allan et al., 1992; Biggadike et al., 2008, 2009a, 2009b). Therefore, the possibility that these conformations interact differently with the promoter region of target genes and effect different transcriptional signatures seems likely.

As stated above, our research group has shown that LABAs augment the activity of 2×GRE BEAS-2B reporter cells and the expression of GC-regulated gene in a cAMP- and PKA-dependent manner (Holden et al., 2011; Kaur et al., 2008a, 2008b). Although the exact molecular mechanisms involved are still unknown, it is possible that the augmentation of GRE-dependent gene transcription is due to the phosphorylation of GR and a consequent change in agonist pharmacodynamics. Indeed, studies published by other investigators have found that phosphorylation of GR at Ser211 induces a conformational change and a concomitant increase in gene transcription (Chen et al., 2008). Although this possibility cannot be excluded, our data have shown that combination of Ind or Fsk did not change the potency or affinity of any GR ligand tested. The affinity of the GR antagonist, Org, was also preserved. Moreover, an analysis of Dex and FF concentration-response curve data by operational model fitting, before and after fractional GR inactivation, demonstrated that Ind had no effect on the efficacy parameter, τ (Black and Leff, 1983), which is also consistent with these findings. Collectively, therefore, these data provide

compelling evidence that agonist pharmacodynamics are unaffected by Ind. According to the operational model, τ is equal to $[R_t]/K_E$, where $[R_t]$ is the total functional receptor concentration and K_E is the concentration of agonist-receptor complexes required to produce half maximal response. As $[R_t]$ is assumed to be invariant in a given, unstimulated tissue, the value of K_E must also be constant if τ is unchanged. On this basis we propose that PKA phosphorylates, and thereby modifies the behaviour of, a substrate(s) that behaves as a molecular rheostat (or volume control), which increases the transcriptional competency of liganded GR and, therefore, the magnitude of luciferase activity, without changing the relationship between fractional GR occupancy and response. This conclusion is consistent with the observation that Ind enhanced GRE-dependent transcription by resetting the system maximum parameter, E_m. Indeed, Mif and Org, which are considered to be selective GR antagonists and were almost inactive in inducing the expression of luciferase activity by themselves, exhibited a very modest but notable enhancement of luciferase activity in the presence of Ind (Figures 4.1E, K, F & L).

In conclusion, the data presented in this chapter demonstrate that in 2×GRE reporter cells, which only respond to GR agonists, the synergistic enhancement of GRE-dependent transcription by a LABA is not due to a change in the potency, affinity or operational efficacy of a GR agonist. Rather, the data are consistent with the idea that a LABA, probably via PKA-dependent phosphorylation, allows liganded GR to drive GRE-dependent transcription to a new maximum level that cannot be achieved by GC alone. Implicit in the idea is that the protein(s) targeted by Ind in some way allows GR to more optimally bind DNA at consensus GRE sites to promote the transcription of the luciferase gene presumably by making it a more effective transcription factor.

Chapter Five: **Pharmacodynamics of Gene Regulation by Glucocorticoids as a Stand-Alone Monotherapy and as a Combination Therapy**

Some of the data discussed in this chapter have been published in two, peer-reviewed manuscripts:

Taruna Joshi, Malcolm Johnson Robert Newton & Mark A. Giembycz (2015). An analysis of glucocorticoid receptor-mediated transcription in human airway epithelial cells identifies unique, ligand-directed, gene expression fingerprints with implications for asthma therapeutics. *British Journal of Pharmacology*, **172** (5), 1360–1378.

Taruna Joshi, Malcolm Johnson, Robert Newton & Mark A. Giembycz (2015). The long-acting β_2-adrenoceptor agonist, indacaterol, enhances glucocorticoid receptor-mediated transcription in human airway epithelial cells in a gene- and agonist-dependent manner. *British Journal of Pharmacology*, **172** (10), 2634–2653.

5.1 Rationale

In the previous chapter, it was established that Ind and the adenylyl cyclase activator, Fsk, synergistically enhanced GRE-dependent transcription of a luciferase reporter, in a manner that was proportional to the intrinsic activity of the GR ligand examined. In this chapter those observations are extended to an analysis of GC-inducible genes using ligands that have intrinsic activity values on the GRE reporter that vary from 1 (full agonism) to 0 (antagonism). The regulation of genes is considerably more complex than of a simple 2×GRE reporter. Hence, to

determine the effect of GCs as a monotherapy and in combination with Ind on the regulation of *bona fide* genes we took advantage of data derived from two previous microarray studies in the pulmonary type II alveolar cell line (A549) and in BEAS-2B cells. Four representative genes were selected for further examination out of which three exhibit potential anti-inflammatory properties (*GILZ, p57^{kip2}* and *CRISPLD2*) and the fourth, a putative side-effect gene (*PDK4*) (Eddleston et al., 2007, Samuelsson et al., 1999, Sugden and Holness, 2002, Vasarhelyi et al., 2014, Wang et al., 2009).

TSC22D3 (*aka GILZ*; glucocorticoid-induced leucine zipper) is reported to inhibit the activity of the pro-inflammatory transcription factors AP-1 and NFκB and was first identified as a Dex responsive gene in T cells, mast cells, epithelial cells, airway smooth muscle cells and A549 cells (Ayroldi and Riccardi, 2009; Ayroldi et al., 2001, 2007; Eddleston et al., 2007; Kaur et al., 2008b; King et al., 2013). In addition, evidence suggests that *GILZ* can also inhibit ras and/or raf signalling complex and, thereby, repress the ERK pathway, which is known to promote inflammatory responses (Ayroldi and Riccardi, 2009; Ayroldi et al., 2007).

CDKN1C (*aka p57^{kip2}*; cyclin dependent kinase inhibitor 1C) is a cyclin-dependent kinase (CDK) inhibitor, which belongs to cip/kip family. *p57^{kip2}* can interfere with DNA replication and, ultimately, cell proliferation; it is induced by combination therapy in ASM and could play a role in attenuating airway remodelling (Dekkers et al., 2012; Samuelsson et al., 1999). *p57^{kip2}* is also reported to repress JNK signalling suggesting that it may also have anti-inflammatory activity (Chang et al., 2003).

CRISPLD2 (cysteine-rich secretory protein LCCL domain containing 2) has been reported to physically bind LPS and so inhibit LPS-induced inflammatory processes mediated via TLR4 (Himes et al., 2014; Wang et al., 2009). Indeed, *CRISPLD2* ultimately protects against septic shock

and LPS-induced endotoxicity by repressing LPS-stimulated IL-6 and TNFα release (Himes et al., 2014; Wang et al., 2009). *CRISPLD2* is secreted by multiple tissues and cells including ASM, airway epithelial cells, T cells, granulocytes, and NK cells.

Many metabolic processes in the human body are regulated by the pyruvate dehydrogenase complex (PDC) to maintain a continuous, balanced supply of ATP (Jeong et al., 2012). The PDC controls the glycolytic or the tricarboxylic acid cycle by oxidising glucose to acetyl-coA in mitochondria (Jeong et al., 2012). Pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP) are key regulators of PDC activity through their ability to inactivate and activate the complex respectively (Jeong et al., 2012). Four PDK isoenzymes (PDK 1, 2, 3 & 4) are expressed in humans in a tissue-specific manner. Of these, *PDK2* and *PDK4* are of considerable interest as they are induced during starvation and in diabetes (Jeong et al., 2012). Moreover, *PDK4* expression is also increased in response to exogenous GCs, which can be detrimental under normal conditions as it can lead to hyperglycemia and fatty liver, which are potential adverse-effects of GCs in asthma therapy (Connaughton et al., 2010; Jeong et al., 2012; Kwon et al., 2004; Sugden and Holness, 2003).

5.2 Hypothesis

A given GC displays a unique gene induction fingerprint and, depending on the gene, it can behave as a super agonist, full agonist, partial agonist or even an antagonist. Furthermore, the gene expression fingerprint of a given GC will depend upon GR density in a target tissue and on its intrinsic efficacy.

5.3 Results

In this chapter, the results are discussed in two sections. The first section involves analyses of *bona fide* gene regulation by a panel of GCs as stand-alone therapy and the second section describes the results obtained for GC-induced gene induction in the presence of the LABA, Ind.

5.3.1 *Determination of GC-Specific Gene Induction Profile*

5.3.1.1 Transactivation of real genes by a panel of GCs

To compare the effect of a panel of GCs with varying intrinsic efficacies on real genes, as opposed to the artificial reporter system described in the previous chapter, 2×GRE BEAS-2B reporter cells were treated with maximally effective concentrations of FF, Dex, DC, GW and HC (determined from the reporter data described in Chapter 3). After 6h, RNA was extracted and real time PCR analysis of the expression of four candidate genes discussed above was performed. Mif and Org, which were very weak agonists in inducing luciferase, were also included in the study to examine their transactivation potential on real genes.

Figure 5.1 shows that the seven ligands studied displayed distinct degrees of agonism that varied in a gene-dependent manner. *PDK4* and *p57*^{kip2} behaved in a manner that resembled the activation of 2×GRE reporter with FF and Dex demonstrating full agonism when compared to DC, GW and HC, which were partial agonists (Figures 5.1A & B, Table 5.1). However, there were notable differences. For example, FF was significantly more effective at inducing the side-effect gene *PDK4* (1.9 fold) than was Dex, while there was no such discrepancy with *p57*^{kip2} (Figures 5.1A & D, Table 5.1). Interestingly, HC, DC and GW weakly induced *PDK4* (Figure 5.1B) but were as effective as FF and Dex in augmenting *CRISPLD2* expression (Figure 5.1D). A similar

pattern of expression was observed with *GILZ* (Figure 5.1C). Surprisingly, Mif and Org, which did not increase luciferase activity (Figure 3.3), were able to very weakly promote the expression of *p57*^{kip2} and *GILZ* (Figures 5.1A & C). Org also induced *PDK4* and *CRISPLD2*, although the effect was very modest (Figures 5.1B & D).

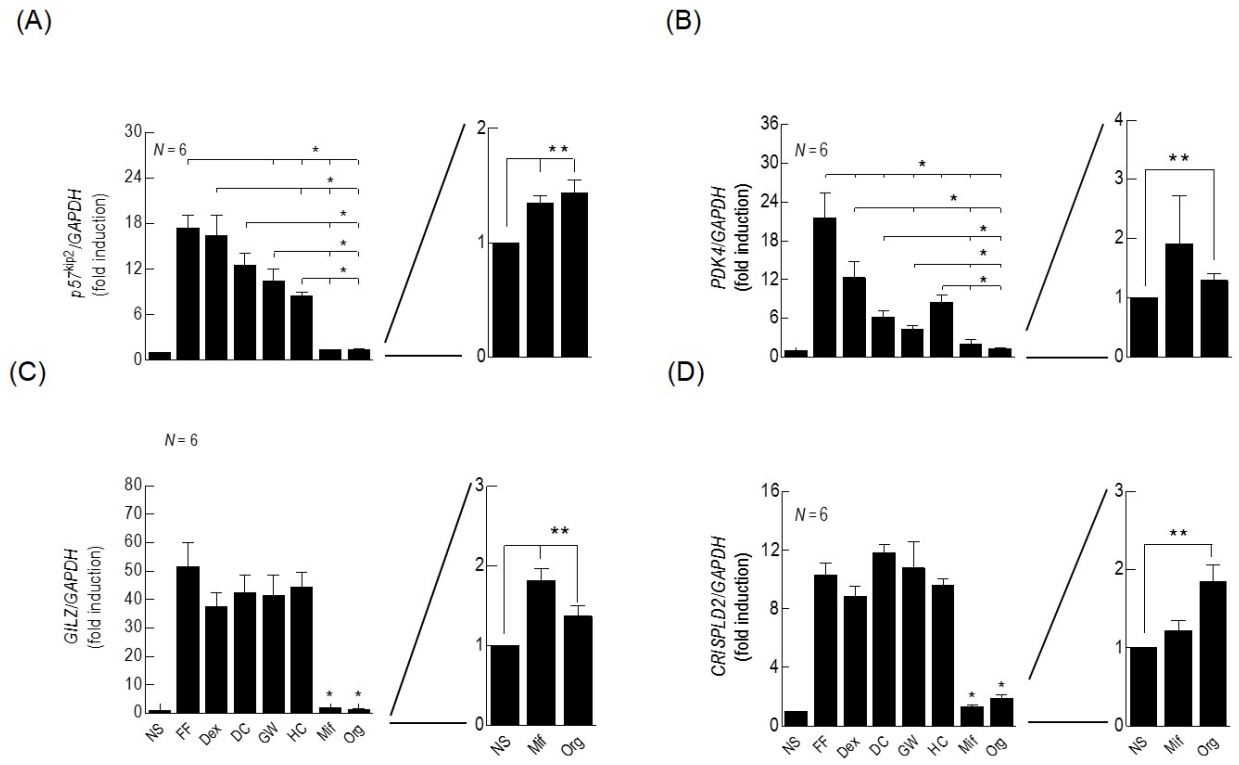


Figure 5.1 Comparative effects of a panel of GR ligands on gene expression.
2×GRE BEAS-2B reporter cells were treated with FF (100nM), Dex (1µM), DC (100nM), GW (1µM), HC (3µM), Mif (1µM) or Org (1µM). At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *p57*^{kip2} (panel A), *PDK4* (panel B), *GILZ* (panel C) and *CRISPLD2* (panel D). Data are the mean ± s.e. mean of *N* independent determinations and are expressed as a ratio to *GAPDH*.**P* < 0.05, significant difference in gene expression relative to FF. ***P* < 0.05, significant induction relative to untreated cells. Data were analyzed by repeated measures, one-way ANOVA/Tukey's multiple comparisons test using untransformed data.

Table 5.1 Relative activities of GCs for the induction of *p57*^{kip2}, *GILZ*, *PDK4* and *CRISPLD2*.

Glucocorticoid[a]	Relative Gene Expression (Dex = 1)			
	***p57*^{kip2}**	***GILZ***	***PDK4***	***CRISPLD2***
Dexamethasone (1μM)[b]	1	1	1	1
Fluticasone Furoate (100nM)	1.07	1.39	1.88	1.18
Des-ciclesonide (100nM)	0.75	1.14	0.48	1.37
GW 870086X (1μM)	0.62	1.11	0.30	1.25
Hydrocortisone (3μM)	0.48	1.19	0.69	1.09
Mifepristone (1μM)	0.02	0.02	0.09	0.04
Org 34517 (1μM)	0.03	0.01	0.03	0.11

[a]Glucocorticoids were used at a concentration that maximally activated the 2×GRE reporter.
[b]For each gene, data are expressed relative to the fold induction produced by Dex, which was assigned a value of 1.

5.3.1.2 Effects of irreversible inactivation of GR on gene expression

To determine the relationship between GR number and GC-induced gene expression, experiments were performed in cells treated with Dex-Mes, which covalently inactivates GR. Initially, Dex-Mes concentration-response curves were constructed in 2×GRE BEAS-2B cells to evaluate the transactivation activity of Dex-Mes on real genes, as was done previously with the luciferase reporter system (Figure 3.8). Cells were exposed to varying concentrations of Dex-Mes for 6h and RNA was then extracted for real-time PCR analysis. Figure 5.2 demonstrates that at concentrations of Dex-Mes up to 10nM, none of the genes were significantly induced. However, at higher concentrations Dex-Mes was active indicating that it can display very weak partial agonist behavior.

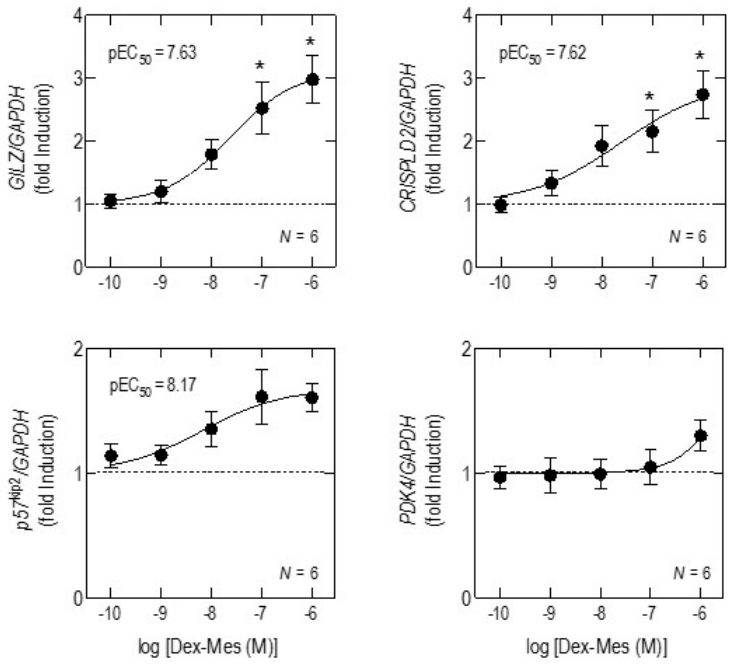


Figure 5.2 Effect of Dex-Mes on gene expression.
2×GRE BEAS-2B reporter cells were treated with Dex-Mes at the concentrations indicated. At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *GILZ* (panel A), *CRISPLD2* (panel B), *p57*^{kip2} (panel C) and *PDK4* (panel D). Data are expressed as the mean ± s.e. mean of *N* independent determinations and are expressed as a ratio to *GAPDH*. The dashed line in each panel defines baseline gene expression. **P* < 0.05, significant induction relative to untreated cells; one-way ANOVA/Tukey's multiple comparison test on untransformed data.

Therefore, in all further experiments, 2×GRE BEAS-2B reporter cells were treated with 10nM Dex-Mes for 30min to inactivate a fraction of the total GR population and then washed in Dex-Mes-free medium to stop the reaction. After a 1h incubation at 37^{o}C in Dex-Mes-free medium, cells were treated for a further 6h with the maximally-effective concentration of FF (100nM) or GW (1μM) and processed for real-time PCR. The concentrations of FF and GW chosen were 100 times greater (300nM and 1μM respectively) than their respective K_A values to ensure >99% GR occupancy as predicted by the *Law of Mass Action*.

As shown in figure 5.3, Dex-Mes inhibited the expression of *GILZ, CRISPLD2*, *p57*^{kip2} and *PDK4* induced by FF by 13%, 38%, 41% and 56% respectively (Figures 5.3A-D). Dex-Mes

produced a similar profile of inhibition when GW was the agonist (*GILZ*: 19%; *CRISPLD2*: 41%; *p57*^{kip2}: 35%; *PDK4*: 58%; Figures 5.3E-H). These data show that *GILZ* was the least sensitive gene to GR inactivation indicating that FF and GW needed to occupy fewer GR to maximally induce *GILZ* when compared to *CRISPLD2, p57*^{kip2} and *PDK4*. On the other hand, because the expression of *PDK4* was most affected by Dex-Mes, FF and GW need to occupy a higher fraction of GR to maximally induce this gene. Therefore, the data imply that there is greater receptor reserve for the induction of *GILZ* than of *PDK4*, and that the relationship between a given level of GR occupancy and magnitude of response is gene dependent.

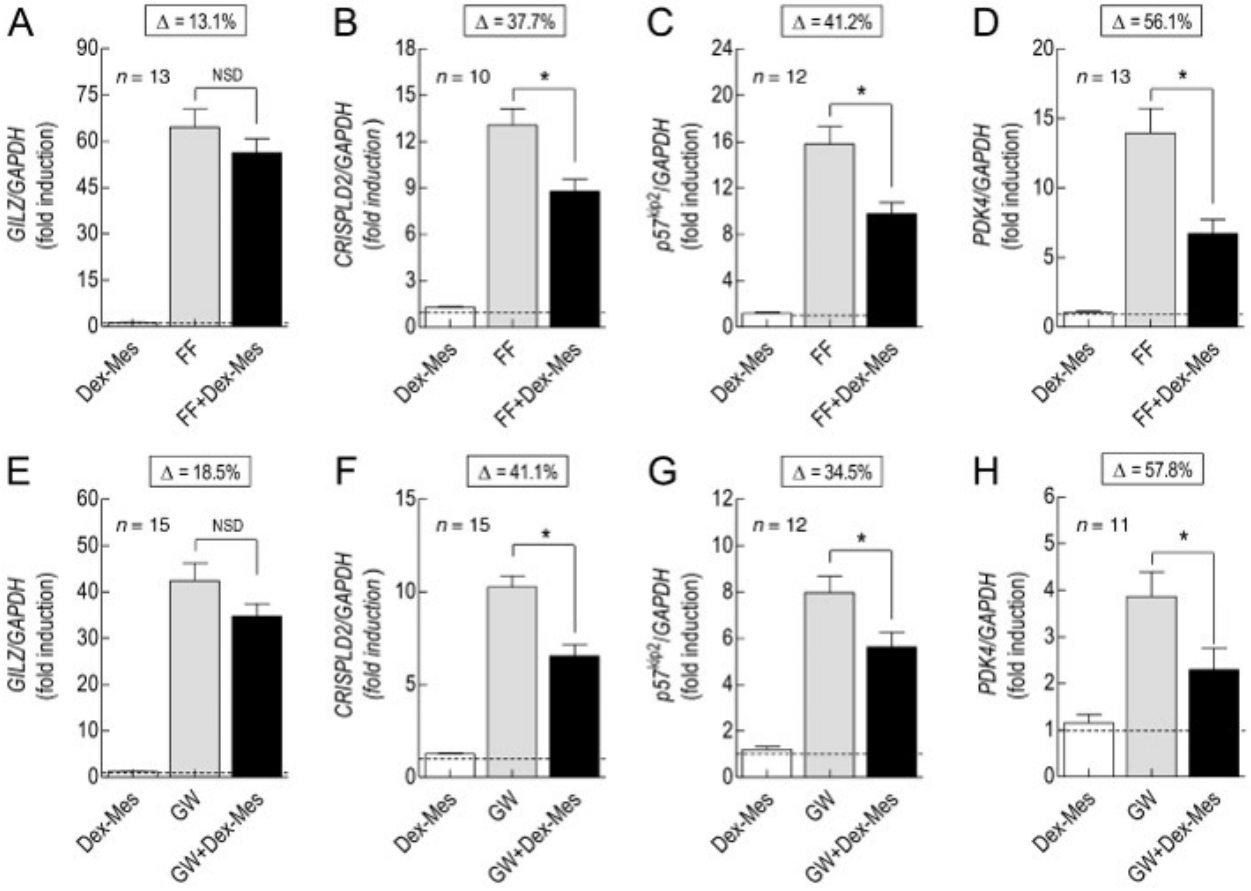


Figure 5.3 Effect of controlled, GR inactivation on FF- and GW-induced gene expression.
2×GRE BEAS-2B reporter cells were treated with Dex-Mes (10nM for 30min) or vehicle. The cells were washed in Dex-Mes-free medium, left to recover for 60min and exposed to FF (300nM) or GW (1μM) at concentrations that produce >99% GR occupancy. At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *GILZ* (panels A & E), *CRISPLD2* (panels B & F), *p57*^{kip2} (panels C & G) and *PDK4* (panels D & H). Data are the mean ± s.e. mean of *N* independent determinations and are expressed as a ratio to *GAPDH*. The horizontal dashed line in each panel defines basal gene expression. The percentage change (Δ) in gene expression effected by Dex-Mes is given above each panel. *$P < 0.05$, significant attenuation of gene expression; repeated measures, one-way ANOVA/Tukey's multiple comparisons test. NSD, not significantly different.

5.3.1.3 Relationship between GR occupancy and GC-dependent gene induction

To determine the relationship between GR occupancy and induction of the four selected candidate genes, concentration-response curves to FF and GW were constructed using 2×GRE BEAS-2B reporter cells. Similar to the luciferase data (Chapter 3), FF was more potent (4.9-6.6 fold) than GW although, the rank order of gene sensitivity was independent of the ligand used, thus *GILZ* > *CRISPLD2* > *p57*^{kip2} > *PDK4* (Table 5.2). GW was equi-effective with FF at inducing *GILZ* and *CRISPLD2* expression, while it behaved as a partial agonist in inducing *p57*^{kip2} (α = 0.71) and *PDK4* (α = 0.26) (Figures 5.4 A-D, Table 5.2). If the affinity of a ligand for a specific receptor is assumed to be a constant value, then the data in figure 5.4 suggest that the number of GRs required to promote a fixed degree of gene expression was dependent upon both the **ligand** as well as the **gene** of interest (see below). These relationships were quantified by using the K_A values of GW (8.3nM) and FF (2.04nM) determined in chapter 3. For both GCs, occupancy–response relationships were hyperbolic confirming the presence of a GR 'reserve' for gene induction (Figure 5.4E–H). Thus, consistent with variations in the calculated K_A/EC_{50} ratios (Table 5.2), the proportion of 'spare' receptors required to produce a given level of response varied in gene-dependent manner (*GILZ* > *CRISPLD2* > *p57*^{kip2} > *PDK4*; Table 5.3). For example, there was a greater GR 'reserve' for the induction of all four genes by FF than by GW (Figure 5.4E–H; Table 5.3). In particular, on *PDK4*, the occupancy–response relationship was very shallow and approached linearity, which is the limiting (and rare) situation of a hyperbola where $EC_{50} \rightarrow K_A$ (Figure 5.4H). Indeed, 100% GR occupancy by GW induced *PDK4* expression by only 26% of the maximum FF-induced response, which, itself, required only 18% GR occupancy (Figure 5.4H). Similar data were found for *p57*^{kip2}, *CRISPLD2* and *GILZ* (Figure 5.4E–G).

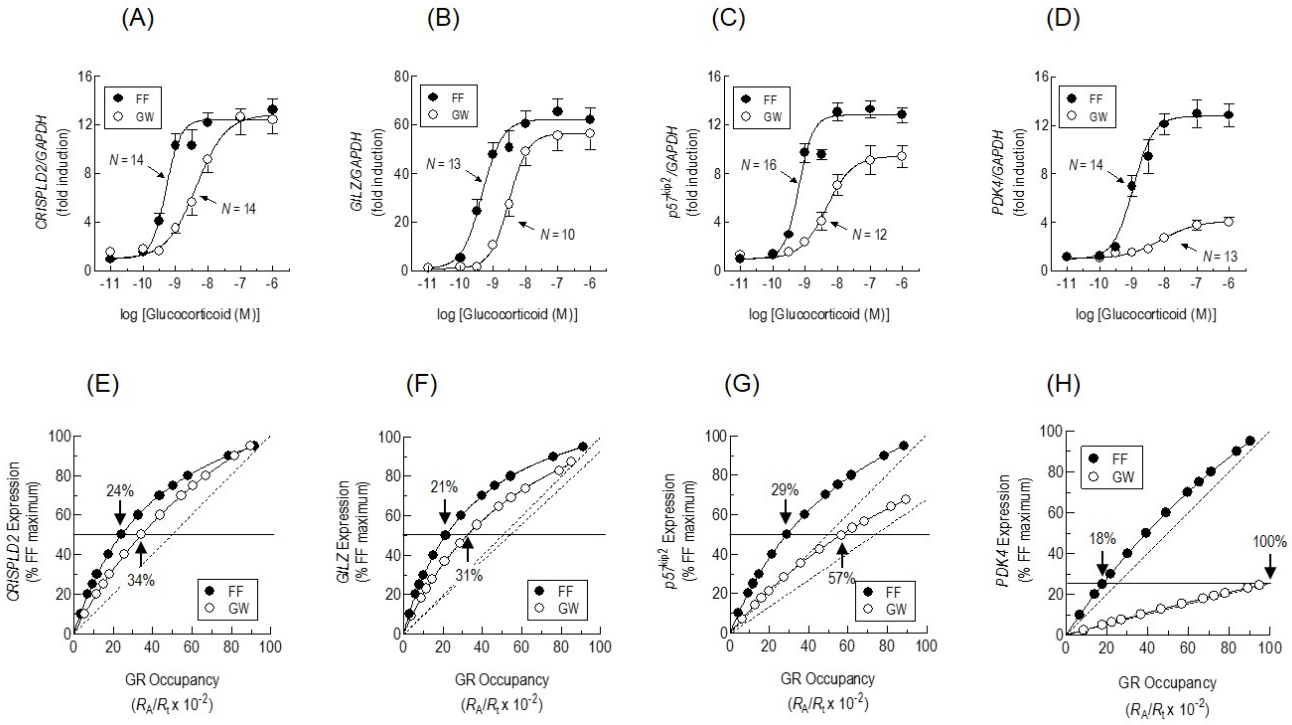


Figure 5.4 Comparative effects of FF and GW on gene expression.
2×GRE BEAS-2B reporter cells were treated with FF and GW (both 100pM to 1µM). At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *CRISPLD2*, *GILZ*, *p57*^{kip2} and *PDK4*. Panels A to D show, as a ratio to *GAPDH*, the concentration-response relationship for the induction of these genes by FF and GW where each data point represents the mean ± s.e. mean of *N* independent determinations. In panels E to H, mean gene expression is shown as a function of fractional GR occupancy according to equation 4 using K_A values for FF (2.04nM) and GW (8.30nM) derived from the experiments shown in figures 3.6A and 3.9B. The dashed line in panels E-H is the line of identity where gene expression is a linear function of GR occupancy. The solid black line in each panel defines a given level of gene expression (50% for *CRISPLD2*, *GILZ* and *p57*^{kip2}; 27% for *PDK4*). The point at which these lines bisect the occupancy-response curves shows the percentage of GRs required to produce the indicated level of response.

Table 5.2 Pharmacodynamic parameters of FF and GW for inducing *GILZ, CRISPLD2, p57^{kip2}* and *PDK4*.

Gene	**Fluticasone Furoate** (pEC_{50})	**Fluticasone Furoate** (max fold induction)	**GW 870086X** (pEC_{50})	**GW 870086X** (max fold induction)	$EC_{50}^{GW}/EC_{50}^{FF}$	K_A^{FF}/EC_{50}^{FF}	K_A^{GW}/EC_{50}^{GW}
GILZ	9.26 ± 0.08 (13)	61.2 ± 3.8	8.57 ± 0.07 (10)	56.5 ± 6.2	4.9	3.7	3.1
CRISPLD2	9.21 ± 0.07 (14)	12.6 ± 0.7	8.45 ± 0.06 (14)	12.6 ± 1.3	5.8	3.3	2.3
p57^{kip2}	9.09 ± 0.05 (16)	13.2 ± 0.6[a]	8.27 ± 0.07 (12)	9.4 ± 0.9	6.6	2.5	1.5
PDK4	8.88 ± 0.08 (14)	13.1 ± 0.9[a]	8.08 ± 0.09 (13)	4.3 ± 0.5	6.3	1.5	1.0

Values in brackets indicate number of determinations. Data calculated from figure 5.4A-D using K_A values of 8.3nM and 2.04nM for GW and FF respectively.

[a] $P < 0.05$, FF and GW maximum fold inductions significantly different from corresponding GW data- Student's two-tailed, unpaired *t*-test.

TABLE 5.3. Relationship between GR occupancy and gene expression.

		Percentage GR Occupancy Required to Produce:			
Gene	**Agonist**	**25% Response**	**50% Response**	**75% Response**	**95% Response**
GILZ	Fluticasone Furoate	8.2	21.4	46.5	91.5
	GW 870086X	11.7	28.5	54.7	85.1
CRISPLD2	Fluticasone Furoate	9.5	24.3	50.3	92.0
	GW 870086X	14.9	34.3	60.7	89.7
p57^{kip2}	Fluticasone Furoate	12.0	29.0	53.0	88.5
	GW 870086X	16.1	36.3	62.4	89.5
PDK4	Fluticasone Furoate	18.0	39.4	65.3	90.5
	GW 870086X	22.5	46.7	72.7	95.0

The K_A of GW and FF were derived by Schild analysis and controlled GR inactivation respectively (Figures 3.6A & 3.9B) and used to calculate the relationship between fractional GR occupancy and gene expression using the mean concentration-response curves shown in figure 5.4A-D. Data show the fraction (%) of GR occupied by FF and GW that produce 25%, 50%, 75% and 95% of maximum gene expression.

5.3.1.4 GW is a competitive antagonist of Dex-induced gene expression

2×GRE BEAS-2B cells were treated for 6h with maximally-effective concentrations of GW (1μM) and Dex (1μM) separately and in combination. After 6h cells were harvested for real-time gene expression analysis. As figure 5.5A shows, GW induced *PDK4* by 4.5 fold, while Dex was a stronger agonist and promoted the expression of *PDK4* by 17.5-fold relative to *GAPDH*. On addition of GW and Dex concurrently, the maximum *PDK4* induction by Dex was reduced from 17.5 to 5.1 fold. A similar effect was observed with $p57^{kip2}$ expression (Figure 5.4B). In contrast, Dex and GW induced *CRISPLD2* and *GILZ* to similar degrees, which were not significantly affected when the two GCs were used in combination (Figure 5.4C & D).

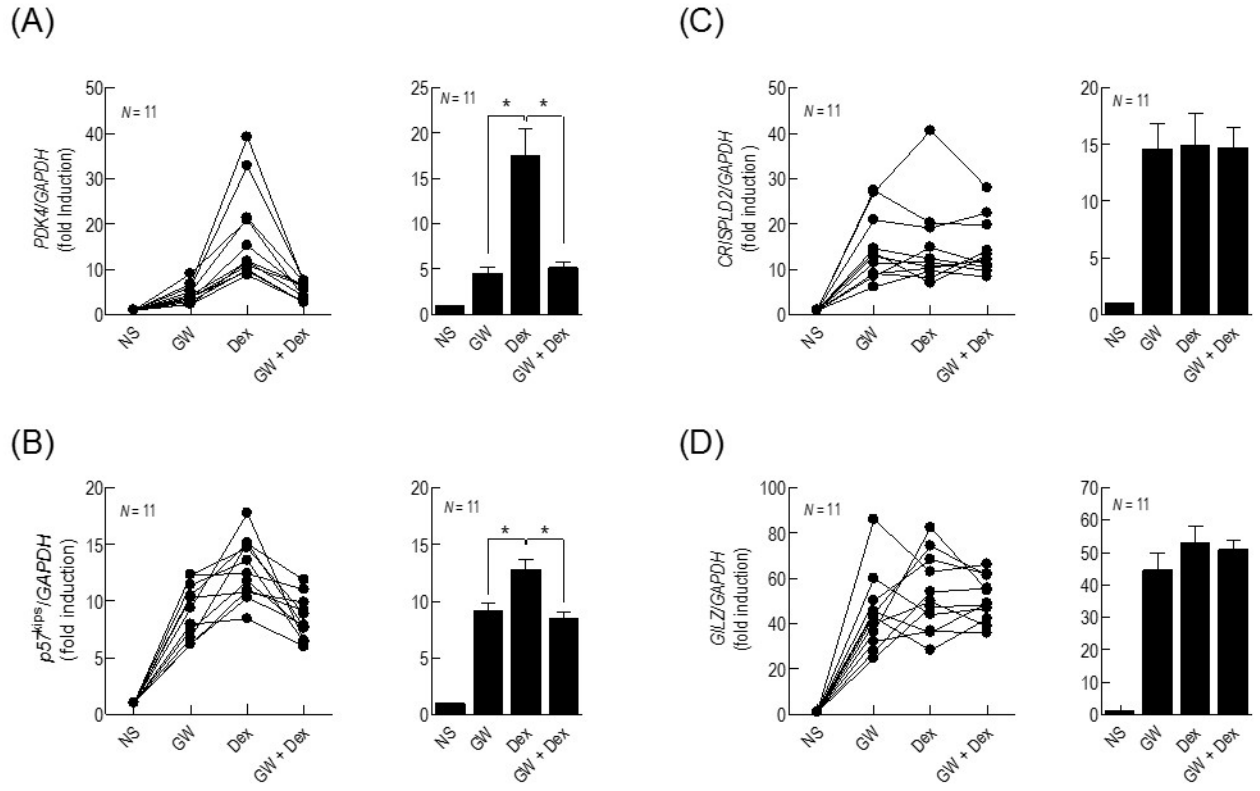


Figure 5.5 Antagonism of Dex-induced gene expression by GW.
2×GRE BEAS-2B reporter cells were treated with Dex (1μM), GW (1μM) or Dex and GW in combination. At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primers pairs specific for *PDK4* (panel A), $p57^{kip2}$ (panel B), *CRISPLD2* (panel C) and *GILZ* (panel D). Individual data sets and the mean ± s.e. mean *N* independent determinations for each gene are shown and are expressed as a ratio to *GAPDH*. **P* < 0.05, significant inhibition of Dex-induced gene induction. Data were analyzed by repeated measures one-way ANOVA/Tukey's multiple comparisons test using untransformed data.

5.3.2 *Effects of Combination Therapy on GR-Mediated Gene Expression*

5.3.2.1 Determination of transactivation potential of GCs in combination with Ind

To determine the effect of Ind on GC-induced, *bona fide* gene expression, 2×GRE BEAS-2B cells were treated with a maximally-effective concentration of GC alone and in the presence of Ind (100nM). At 6h, mRNA was harvested and processed for real time PCR analyses.

As shown in figure 5.6A, the interaction between Ind and GR agonists varied in a gene-dependent manner. The effect of GCs on *PDK4* was similar to that found on the 2×GRE reporter with Dex, GSK, DC and GW demonstrating increasing partial agonism relative to FF (Figure 5.6; Table 5.4). Ind (100nM) significantly augmented the GR-mediated expression of *PDK4* but the same relative degrees of agonism were preserved (Figure 5.6A; Table 5.4). Mif had no effect on the expression of *PDK4*, but produced significant, albeit weak, agonism in cells exposed concurrently to Ind (Figure 5.6A). In contrast, Org did not induce *PDK4* in the absence or presence of Ind (Figure 5.6A).

Similar data were obtained for *p57*^{kip2} although there were differences (Figure 5.6B; Table 5.4). In particular, Ind was able to induce p57^{kip2} weakly (~2 fold) by itself. Moreover, FF was significantly more effective at inducing *PDK4* than Dex in the absence and presence of Ind, whereas with *p57*^{kip2} there was no discrepancy (Figure 5.6B). Furthermore, Ind rendered Org a very weak partial agonist on *p57*^{kip2} (Fig. 5.6B). Figure 5.7 shows that the relationship between the ability of each GR ligand in inducing *PDK4* and *p57*^{kip2} (expressed as fold induction) in the absence and presence of Ind was linear. Moreover, figure 5.7C and D demonstrates that the relationship was saturated with high efficacy agonists when the effect of Ind was expressed as a fold enhancement.

The *CRISPLD2* expression profile was completely different from *PDK4* and *p57*^{kip2}. GSK, DC and GW which were partial agonists at inducing *PDK4* and *p57*^{kip2}, were full agonists on *CRISPLD2* being as effective as FF and Dex (Figure 5.6C). Interestingly, Ind by itself robustly induced *CRISPLD2*

expression (~10-fold) and synergistically augmented the expression of this gene induced by FF, Dex, GSK, DC and GW to similar degrees (Figure 5.6C). Ind also elevated *CRISPLD2* expression in combination with Mif (~3-fold) and Org (~2.5 fold), which by themselves were very weak agonists (~1.3 fold inductions).

GILZ was the final GC-inducible gene examined and was found to be induced equally by steroidal (FF, Dex, DC, GW) and non-steroidal (GSK) GR ligands except for Mif and Org (Figure 5.6D). Surprisingly, and in contrast to *CRISPLD2*, Ind had no effect by itself on *GILZ* expression and did not augment the expression of *GILZ* in response to any of the GR ligands tested (Figure 5.6D).

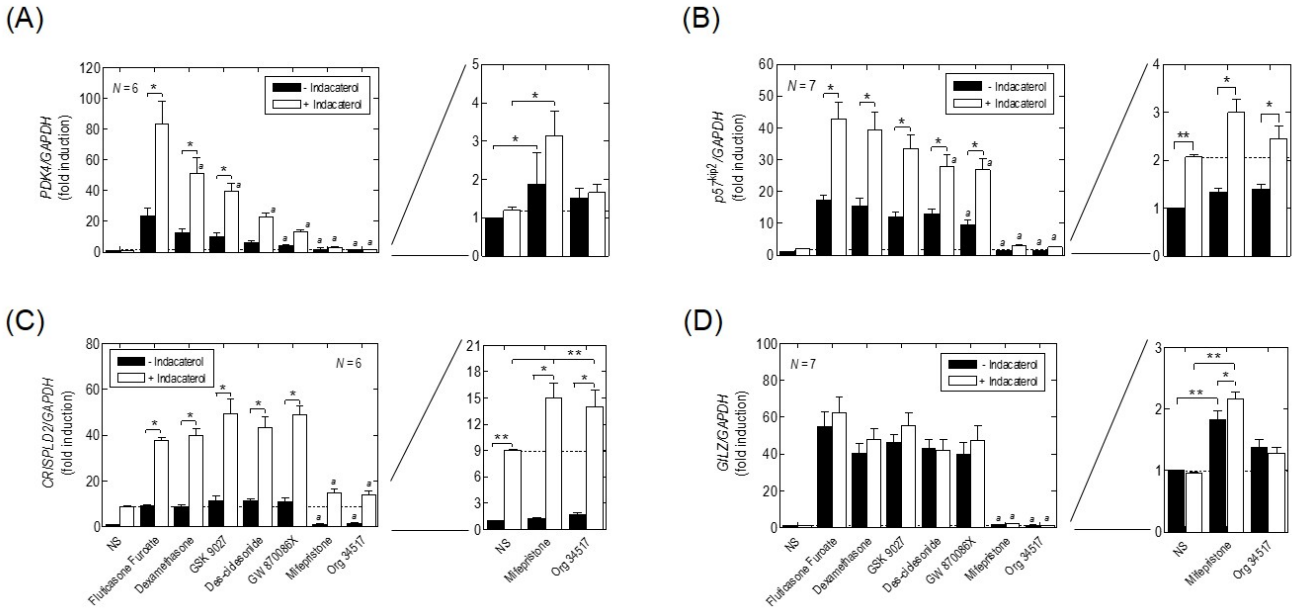


Figure 5.6 Comparative effects of Ind on gene expression produced by a panel of GR ligands. 2×GRE BEAS-2B reporter cells were treated with fluticasone furoate (100nM), dexamethasone (1μM), GSK 9027 (3μM), des-ciclesonide (100nM), GW 870086X (1μM), mifepristone (1μM) or Org 34517 (1μM) in the absence and presence of indacaterol (100nM). At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *PDK4* (panels A), *p57*^{kip2} (panels B), *CRISPLD2* (panels C) and *GILZ* (panels D). Data are the mean ± s.e. mean of *N* independent determinations and are expressed a ratio to *GAPDH*. **P* < 0.05, significant difference in gene expression relative to fluticasone furoate. ***P* < 0.05, significant induction relative to untreated cells. Data were analyzed by repeated measures, one-way ANOVA/Tukey's multiple comparisons test.

Table 5.4 Relative activities of GR agonists for the induction of *p57^{kip2}*, *GILZ*, *PDK4* and *CRISPLD2* and the effect of Ind.

Glucocorticoid	Relative Gene Expression (*FF = 1*)[a]							
	p57^{kip2}		*GILZ*		*PDK4*		*CRISPLD2*	
	-Indacaterol	*+Indacaterol*	*-Indacaterol*	*+Indacaterol*	*-Indacaterol*	*+Indacaterol*	*-Indacaterol*	*+Indacaterol*
Fluticasone Furoate (100nM)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Dexamethasone (1μM)	0.89	0.92	0.73	0.77	0.51	0.61	0.94	1.07
GSK 9027 (3μM)	0.67	0.78	0.83	0.89	0.40	0.47	1.23	1.39
Des-ciclesonide (100nM)	0.74	0.64	0.78	0.67	0.24	0.27	1.27	1.19
GW 870086X (1μM)	0.53	0.62	0.72	0.76	0.15	0.15	1.20	1.37
Mifepristone (1μM)	0.02	0.05	0.02	0.02	0.04	0.03	0.03	0.20
Org 34517 (1μM)	0.02	0.04	0.01	0.01	0.02	0.01	0.08	0.17

[a]Relative gene expression calculated from the data shown in figure 5.6. Effects produced by indacaterol alone have been subtracted. Each GR agonist was used at a concentration that maximally activated the 2×GRE reporter. For each gene, data are expressed relative to the fold induction produced by fluticasone furoate (FF), which was assigned a value of 1. Indacaterol was used at a concentration of 100nM and added concurrently with the GR ligand.

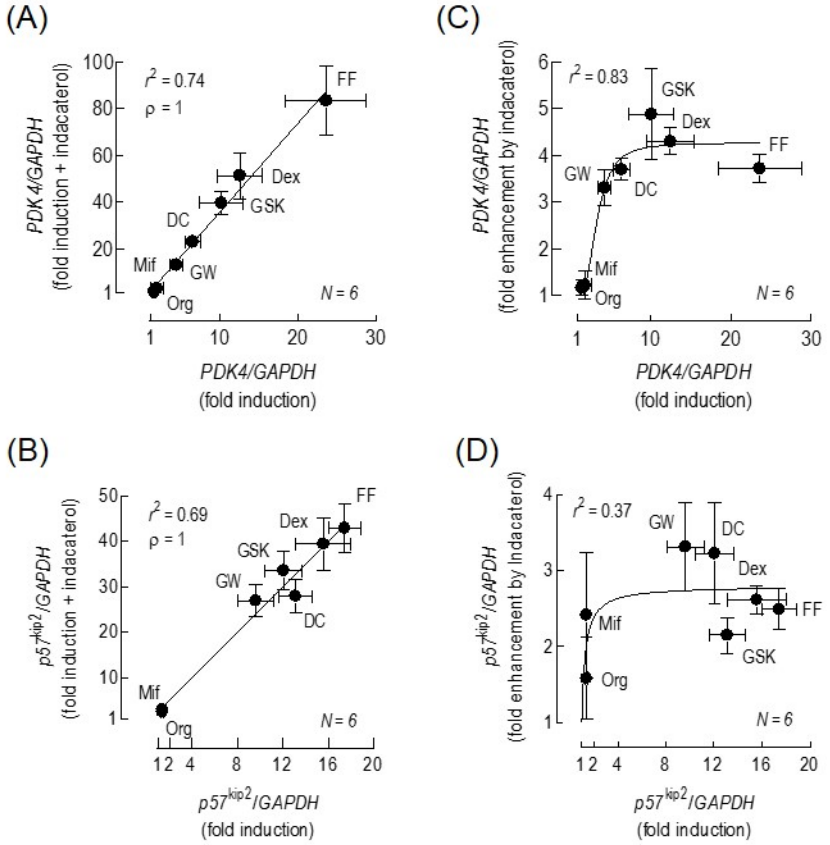


Figure 5.7 Effect of GR agonist intrinsic activity on the ability of Ind to enhance the expression of *PDK4* and *p57*^{kip2}.

Panels A and B show a linear relationship between the expression of *PDK4* and *p57*^{kip2} (data taken from figure 5.6 and shown as fold-induction) produced by seven GR agonists in the absence and presence of indacaterol (100nM). Panel C and D show that the fold enhancement by Ind of the maximal transcription produced by each GR agonist was a saturable function of agonist intrinsic activity. Key: FF, fluticasone furoate; Dex, dexamethasone; DC, des-ciclesonide; GW, GW 870086X; GSK, GSK 9027; Mif, mifepristone; Org, Org 34517.

5.3.2.2 Effect of Ind on the concentration-response relationship of FF- and GW-induced gene expression

To further investigate GR-mediated gene expression in combination with Ind, the strongest (FF) and weakest (GW) agonists were selected and studied in a quantitative manner. Both of these agonists increased the expression of *GILZ, PDK4, CRISPLD2* and *p57*^{kip2} (Figure 5.8). Similar to the reporter data (chapters 3 & 4), FF was more potent (3.3 to 6.9 fold) than GW at inducing all four genes. Moreover, the sensitivity of these genes to FF varied (highest to lowest: *GILZ* = *CRISPLD2* > *p57*^{kip2} > *PDK4* (Table 5.5).

Ind in combination with FF and GW, profoundly augmented the expressions of *CRISPLD2*, *PDK4* and *p57*^{kip2}, without changing the potency of either GR agonist. Therefore, because Ind did not modify the affinity of either FF or Dex (Figure 4.7), the K_A/EC_{50} ratios and, therefore, receptor reserves calculated in figure 5.4 and Tables 5.3 and 5.4 were preserved. Although these data mimicked the effect of Ind on the 2×GRE BEAS-2B reporter (Figure 4.1), their interpretation is more complex because *CRISPLD2* and, to a lesser extent, *p57*^{kip2}, were induced by Ind alone (Fig. 5.8). Nevertheless, the concentration-response curves showed that the combination of Ind (100nM) and a maximally-effective concentration of either FF or GW augmented the expression of *CRISPLD2*, *p57*^{kip2} and *PDK4* to a level that was significantly greater than the sum of their individual effects. Relative to FF ($\alpha = 1$), GW was a full agonist on *CRISPLD2* and *GILZ*, and a partial agonist on both *p57*^{kip2} ($\alpha = 0.74$) and *PDK4* ($\alpha = 0.2$). In contrast, using the same cDNA, Ind did not significantly affect glucocorticoid-induced *GILZ* expression under identical experimental conditions (Figure 5.8E; Table 5.5).

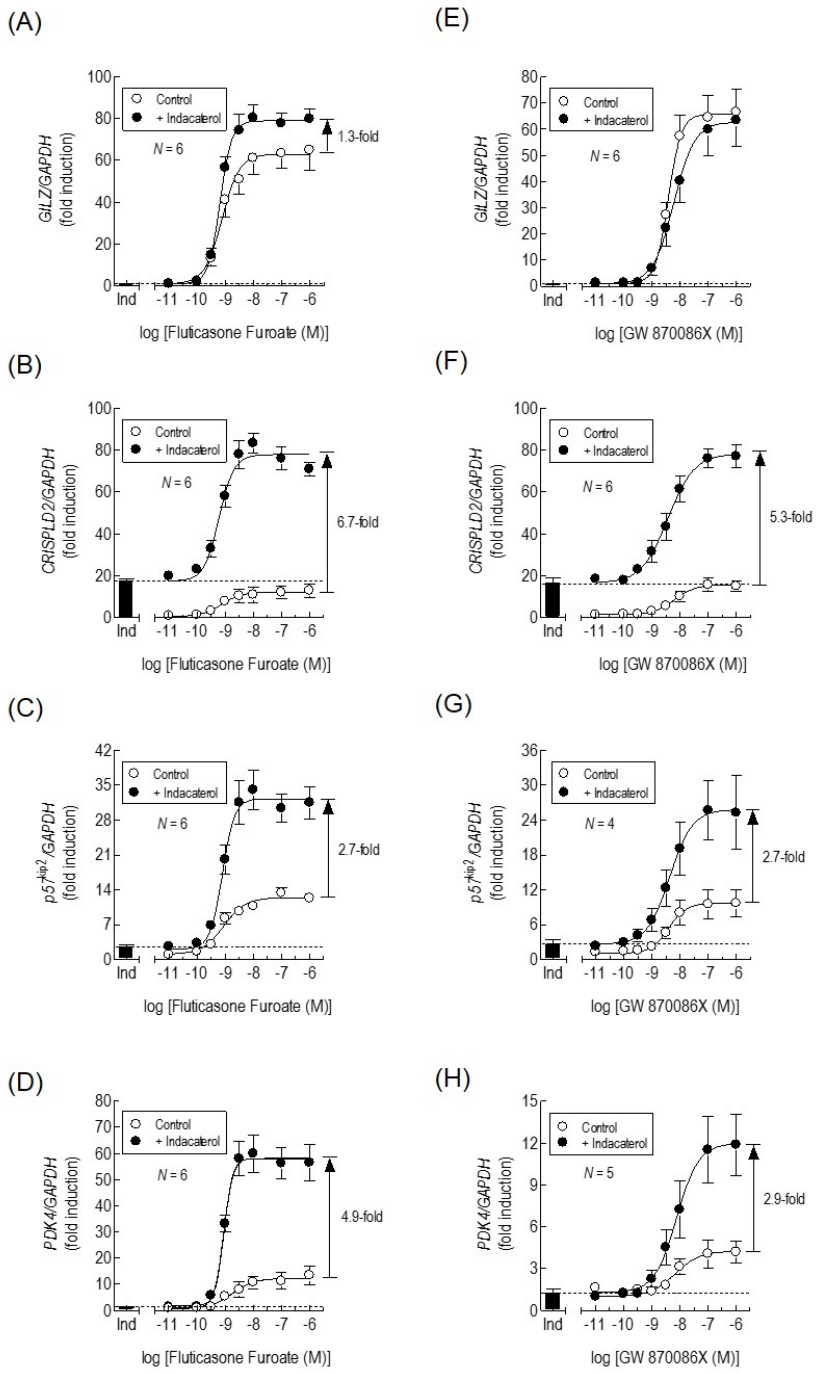


Figure 5.8 Effect of Ind on the ability of FF and GW to promote gene expression.

2×GRE BEAS-2B reporter cells were treated with fluticasone furoate (panels A to D) and GW 870086X (panels E to H) in the absence and presence of a maximally-effective concentration of indacaterol (100nM). At 6h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *GILZ*, *CRISPLD2*, *p57*^{kip2} and *PDK4*. Data are expressed as a ratio to *GAPDH* and presented as concentration-response curves. Each bar and data point represent the mean ± s.e. mean of *N* independent determinations. The horizontal dashed line in each panel defines gene expression produced by indacaterol alone. Quantification and statistical analyses of these data are presented in Table 5.5. The vertical arrows in each panel show the fold enhancement by Ind of gene expression produced by a maximally-effective concentration of GR agonist.

5.3.2.3 'Steroid-sparing' effects of Ind

Although glucocorticoids are used widely to treat inflammation, they can produce significant side-effects with chronic use. Therefore, any intervention that can reduce the dose of glucocorticoid given to a patient without compromising clinical efficacy – a so-called "steroid-sparing" effect - is highly desirable. The data in figure 5.8 show that Ind was steroid sparing in gene-dependent manner. Thus, Ind exerted steroid sparing effects on the transcription of *p57*^{kip2} and *PDK4* but not on *GILZ*. Figure 5.9 illustrates the interaction between Ind and FF in more detail, using *p57*^{kip2} induction as an example. Thus, at the pEC_{95}, FF increased gene expression by 11.5 fold while Ind (100nM), which was a weak inducer of *p57*^{kip2} (~2.5 fold), produced two main effects. First, the same degree of gene expression was achieved at a concentration of FF that was 10-fold lower (Figure 5.9, green line). Second, the effect of the same concentration of FF was enhanced by 270% from 11.5 fold to 31.5 fold (Figure 5.9, pink line).

Table 5.5 Effect of Ind on the potency and ability of FF and GW to promote gene expression.

Gene	*GILZ*		*CRISPLD2*		*p57*^{kip2}		*PDK4*	
Treatment	pEC_{50} (M)	Maximum Induction (fold)	pEC_{50} (M)	Maximum Induction (fold)	pEC_{50} (M)	Maximum Induction (fold)	pEC_{50} (M)	Maximum Induction (fold)
Indacaterol[c]	ND	0.88 ± 0.1	ND	17.5 ± 1.1[b]	ND	2.60 ± 0.3	ND	1.30 ± 0.2
Fluticasone Furoate	9.12 ± 0.08	62.6 ± 7.9	9.12 ± 0.06	12.0 ± 1.1	9.01 ± 0.09	12.5 ± 0.7	8.81 ± 0.11	11.8 ± 1.4
Fluticasone Furoate +	9.16 ± 0.07	80.0 ± 5.5	9.15 ± 0.10	78.6 ± 3.8[a]	9.20 ± 0.02	33.9 ± 1.3[a]	9.10 ± 0.07	58.0 ± 6.5[a]
Indacaterol[c]	ND	0.77 ± 0.1	ND	16.4 ± 2.7	ND	2.60 ± 0.8	ND	1.30 ± 0.3
GW 870086X	8.44 ± 0.06	65.8 ± 8.2	8.28 ± 0.07	14.9 ± 2.8	8.41 ± 0.12	9.70 ± 2.5	8.29 ± 0.23	4.20 ± 0.8
GW 870086X + Indacaterol	8.26 ± 0.12	62.7 ± 9.9	8.37 ± 0.20	79.5 ± 5.2[a]	8.37 ± 0.10	25.9 ± 6.1[a]	8.08 ± 0.17	12.2 ± 2.3[a]

Data were calculated from the concentration-response curves shown in figure 5.8 and represent the mean ± s.e. mean of six independent determinations with the exception of GW 870086X on *p57*^{kip2} and *PDK4* where $N = 4$ and 5 respectively.
[a]$P < 0.05$, significant enhancement by indacaterol of fluticasone furoate- and/or GW 870086X-induced gene expression - Student's two-tailed, unpaired *t*-test.
[b]$P < 0.05$, significant induction of gene expression by indacaterol alone relative to untreated cells - Student's two-tailed, unpaired *t*-test.
[c]A fixed concentration of indacaterol (100nM) was added concurrently with the glucocorticoid and gene expression measured at 6h. ND, not determined.

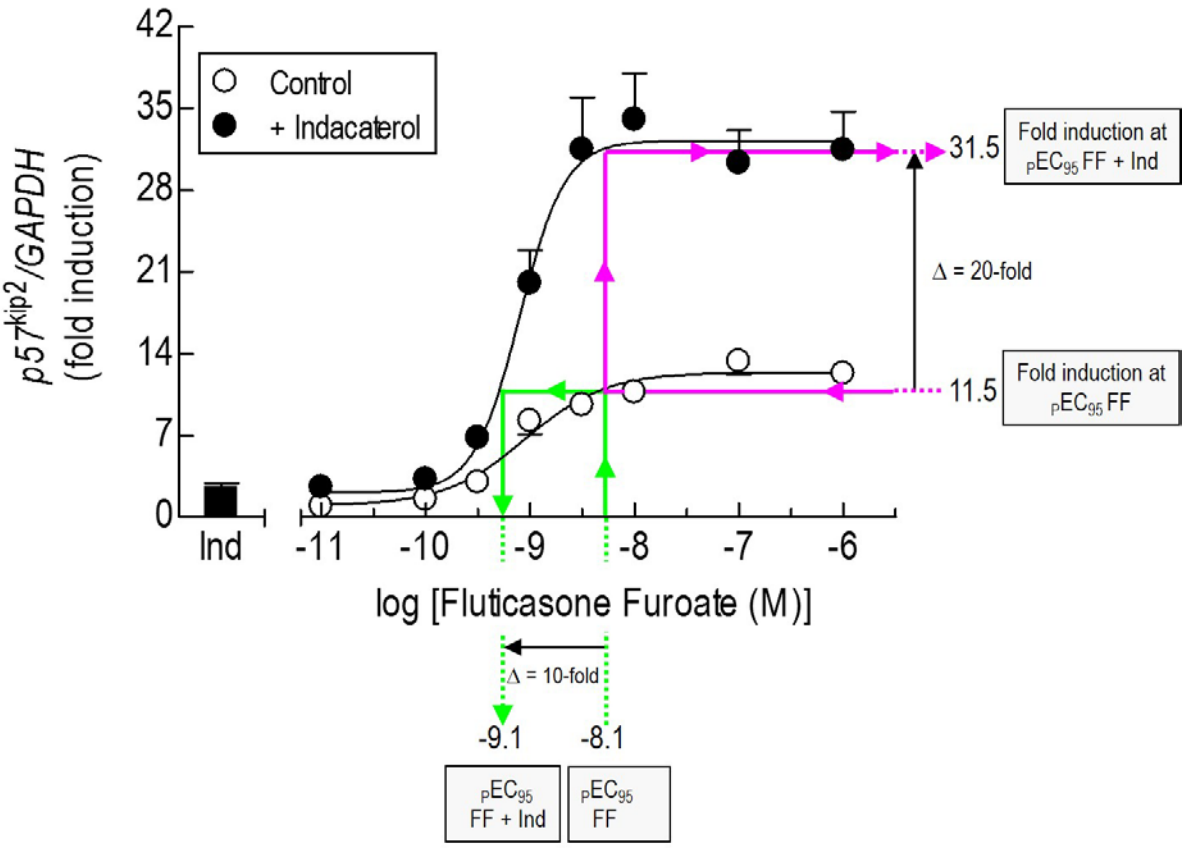


Figure 5.9 Effect of Ind on the expression of *p57*^{kip2} produced by FF.
The graph in figure 5.8C has been redrawn to illustrate that the addition of indacaterol (100nM) to FF (10pM to 1µM) enhanced the expression of a representative glucocorticoid-inducible gene, *p57*^{kip2}, in a "steroid-sparing" manner. In these cells, indacaterol had a negligible activity on the expression of *p57*^{kip2} (black bar) but markedly augmented the effect of FF from 11.5 to 31.5 fold at the pEC_{95} (pink line). Indacaterol was "steroid-sparing" in these cells. Thus, in the presence of indacaterol, an 11.5 fold increase in *p57*^{kip2} mRNA levels was produced at a concentration of FF that was 10 fold lower (green line). Key: FF, fluticasone furoate, Ind, indacaterol.

5.4 Discussion

GCs are administered as a monotherapy for mild-to-moderate asthma as anti-inflammatory compounds. They passively diffuse into pulmonary cells and bind with the dormant GR that is associated with multiple chaperone proteins. Once a GC-GR complex is formed, it translocates to the nucleus and dissociates from the chaperones, to induce multiple genes by binding to DNA on GREs. GCs elicit anti-inflammatory activity by either suppressing the expression of pro-inflammatory genes (transrepression) or by inducing the expression of anti-inflammatory genes (transactivation). Currently, transactivation and transrepression are believed to be equally important mechanisms for GC activity that operate in-parallel (King et al., 2013).

In moderate-to-severe asthma, GCs generally do not always provide control and, instead, a GC/LABA combination therapy has become firmly established as a clinically-superior treatment option. Combination therapy contains a LABA, which, in addition to causing bronchodilation, may potentiate the anti-inflammatory effects of a GC in an additive or synergistic manner (Giembycz et al., 2008; Newton et al., 2010). Currently, the exact molecular mechanism that accounts for the synergistic effects of a GC and a LABA in a combination therapy is unclear. However, our research group has reported previously that LABAs can augment GC-inducible gene expression beyond the maximum effect produced by a GC alone and have speculated that this mechanism could contribute to the therapeutic activity of these drugs (Holden et al., 2007, 2011, 2014; Kaur et al., 2008b; Moodley et al., 2013; Rider et al., 2013). The present study was designed to extend those observations by examining the effects of a LABA, Ind, on the pharmacodynamics of gene expression produced by a panel of GR ligands displaying varying degrees of activity from, so-called, “super-agonism” to antagonism.

5.4.1 *Effect of GCs on Gene Expression*

A central finding of the experiments in this chapter was that the panel of GR ligands studied did not behave equally in promoting gene expression (i.e. the intrinsic activity values of the GCs tested varied in gene-dependent manner). Notably, the experiments identified GR ligands that behaved as a super agonist (FF), full agonist (Dex), partial agonist (GW, HC, DC, GSK, Mif) and antagonist (Org), depending on the gene of interest. As discussed in more detail below, these findings have significant implications for asthma and other inflammatory disorders for which GCs are a mainstay therapy (Kaur et al., 2008a; Kelly et al., 2012; King et al., 2013; Rider et al., 2013).

The 2×GRE reporter described in chapters 3 and 4 is an artificial system and is unlikely to be a reliable model for understanding the complexities of *bona fide* gene transactivation. To determine the extent to which the results obtained with the GRE reporter can be applied to gene regulation, the ability of a panel of GR agonists to induce multiple genes was compared in the absence and presence of Ind. Genes with anti-inflammatory (*GILZ, p57*^{kip2}, *CRISPLD2*) and putative, adverse-effect (*PDK4*) potential were studied. Surprisingly, distinct patterns of gene expression were revealed depending upon the GC and gene studied. *p57*^{kip2} and *PDK4* were induced with a rank order of agonism that mirrored the induction of the 2×GRE BEAS-2B reporter. Thus, GW and DC were partial agonists in inducing *PDK4* and *p57*^{kip2}. Moreover, consistent with classical receptor theory and a recent study by Uings et al., (2013), GW antagonized gene expression induced by the stronger agonist, Dex.

Despite these similarities, marked differences were also apparent. Strikingly, on *PDK4*, Dex ($\alpha = 0.53$), the endogenous ligand, HC ($\alpha = 0.37$), DC and GW ($\alpha < 0.25$) were all partial agonists compared to FF. In contrast, Dex and FF were equi-effective at inducing *p57*^{kip2} whereas DC and

GW displayed significant agonism. Studies on *GILZ* and *CRISPLD2* revealed another profile of gene expression where FF, Dex, HC, DC and GW displayed comparable degrees of agonism. Therefore, an analysis of just four genes illustrates that a given GC expresses a unique gene induction 'fingerprint'. These finding may have therapeutic relevance because 0.1-1% (and, potentially, up to 10%) of human genome is regulated by GCs in a positive manner (Gertz et al., 2013; Reddy et al., 2009; So et al., 2007). Furthermore, GR density varies among different tissues (Pujols et al., 2002; Su et al., 2004). This difference in GR number has important pharmacodynamic implication because the efficacy of an agonist is the product of its intrinsic efficacy and receptor number. Thus, GR density will determine in a target tissue whether a given agonist will behave as a full agonist, a partial agonist or an antagonist in inducing a specific gene.

The gene-dependent difference in GC intrinsic activity suggested that the relationship between GR occupancy and transcription was not uniform. To test this idea, cells were subjected to controlled, fractional, GR inactivation with Dex-Mes and the ability of FF and GW to promote gene expression assessed. As shown in figure 5.4, gene expression induced by FF and GW exhibited different sensitivities to Dex-Mes (*PDK4* > *p57*kip2 > *CRISPLD2* > *GILZ*). These findings provide evidence that the ability of agonist-bound GR to promote transcription is, indeed, gene-dependent. Experiments published by Uings *et al*. (2013) revealed that the potency of Dex to induce a panel of genes in A549 cells varied up to 20-fold, which is consistent with this conclusion. The construction of occupancy–response plots corroborated those data and showed, in addition, that FF had a higher intrinsic efficacy (i.e. there was a greater GR 'reserve') than GW for the induction of *GILZ*, *CRISPLD2*, *PDK4* and $p57^{kip2}$. Furthermore, the intrinsic efficacy of each GC varied in a gene-dependent manner indicating that a given cell interprets equivalent degrees of GR occupancy differently. In its simplest form, this may relate to variations in the ability of

agonist-bound GR to interact with DNA in the promoter(s) of target genes. Indeed, it is believed that the 3D-conformation adopted by activated nuclear hormone receptors including GR is agonist-dependent (Allan et al., 1992; Biggadike et al., 2008, 2009a). Clearly, this could influence its interaction with obligatory co-activators and/or co-repressors and, therefore, its ability to bind DNA and promote gene transcription. Similarly, it is well established that GC-inducible promoter regions through which ligand-bound GR increases gene transcription are variable (Newton et al., 2010). Thus, in addition to epigenetic and other undefined regulatory mechanisms, it seems probable that the 3D-conformation of activated GR and the promoter context of the gene of interest will dictate the 'transcriptional competency' of a given GC.

5.4.2 *Effect of Ind on GC-Induced Gene Expression*

The experiments in chapter 4 center on the pharmacodynamics of GR-mediated reporter activation and its enhancement by a LABA. However, as stated above, this construct, which is driven by two, simple GREs, cannot accurately model the regulation of *bona fide* genes, which are controlled in a complex manner. To determine the extent to which the results obtained with the GRE reporter can be applied to gene regulation, the ability of a panel of GR agonists to induce *GILZ, p57*^{kip2}, *CRISPLD2* and *PDK4* was compared in the absence and presence of Ind. As for GC alone, the behavior of these agonists varied in a gene-dependent manner in presence of Ind. Thus, on *PDK4* and, to a lesser degree, *p57*^{kip2}, FF, Dex, GSK, DC and GW displayed increasing partial agonism and this was reproduced in the presence of Ind, which significantly enhanced the expression of these two genes. In contrast, the same GR agonists were equi-effective at inducing *CRISPLD2* and *GILZ*. However, while Ind augmented *CRISPLD2* expression induced by all

agonists to a similar degree, it failed to affect *GILZ*. This variable profile of gene expression between GR agonists prompted a more comprehensive concentration-response analysis focusing on FF and GW, which displayed marked, gene-dependent differences in agonist activity. On *PDK4,* Ind was inactive but significantly potentiated the maximal effect produced by FF and GW (a partial agonist on this gene) without affecting their potency. Thus, *PDK4* behaved identically to the reporter.

However, the interpretation of these results is not straightforward. Transcription factor binding site mining using MatInspector® software (Genomatix, Munich, Germany) identified putative GRE and CRE sites in the promoter of the *PDK4*, *CRISPLD2, GILZ* and $p57^{kip2}$ genes, which is consistent with the results of other studies (Connaughton et al., 2010; Eddleston et al., 2007; Jeong et al., 2012; Montminy et al., 1986; Zhang et al., 2005). Therefore, the mechanism of synergistic augmentation of gene expression and of the GRE reporter might not be identical. Certainly, the regulation of *CRISPLD2* and $p57^{kip2}$ is more complicated than the reporter as these genes are induced by Ind alone. This is presumably due to the phosphorylation and binding of CREB (or another cAMP-activated transcription factor) to the putative, accessible CRE sites in the promoters of the *CRISPLD2* and $p57^{kip2}$ genes. Thus, numerous processes could account for the supra-additive effect produced by Ind and GR agonists on $p57^{kip2}$ and *CRISPLD2* that are not mutually exclusive. For example, it can be explained by positive co-operativity between GR and CREB as has been reported for the somatostatin gene (Liu et al., 1994). Equally, there is the possibility of independent but concurrent gene activation through GRE-, CRE- and/or composite sites which vary in number and arrangement in different gene promoters.

The final gene studied, *GILZ,* was strongly induced by FF and GW; however it was not significantly affected by Ind alone, and Ind failed to augment the effect of any GR agonist tested

confirming that the ability of a LABA to enhance GR-mediated transcription is gene-dependent and, currently, cannot be predicted.

Collectively, the findings discussed earlier are relevant to understanding how a GC and a LABA interact at a molecular level to provide asthma control. It has been reported that LABAs enhance nuclear translocation of the GC-GR complex and that this effect may lead to superior clinical outcomes (Eickelberg et al., 1999; Haque et al., 2013; Usmani et al., 2005). However, it is difficult to accept this translocation hypothesis with the data reported herein. For instance, it cannot explain how Ind augmented GR-mediated gene expression produced by a maximum effective concentration of FF that would presumably promote the translocation of all GR to the nucleus. Moreover, ability of a LABA to enhance GR translocation would be expected to upregulate all GC inducible genes rather than selected populations. Evidence against the translocation hypothesis has also been reported in another study (Lovén et al., 2007). Thus, alternative explanations should be considered such as the phosphorylation of GR by PKA or other cellular kinases, which could make it more susceptible to positive, co-operative interactions with CREB, ATF-1, CBP/p300 and CREM, at some, but not all, gene promoters (Chen et al., 2008; Galliher-Beckley and Cidlowski, 2009; Zhou and Cidlowski, 2005). A role for transcriptional co-activators or co-repressors is also possible. In this respect, a study done with the progesterone receptor reported that 8-Br-cAMP (a cAMP analogue) augmented gene expression by promoting the dissociation of the transcriptional co-repressors: NCoR and SMRT, which made the ligand-bound receptor more transcriptionally competent (Chen et al., 2014; Wagner et al., 1998). This effect was extended to partial agonists and, interestingly, antagonists which now demonstrated very weak agonism due to increased transcriptional accessibility (Wagner et al., 1998). Such a mechanism involving transcriptional co-repressors is highly plausible specifically because PKA-targeted proteins may only regulate a

subset of genes, which would explain the observation that Ind did not significantly augment *GILZ* expression. Another possible explanation for the lack of effect of Ind on *GILZ* expression is that CRE sites were, for some reason, inaccessible to CREB or a related transcription factor. Currently, however, little is known of the molecular mechanisms that allow a LABA and a GC to promote gene expression in a synergistic manner. Clearly this is an area of research ripe for further studies as understanding mechanism could rationally guide the development of new ICS/LABA combination therapy in the future.

5.4.3 *Overall Conclusion*

The data presented in this chapter show that in the human airway epithelial cell line, BEAS-2B, Ind augmented GR-mediated transcription in a gene- and agonist-dependent manner by a mechanism that was associated with an increase in the system maximum parameter, E_m, without any changes in agonist potency, efficacy or affinity. Ind was also "steroid-sparing" in a gene-dependent manner and was able to convert GR antagonists into very weak partial agonists. The agonist-dependent differences in GR-mediated transactivation and the enhancement by Ind raise an important question: how much agonism is required in a clinically effective GC/LABA combination therapy? The answer to this question is unclear. High efficacy GR agonists such as FF may be preferred as they should induce all genes of the GC transcriptome that lead to improved clinical outcomes that are also susceptible to further up-regulation by a LABA. However, it is clear that a strong GR agonist would also promote the expression of side-effect genes (e.g. *PDK4*) that could also be further up-regulated by a LABA. Thus, alternatively, partial agonists such as GW or DC might be more desirable from a safety point of view as they should *trans*activate many therapeutically-relevant genes that can be further enhanced by LABA, but with a more favourable side-effect profile providing a 'safer' medicine.

Chapter Six: **General Conclusions**

Glucocorticoids are the mainstay therapy for asthma treatment due to their pleiotropic anti-inflammatory potential, which can affect multiple cell types responsible for asthma symptoms and exacerbations. Despite being such an important treatment option, not much work has been done to compare the transactivation potential of different, commercially available, clinically relevant GCs. It has always been assumed that GCs are therapeutically equivalent. However, there is no evidence to support this assumption and the transcriptional competence of clinically used GCs have never been compared head-to-head. Also, pharmacodynamics principles and drug-receptor concepts, which are well established for GPCRs, have never been extended and applied to the study of nuclear hormone receptors, specifically GR. This thesis addresses this gap in knowledge using a panel of clinically relevant GCs alone and in combination with the ultra LABA, Indacaterol.

Studies performed in chapter 3, compared the pharmacodynamics of a panel of six different GCs to promote gene expression in the human airway epithelial cell line BEAS-2B. Additionally, these synthetic GCs were compared to the endogenous ligand, HC, and a non-steroidal GR agonist, GSK 9027, to induce transcription of an artificial luciferase reporter construct. The results presented in this chapter demonstrated that the panel of GCs tested did not induce luciferase activity equally. Transcriptional differences were observed in terms of intrinsic activity (i.e., E_{max}) and potency (i.e. EC_{50} values). Thus, the GCs tested behaved as super agonists (relative to HC), full agonists, partial agonists and inactive ligands in inducing luciferase activity. Using CBX as the selective inhibitor of the 11β-HSD-2 enzyme, the data confirmed that the differences in agonism were not due to the metabolism of GCs by 11β-HSD-2. Additionally, concentration-response curves of FF, Dex, DC and GW, when constructed in the absence and presence of a

reversible GR antagonist Org, suggested that the GC induced luciferase activation was GR specific, which overruled the possibility of any cross-receptor effects due to other nuclear hormone receptors present in the reporter cell line that can bind to and activate conventional GREs. When affinity calculations were performed for GW using three different methods, similar results ($pK_{A(B)}$ ~ 8) were obtained, which gives confidence that the derived affinity value is accurate.

Application of the operational model of agonism in cells before and after fractional irreversible GR inactivation with Dex-Mes indicated that no receptor reserve existed for any GC tested for the induction of luciferase activity, which indicated that 100% GR occupancy is required for maximum expression of luciferase gene ($K_A = EC_{50}$). This observation is considered very rare and unusual by pharmacologists. Even for super agonists such as FF and Dex there was no receptor reserve for the induction of luciferase activity. Also, operational model fitting estimated that system's (or BEAS-2B cell's) capacity (E_m) to produce maximum luciferase induction was much higher than the E_{max} values of luciferase induction by FF and Dex, which suggests that even FF and Dex were acting as partial agonists in inducing the artificial reporter construct.

Chapter 4 described the effect of combining the ultra LABA, Ind to the panel of six different GCs and a non-steroidal GR agonist GSK 9027. It is well established and recommended by GINA that for the adequate control of moderate to severe asthma, GCs are to be combined with a LABA. However, the effect of a LABA on the ability of a given GC to enhance gene expression has never been studied. Our data demonstrated that Ind mediated enhancement of luciferase expression was GC dependent, which varied with the intrinsic activity of the GC tested. Ind produced a greater enhancement of transcription in combination with full agonists such as FF and Dex, when compared to the partial agonists GW, GSK and DC. It was noteworthy, that in combination with Ind, inactive ligands such as Mif and Org produced very weak luciferase

induction. However, Ind did not affect the affinity or potency of any GCs tested. In conclusion, the results presented in chapter 4 demonstrate that in 2×GRE reporter cells, the synergistic enhancement of GRE-dependent transcription by Ind was not due to a change in the potency, affinity or operational efficacy of a GR agonist. We propose that Ind, *via* PKA-dependent phosphorylation, allows liganded GR to more optimally bind DNA at consensus GRE sites to drive GRE-dependent transcription to a new maximum level that cannot be achieved by GC alone.

To confirm whether findings made with the GRE reporter are not just an artifact, we performed a pharmacodynamics analysis of GC-induced *bona fide* gene expression. Our previous unpublished microarray studies allowed us to select four GC-regulated candidate genes to test whether the data obtained with the luciferase construct resembled GC-induced gene expression. These were the potential, anti-inflammatory genes *GILZ, p57^{kip2}*, *CRISPLD2* and an adverse effect gene *PDK4*. The transcriptional regulation of bona fide genes is much more complicated than a luciferase reporter construct containing two repeats of simple GRE sites. Cells were treated with the panel of seven different GCs (including HC) to pharmacodynamically compare the transactivation potential of GCs as a monotherapy. In another set of experiments the same GCs were combined with Ind to understand the effect of combination on GC-mediated gene expression. GCs produced unique gene induction profiles, which were specific to the GC tested and the gene studied. The pattern of induction of *PDK4* and *p57*^{kip2} was similar to luciferase expression where the rank order of agonism exhibited by different GCs was FF > Dex > DC > GW> Mif > Org. In contrast, *GILZ* and *CRISPLD2* were equi-effectively induced by FF, Dex, GW, DC and GSK.

In combination with Ind, the GC-induced gene induction profile remained same but the ability of Ind to enhance gene expression depended on the GC used. The combination of Ind with GC also added another level of complexity towards gene regulation because *p57^{kip2}* and *CRISPLD2*

were also induced by Ind itself, unlike GILZ and *PDK4*, which are strictly GC regulated. Surprisingly, Ind did not produce any enhancement of GC-induced expression of *GILZ* with any of the GCs tested, which could be due to differences in the promoter structure of *GILZ* versus other genes. In case of *GILZ* there is a possibility that a CRE site, which has been identified in its promoter, is inaccessible for Ind induced, PKA-dependent, CREB binding. Thus, this could inhibit CREB from interacting with other co-activators such as ATF-1 and CBP/p300, which play important role in enhancing transcriptional competence by interacting with the basal transcriptional machinery as well as gene enhancers. Therefore, the mechanism of synergistic augmentation of gene expression and of the GRE reporter might not be identical.

Following fractional, irreversible GR inactivation by Dex-Mes, FF- and GW-induced transcription varied with the gene studied. *GILZ* induction by FF and GW was least affected by GR inactivation, while *PDK4* induction was the most sensitive to this intervention. Thus, the data suggests that *PDK4* expression requires more GR occupancy and has lower GR reserve, and hence is more sensitive towards Dex-Mes mediated irreversible GR inactivation. It is noteworthy, that GR occupancy calculations confirmed that GW occupied all the available GR to maximally induce *PDK4* and, even then, the maximum expression was significantly lower than the maximum induction of *PDK4* by FF. Thus, similar to the luciferase data, GW had no GR reserve for inducing *PDK4*. Also, in comparison to other genes, *PDK4* expression by FF and GW required the highest GR occupancy. Taken together, GR occupancy as well as reserve are gene and GC dependent.

Therefore, in conclusion, an analysis of just four genes illustrates that a given GC expresses a unique gene induction ‘fingerprint’. This study also suggests that clinically used GCs might not be therapeutically equivalent. Performing the gene induction ‘fingerprint’ studies could help in making informed decisions about a new therapy’s potential in inducing beneficial anti-

inflammatory genes *versus* adverse effect genes and this, in turn, could rationally guide the development of new ICS/LABA combination therapy in the future.

Chapter Seven: **Limitations and Future Studies**

The experiments and data discussed in this thesis warrants further investigation in some areas as follows:

1. One of the limitations of the present work is that it was performed using 2×GRE BEAS-2B reporter cells and not primary airway epithelial cells or other airway cell types relevant to asthma pathogenesis and treatment. Confirmation that the results presented in this thesis are broadly representative of other cell types is necessary. In this respect, our real-time PCR data were consistent with previously-published material from our research group with primary ASM cells, A549 cells and human primary epithelial cells which gives confidence that many of our conclusions are valid (Kaur et al., 2008a; Moodley et al., 2013; Rider et al., 2015). In addition, these studies were conducted in isolated cells as a monolayer immersed in culture medium, which does not mimic human physiology. Experiments could be performed in a more physiologically-relevant system, such as air-liquid interface cultures. However, a limitation of this approach is the cellular heterogeneity of the system, which makes meaningful pharmacodynamics analyses difficult.

2. Arguably the most important set of experiments is to determine if the gene expression changes found here in BEAS-2B cells are replicated in the airways of human subjects *in vivo* in response to ICS and LABA alone and in combination. Previous studies have reported gene expression changes in bronchial biopsy samples and mucosal brushings (from which epithelial cells can be harvested) obtained from normal and asthmatic subjects given inhaled budesonide (Essilfie-Quaye et al., 2011; Kelly et al., 2012; Leigh et al., 2014). To extend those studies by performing a head-

to-head comparison of FF and GW (high and relatively low efficacy GR agonists respectively) using a cross-over design protocol could be instructive.

3. Another limitation of this study was that one LABA, Ind, was studied on the expression of only four candidate genes. Clearly, further experiments could be performed with other LABAs, in combination with a larger panel of GR ligands for pharmacodynamic analysis. In addition, microarray gene profiling could be performed to "look" for differences in gene expression signatures between partial agonists (e.g. GW, DC and Mif) and non-steroidal agonists (e.g. GSK) in absence and presence of a LABAs. Such studies may enable the development of customized asthma drugs based on gene expression fingerprints, which could selectively induce desirable genes at the expense of those that produce side-effects.

4. All the experiments reported in thesis used mRNA transcripts as an index of transcription. Extending those data to include protein expression by western blotting or ELISA-based method is essential to confirm that our data could be functionally relevant.

5. The molecular mechanisms responsible for the synergy between a GC and a LABA remains unknown and requires considerable further work. Two approaches are suggested here: one possibility could be to identify the REs (some of which could be composite or allied) targeted by GR and LABA-regulated TFs (the major ones are CREB, CREM and ATF-1) on the gene (*PDK4, CRISPLD2, GILZ and* $p57^{kip2}$) promoters by performing chromatin immunoprecipitation (ChIP), which can be further analysed by real-time PCR or ChIP Seq. These sites can be cloned in to a luciferase reporter plasmid and transfected into BEAS-2B or any other airway cells, to study the

effects of GC alone, LABA alone and GC/LABA combination. Additionally, these sites can be mutated to further understand the involvement of these REs in GC-regulated gene expression or GC/LABA synergism.

A second approach is to identify the downstream targets of PKA that could regulate synergy. This could be achieved by using phospho-specific flow cytometry. This method measures multiple kinase signalling pathways and the phosphorylation state of intracellular proteins at the single cell level as well as in heterogeneous cell populations (Hawley and Hawley, 2011; Perez, Krutzik and Nolan, 2004). Thus, many phosphorylation events can be analysed simultaneously per cell (Hawley and Hawley, 2011; Perez, Krutzik and Nolan, 2004). After identification of the substrates phosphorylated by LABA or a GC or both, they could be silenced by siRNA to further assess their involvement in GC and LABA regulated synergistic gene expressions. Further, phospho-flow is ideal for analysing blood samples, which would allow comparisons to be made from asthmatic patients and normal healthy individuals.

6. Changes in GR phosphorylation has also been attributed to GC-dependent gene expression or suppression (Chen et al., 2008). GR phosphorylation is reported to be regulated by serine/threonine protein phosphatase 5 (PPP5) which is typically associated with GR in the cytoplasm (Hinds and Sánchez, 2008; Zhang et al., 2009). Silencing *PPP5* after treating cells with GC or LABA or combination could help understand the significance of this phosphatase in GR activation, translocation and gene expression. These experiments could also be extended to other immunophilins as well as chaperone proteins associated with GR such as FKBP51, FKBP52, hsp70, and hsp90 (Vandevyver et al., 2012).

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APPENDIX A: GLOSSARY OF PHARMACODYNAMIC TERMS

α. See intrinsic activity

Agonist. A molecule that produce a response by binding to and activating a receptor.

Affinity. (*aka* the reciprocal of the equilibrium dissociation constant, K_A): The tenacity to which a ligand binds to a receptor. Arithmetically, it is the molar concentration of ligand that binds to 50% of the receptor population at equilibrium. Agonist and antagonist affinity are typically denoted $1/K_A$ and $1/K_B$ respectively and are often expressed in $\log_{10}$ format (i.e. pK_A, pK_B).

Antagonist. A molecule that interferes with the ability of an agonist to bind to and activate a receptor.

Efficacy. See intrinsic efficacy

***K*A.** See affinity

***K*B.** See affinity

***K*E**. The concentration of agonist-receptor complexes ([AR]) producing 50% of the maximal effect.

Intrinsic Activity (α). A scale of agonism in which the maximal response of an agonist at a given receptor is expressed as a fraction of the response produced by a reference "full" agonist at the same receptor). Values of α less than one indicate partial agonism.

Intrinsic Efficacy. A term used to quantify the ability of an agonist to produce response. It is ***solely*** an agonist-dependent parameter. However, response is also dependent upon receptor number and efficiency of receptor-effector coupling, which are furnished by a tissue. Thus, the **efficacy** of an agonist to produce response in a given tissue is the product of intrinsic efficacy and receptor number.

Mass Action. A law that states that the rate of a simple reaction is proportional to the concentration (mass) of initial reactants. It was originally used to explain chemical reactions but has been applied to pharmacology because in many cases an agonist interacts with a receptor in a simple, reversible manner. Thus, [A] + [R] ↔ [AR], where [A], [R] and [AR] are the concentrations of agonist, receptor and agonist-receptor complexes respectively.

Operational Model. A mathematical description of how an agonist produces a physiological response. The model defines both the affinity (K_A) and efficacy (τ) of an agonist. The parameter, τ, is a ratio of $[R_t]/K_E$, where $[R_t]$ is the total receptor concentration in a target tissue, and K_E is the concentration of agonist-occupied receptor [AR] complexes necessary to produce half maximal response. Logically, a physiological effect produced by an AR complex depends on the intrinsic efficacy of the agonist ***and*** the efficiency of receptor-effector coupling. As such, K_E is both an agonist and tissue-dependent parameter. Accordingly, the same magnitude of response can be

produced in a system where [R_t] and K_E are low (i.e. AR is very productive) or where [R_t] and K_E are high (i.e. AR is less productive).

Partial Agonist. An agonist that binds to a receptor and produces response that is less than that of a full agonist (and the system maximum).

Potency. The concentration a compound that produces a defined biological response. Typically, this is expressed as an EC_{50} value, which refers to the concentration required to produce 50% of the maximal effect.

Receptor Reserve (*aka* spare receptors). A term used to describe a system where an agonist produces the maximum tissue response by activating only a fraction of the total receptor population. Clearly, in such systems, all receptors will be bound and activated by the agonist, but the stimulus produced by only a fraction of the receptor population is sufficient to produce response equal to the system maximum. The remaining receptors will produce stimulus that is surplus to requirement and constitute a "reserve" or are said to be "spare". It follows that in such systems, receptor inactivation has no effect on the maximum agonist-induced response unless the reserve is depleted.

***R*$_t$.** The total number of receptors on or within a given cell type.

Schild Analysis. A procedure that measures the affinity of an antagonist (K_B) for a given receptor. Typically, this constant is calculated from agonist concentration-response curves constructed in the absence and presence of increasing concentrations of an antagonist. The K_B of an antagonist for a given receptor will be the same for a range of agonists that interact with the same receptor as the antagonist.

Super Agonist. An agonist that has higher intrinsic efficacy at a given receptor than the naturally-occurring, endogenous activating ligand and can produce a greater maximum response especially when receptor number is limiting.

System Maximum (E_m). A term that refers to the maximum pharmacological response that can be produced in a given system. Typically, response equivalent to the E_m is produced by full agonists.

Tau (τ). A dimensionless parameter, also known as the "transducer ratio", which defines the efficacy of an agonist at a receptor in a given tissue. It is a measure of the efficiency of transduction of agonist-occupied receptors into pharmacological effect and is the ratio of [R_t]/K_E (see Operational Model of Agonism).

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