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REVIEW ARTICLE

Understanding how long-acting β_2-adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids in asthma – an update

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In moderate-to-severe asthma, adding an inhaled long-acting β_2-adenoceptor agonist (LABA) to an inhaled corticosteroid (ICS) provides better disease control than simply increasing the dose of ICS. Acting on the glucocorticoid receptor (GR, gene NR3C1), ICSs promote anti-inflammatory/anti-asthma gene expression. *In vitro*, LABAs synergistically enhance the maximal expression of many glucocorticoid-induced genes. Other genes, including dual-specificity phosphatase 1(DUSP1) in human airways smooth muscle (ASM) and epithelial cells, are up-regulated additively by both drug classes. Synergy may also occur for LABA-induced genes, as illustrated by the bronchoprotective gene, regulator of G-protein signalling 2 (RGS2) in ASM. Such effects cannot be produced by either drug alone and may explain the therapeutic efficacy of ICS/LABA combination therapies. While the molecular basis of synergy remains unclear, mechanistic interpretations must accommodate gene-specific regulation. We explore the concept that each glucocorticoid-induced gene is an independent signal transducer optimally activated by a specific, ligand-directed, GR conformation. In addition to explaining partial agonism, this realization provides opportunities to identify novel GR ligands that exhibit gene expression bias. Translating this into improved therapeutic ratios requires consideration of GR density in target tissues and further understanding of gene function. Similarly, the ability of a LABA to interact with a glucocorticoid may be suboptimal due to low β_2-adrenoceptor density or biased β_2-adrenoceptor signalling. Strategies to overcome these limitations include adding-on a phosphodiesterase inhibitor and using agonists of other Gs-coupled receptors. In all cases, the rational design of ICS/LABA, and derivative, combination therapies requires functional knowledge of induced (and repressed) genes for therapeutic benefit to be maximized.

Abbreviations

AHR, airways hyperreactivity; AP-1, activator protein 1; ASM, airways smooth muscle; C/EBP, CCAAT-enhancer binding protein; COPD, chronic obstructive pulmonary disease; CRE, cAMP response element; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HBE, human bronchial epithelial; HRV, human rhinovirus; ICS, inhaled corticosteroid; LABA, long-acting β_2-adrenoceptor agonist; PEPCK, phosphoenolpyruvate carboxykinase; PKA, protein kinase A

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Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
Adenosine A_{2B} receptor	Phosphodiesterase 4 (PDE4)
β_2-adrenoreceptor	
Prostacyclin IP receptor	
Nuclear hormone receptors[b]	
Glucocorticoid receptor (GR) / NR3C1	

LIGANDS	
Beclomethasone dipropionate	Forskolin
Budesonide	Mifepristone (RU486)
Ciclesonide	Roflumilast
Dexamethasone	Salmeterol
Fluticasone furoate	
Fluticasone propionate	
Formoterol	

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c).

Introduction

In 1994, a clinical study was published that fundamentally transformed the treatment algorithm for the management of asthma (Greening *et al.*, 1994). The salient finding was that asthmatic subjects who were symptomatic despite maintenance therapy with a standard dose of the inhaled corticosteroid (ICS), beclomethasone dipropionate, were controlled by the addition of the long-acting β_2-adrenoceptor agonist (LABA), salmeterol, but not by increasing the dose of ICS (Greening *et al.*, 1994). The clinical superiority of an ICS and LABA in combination relative to ICS alone, irrespective of dose, was subsequently and independently corroborated using several outcome measures including, symptom score, lung function, use of rescue medication and exacerbation frequency (Pauwels *et al.*, 1997; Shrewsbury *et al.*, 2000; O'Byrne *et al.*, 2001; Frois *et al.*, 2009; Ducharme *et al.*, 2010a; Ducharme *et al.*, 2010b; Sears, 2011). Today, ICS/LABA combination therapies are entrenched in all national and international asthma treatment guidelines and are a recommended option for patients whose symptoms remain uncontrolled by ICS monotherapy (www.ginasthma.org). Furthermore, patients with chronic obstructive pulmonary disease (COPD), who present with a high level of inflammation and suffer frequent exacerbations, may also respond more favourably to an ICS/LABA combination therapy relative to a LABA alone (Celli *et al.*, 2008; Nannini *et al.*, 2013; Kew *et al.*, 2014).

Despite these clinical data, a mechanistic basis for the superiority of ICS/LABA combination therapies remains unclear. In 2008, we reviewed ways that LABAs and ICSs may interact to deliver superior clinical outcomes in asthma and COPD (Giembycz *et al.*, 2008). In particular, we discussed evidence that LABAs, in addition to directly improving airway calibre, may enhance the anti-inflammatory activity of ICSs (Giembycz *et al.*, 2008). Furthermore, as glucocorticoids act via the glucocorticoid receptor (GR; gene NR3C1) (Newton, 2000; Clark and Belvisi, 2012), a transcription factor, we argued that LABAs enhance GR-dependent gene expression above the maximum level achievable by ICS alone (Giembycz *et al.*, 2008). However, like other cAMP-elevating agents (Mayr and Montminy, 2001; Sands and Palmer, 2008), LABAs also have a major impact on gene expression, and this necessitates refinement of our original hypothesis. While the LABA-induced transcriptome is not, *per se*, anti-inflammatory, the ability of ICSs to enhance the expression of LABA-inducible genes may be clinically relevant. Thus, the therapeutic activity of ICS/LABA combination therapies will reflect the ability of each component to promote gene expression alone, as well as to enhance, or modify, gene expression produced by its respective companion.

ICS/LABA interactions

Non-interacting, independent effects of glucocorticoids and LABAs

Multiple mechanisms could account for the clinical efficacy of ICS/LABA combination therapies. Perhaps the most straightforward is that LABAs and ICSs each elicit a collection of independent, non-interacting responses that combine additively to provide clinical benefit (Figure 1A). At a basic level, this is illustrated by the ability of LABAs and ICSs to promote bronchoprotection and suppress inflammation respectively (Johnson, 2004). However, as LABAs lack anti-inflammatory activity in asthma, additivity is unlikely to account for their superiority when they are combined with an ICS.

Additive effects of glucocorticoids and LABAs

Independent, additive effects may occur for any response that is regulated by both a glucocorticoid and a LABA (Figure 1B). Induction of the dual-specificity phosphatase, DUSP1, which inactivates MAPKs (Abraham and Clark, 2006), provides an example (Figure 1B). DUSP1 mRNA is rapidly and transiently induced by LABAs and other cAMP-elevating agents (Burgun *et al.*, 2000; Kaur *et al.*, 2008; Korhonen *et al.*, 2013). Similarly, GR binding sites in the DUSP1 promoter allow glucocorticoids to induce, or transactivate, DUSP1 transcription (Johansson-Haque *et al.*, 2008; Shipp *et al.*, 2010; Tchen *et al.*, 2010). This mediates inhibition of MAPK signalling and inflammatory gene expression (Kassel *et al.*, 2001; Lasa *et al.*, 2002; Abraham *et al.*, 2006; Issa *et al.*, 2007; Shah *et al.*, 2014). However, LABAs and glucocorticoids independently promote DUSP1 mRNA expression, and their effects combine in an essentially additive manner (Kaur *et al.*, 2008; Manetsch *et al.*, 2012; Manetsch

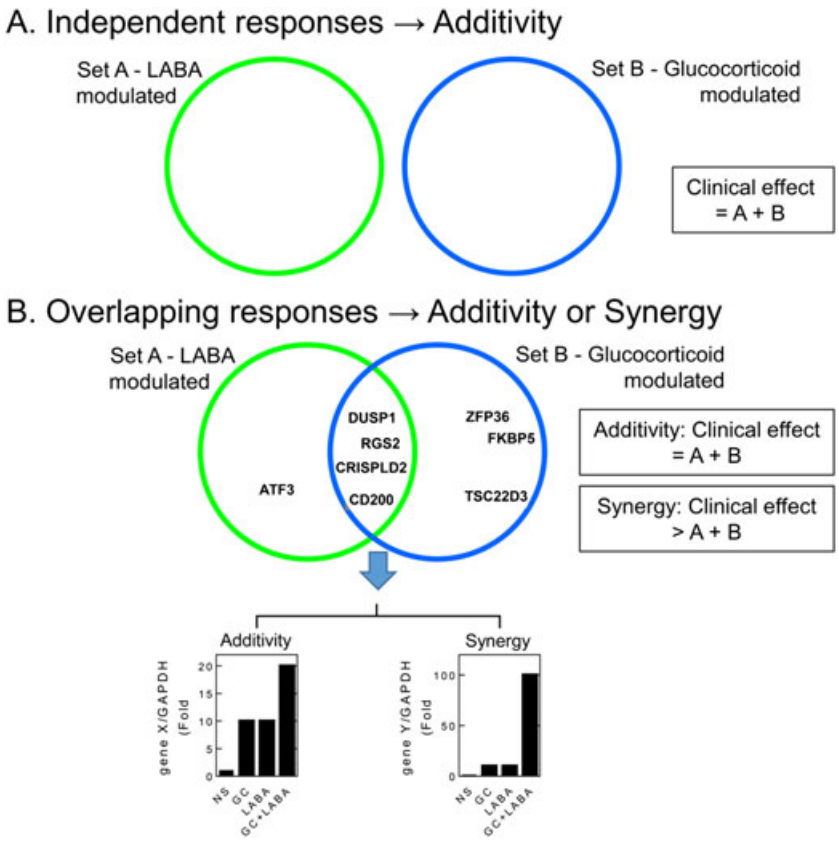

Figure 1

Additive and/or synergistic effector responses induced by LABAs and glucocorticoids (GCs). A. LABAs and GCs may each induce a set of responses (sets A and B respectively) that do not interact. The net effect of a LABA/GC combination treatment would be the sum of the responses produced by each drug alone (A + B). B. While LABAs and GCs each induce a set of responses (sets A and B respectively), a number of these responses may be modulated by both LABAs and GCs. In these situations, where A ∩ B, there may be no interaction between each response, and the net effect is one of simple additivity. This is shown for a hypothetical gene (gene X) that was induced by LABA or GC. In this situation, the overall effect is the sum of the responses produced by each drug (A + B). Alternatively, responses in the overlap region, A ∩ B, may show interaction between the two drugs. This is shown for a hypothetical gene, gene Y, which is modestly induced by maximally effective concentrations of a LABA and by a GC, but together, there is a large induction of gene expression. As this is greater than the sum of the components, this can be described as synergy. In this situation, the overall effect of each drug is greater than the simple sum of A + B.

et al., 2013; BinMahfouz *et al.*, 2015). Similar effects may also be observed in respect of the repression of inflammatory gene expression (see below). Thus, responses induced by LABAs and glucocorticoids can overlap (Figure 1B), but may result in simple additivity (Figure 1B and 2A).

However, glucocorticoids and LABAs activate their cognate receptors to produce adverse effects in off- and on-target tissues. These could be exaggerated by combination therapies and is, again, illustrated by DUSP1. Thus, osteoblast proliferation and the restoration, or maintenance, of bone mass involve ERK, and the up-regulation of DUSP1, by inactivating ERK, may promote osteopaenia (Horsch *et al.*, 2007). Such effects may be relevant in patients taking high dose combination therapy, where systemic exposure is likely.

Opposing effects of glucocorticoids and LABAs

A further example of interaction is where responses induced by one component of an ICS/LABA combination therapy are diminished by the other. In cell-based assays, using airway smooth muscle (ASM) and either transformed or primary bronchial epithelial cells, LABAs induce, and/or potentiate, the expression of certain inflammatory genes, including IL6, CXCL5 and CXCL8, and these effects are suppressed by glucocorticoids (Ammit *et al.*, 2000; Korn *et al.*, 2001; Ammit *et al.*, 2002; Faisy *et al.*, 2002; Edwards *et al.*, 2007; Holden *et al.*, 2010). Likewise, expression of TRPV1 receptors and several GPCRs that mediate inflammatory responses or bronchoconstriction are up-regulated by β_2-adrenoceptor agonists and down-regulated by glucocorticoids (Katsunuma *et al.*, 1999; Mak *et al.*, 2000; Faisy *et al.*, 2004; Liu *et al.*, 2015). Thus, in airway diseases, many undesirable effects of LABAs are attenuated by glucocorticoids. Conversely, it is also possible that desirable effects elicited by one component of an ICS/LABA therapy could be attenuated with the combination.

Synergistic induction of gene expression by glucocorticoids and LABAs

Clinical data in asthma suggest that ICSs and LABAs can interact in a synergistic manner, such that outcome measures, including improved lung function or reduced exacerbation

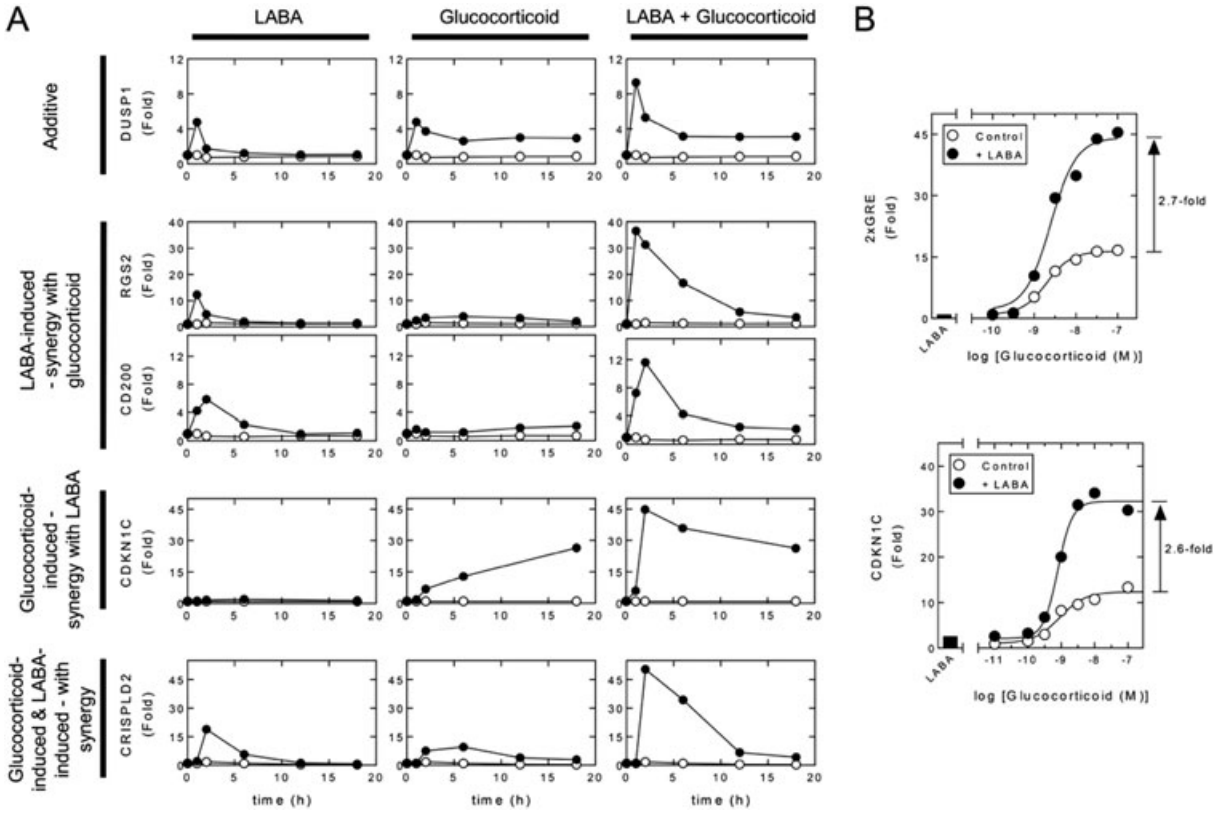

Figure 2

Patterns of mRNA expression induced by LABAs and GCs. A. Overview real-time PCR data showing the effect of maximally effective concentrations of LABA (10 nM, formoterol) and GC (1 μM, dexamethasone) on the mRNA expression of various genes in human bronchial epithelial, BEAS-2B, cells. Experimental data from BinMahfouz *et al.* (2015) represent expression of the indicated gene/GAPDH expressed as fold of untreated (at 1 h) plotted as a mean. B. A maximally effective concentration of LABA (indacaterol, 100 nM) enhanced the activation of a simple 2 × GRE reporter induced by GC (fluticasone furoate) (upper panel). Luciferase data expressed as fold of untreated are plotted as means. Data are taken from Joshi *et al.* (2015b). Alternatively (lower panel), BEAS-2B cells were harvested for RNA and gene expression of CDKN1C and GAPDH analysed by real-time PCR. Data for CDKN1C/GAPDH expressed as fold of untreated are plotted as means.

frequency produced by the two drugs in combination, are superior to the sum of their individual effects. Indeed, LABAs enhance ICS efficacy and lead to asthma control, whereas increasing the dose of ICS may not (Frois *et al.*, 2009; Ducharme *et al.*, 2010a; Ducharme *et al.*, 2010b). While rationalization of these data requires that ICSs and LABAs interact in a way that cannot be reproduced by merely increasing the dose of ICS (Giembycz *et al.*, 2008; Newton *et al.*, 2010b), descriptions of such interactions have been inconsistent. Nevertheless, an increasing number of responses are expressed in a synergistic manner when a glucocorticoid is combined with a LABA. One example is induction of the regulator of G-protein signalling, RGS2 (Holden *et al.*, 2011; Holden *et al.*, 2014) (Figure 2A). Thus, in ASM and bronchial epithelial cells, RGS2 mRNA and protein are elevated by LABAs, and other cAMP-elevating agents, as well as by glucocorticoids (Pepperl *et al.*, 1998; Tsingotjidou *et al.*, 2002; Wang *et al.*, 2004; Chivers *et al.*, 2006; Holden *et al.*, 2011; Holden *et al.*, 2014). In combination, the response to maximally effective concentrations of LABA and glucocorticoid produces profound synergy at the level of both RGS2 mRNA and protein (Figures 2A and 3A–B) (Holden *et al.*, 2011; Greer *et al.*, 2013; Moodley *et al.*, 2013; Holden *et al.*, 2014; BinMahfouz *et al.*, 2015; Joshi *et al.*, 2015b). Thus, the level of RGS2 expression achieved by maximally effective concentrations of LABA plus glucocorticoid is considerably greater than the simple sum of the responses produced by each component alone (Figures 2A and 3A–B). Functionally, RGS2 is a GTPase-activating protein that terminates signalling from GPCRs that signal via Gq (Heximer, 2004; Kimple *et al.*, 2009) (Figure 3C). Such receptors include those mediating ASM contraction and pulmonary leukocyte recruitment. Thus, by reducing Gq-dependent signalling, RGS2 expression should be beneficial in asthma and COPD. Indeed, up-regulation of RGS2 in mice is bronchoprotective, whereas *RGS2*-deficiency promotes airways hyperreactivity (AHR), mucin expression and airways remodelling (Holden *et al.*, 2011; Xie *et al.*, 2012; Liu *et al.*, 2013; Jiang *et al.*, 2014). Thus, the synergy that is achieved by LABAs and glucocorticoids at inducing RGS2 expression not only provides some explanation for the therapeutic activity of ICS/LABA combination therapies, but the relatively modest response to glucocorticoid alone clearly illustrates why this effect may not be achieved by simply increasing the dose of ICS (Figure 3).

Other genes with anti-inflammatory potential, including the surface marker, CD200 and CRISPLD2, are also synergistically up-regulated by glucocorticoid/LABA combinations

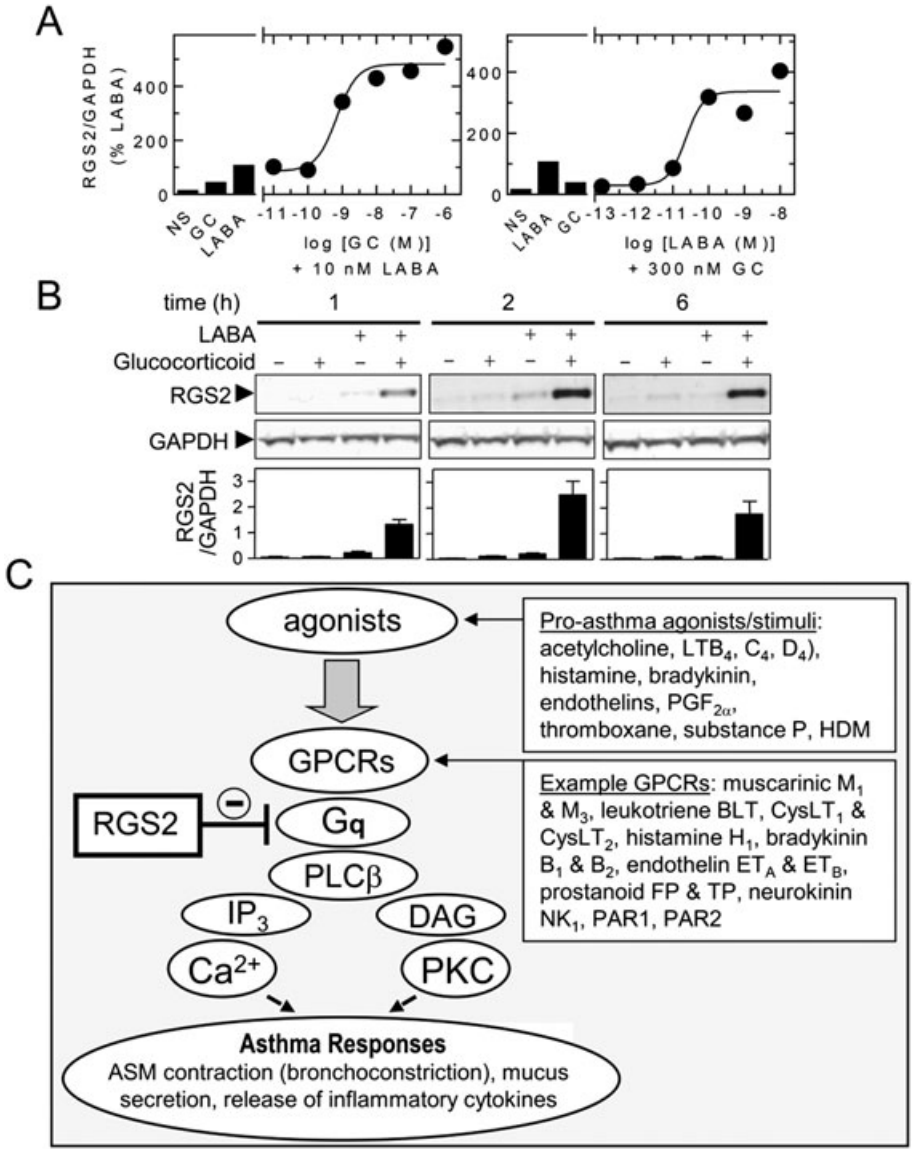

Figure 3

RGS2 expression is synergistically induced by LABA and GC. A. BEAS-2B cells were treated with: in the left panel, GC alone (budeosnide, 1 μM), LABA alone (formoterol, 10 nM) or the indicated concentrations of GC (budesonide) in the presence of LABA (formoterol, 10 nM); in the right panel, LABA alone (formoterol, 10 nM), GC alone (budeosnide, 300 nM) or the indicated concentrations of LABA (formoterol) in the presence of GC (budesonide, 300 nM). After 2 h, the cells were harvested for RNA and real-time PCR analysis of RGS2 and GAPDH mRNA. Data, normalized as RGS2/GAPDH, are expressed as a percentage of the LABA (formoterol, 10 nM) alone and are plotted as means. Data are derived from experiments in Holden *et al.* (2014). B Human bronchial epithelial, BEAS-2B, cells were treated with LABA (salmeterol, 100 nM) and GC (dexamethasone, 1 μM) for the times indicated prior to western blot analysis of RGS2 and GAPDH. Data are expressed as RGS2/GAPDH and plotted as means ± SEM. Data are derived from experiments in Holden *et al.* (2014). C. Schematic showing possible roles for RGS2 in reducing 'pro-asthma' responses. Agonists/activators of GPCRs that couple via Gq lead to phospholipase Cβ activation and the production of inositol(1,4,5)trisphosphate (IP_3) and DAG. This increases $[Ca^{2+}]_c$ and activates protein kinase C (PKC) to promote ASM contraction, mucus secretion, release of inflammatory mediators, cytokines and other pro-asthma responses. RGS2 is a GTPase activating protein that enhances the GTP hydrolysis of $G\alpha_q$ and thereby inactivates signalling by G_q. HDM = house dust mite.

(Moodley *et al.*, 2013; BinMahfouz *et al.*, 2015; Joshi *et al.*, 2015b) (Figure 2A). CD200 is a transmembrane glycoprotein expressed on haematopoietic and non-haematopoietic cells, which by binding its cognate receptor, CD200R, on alveolar macrophages, down-regulates cytokine production (Snelgrove *et al.*, 2008). This interaction also suppresses AHR in a murine model of allergic asthma (Vaine and Soberman, 2014; Lauzon-Joset *et al.*, 2015). Similarly, the secreted protein, CRISPLD2, attenuates gene toll-like receptor-4-mediated, pro-inflammatory signalling by binding and inactivating lipopolysaccharide/lipid A (Wang *et al.*, 2009; Himes *et al.*, 2014; Vasarhelyi *et al.*, 2014).

The widespread changes in gene expression produced by cAMP and glucocorticoids will also promote expression of

adverse-effect genes (Gonzales *et al.*, 2002; Wade *et al.*, 2006). In cell-based experiments, cAMP-elevating stimuli, including LABAs, synergize with glucocorticoids to up-regulate expression of pyruvate dehydrogenase kinase, isoform 4 (gene PDK4) and phosphoenolpyruvate carboxykinase (PEPCK, gene PCK2), which encode proteins involved in glucose and carbohydrate metabolism (Hanson and Reshef, 1997; Jitrapakdee, 2012; Oh *et al.*, 2013; Joshi *et al.*, 2015b). This illustrates a concern that combination therapies enhance known glucocorticoid-induced adverse effects (Schacke *et al.*, 2002). Furthermore, such data highlight the potential advantage of selecting each component of an ICS/LABA combination therapy to maximally induce the expression of clinically beneficial genes, in relevant target tissues, over those genes responsible for adverse effects.

Patterns of cooperatively regulated gene expression

The superiority of ICS/LABA combination therapy requires each component to contribute to overall clinical efficacy in a way that is more than simple additivity (Giembycz *et al.*, 2008). In this respect, three, possibly four, general patterns of glucocorticoid/LABA-dependent gene expression can be envisaged. (i) As shown by RGS2 and CD200, there are genes whose expression are primarily induced by the LABA, but are not, or are only modestly induced, by the glucocorticoid. However, in combination, the glucocorticoid enhances, and/or prolongs, expression induced by the LABA (Figure 2A) (Holden *et al.*, 2011; Holden *et al.*, 2014; BinMahfouz *et al.*, 2015; Joshi *et al.*, 2015b). (ii) There are genes induced by glucocorticoids, which are either not, or are only minimally, induced by the LABA, yet show LABA-dependent enhancement of the glucocorticoid response (Figure 2A). Luciferase expression from a simple, albeit artificial, 2 × glucocorticoid response element (GRE)-dependent reporter provides a good example (Figure 2B) (Kaur *et al.*, 2008; Joshi *et al.*, 2015b). Similarly, the cell cycle kinase inhibitor, CDKN1C (*aka* p57KIP2), and the metabolic gene, PDK4, show this pattern of regulation in BEAS-2B cells (Figure 2B) (Kaur *et al.*, 2008; Rider *et al.*, 2011; Joshi *et al.*, 2015b; Rider *et al.*, 2015b). Evidence for such behaviour is also observed functionally. In T-lymphocytes, LABAs augment GR-mediated apoptosis in a protein kinase A (PKA)-dependent manner (Ji *et al.*, 2007; Ji *et al.*, 2008), but are, by themselves, inactive (Pace *et al.*, 2004). Because glucocorticoid-induced apoptosis may involve GR-mediated gene expression (Wu *et al.*, 2013), such data are consistent with LABAs enhancing glucocorticoid-induced transactivation. (iii) Genes, such as CRISPLD2, show mRNA expression that is independently induced by glucocorticoids and LABAs and reveal synergism when both drug classes are combined (Figure 2A) (BinMahfouz *et al.*, 2015; Joshi *et al.*, 2015b). Within this group is, arguably, a subset of genes (iv), where expression is induced by a glucocorticoid and LABA in combination, but not by either component individually (Giembycz and Newton, 2015). Currently, genes regulated in this way have not been described.

Such groupings represent extremes of what should be considered as a continuum of gene expression. Furthermore, the exact details of expression will depend on multiple external factors, including cell line or type and the treatment time. For example, in bronchial epithelial BEAS-2B and ASM cells, the glucocorticoid-dependent induction of RGS2 mRNA is modest (Holden *et al.*, 2011; Holden *et al.*, 2014), whereas in A549 type II adenocarcinoma cells and primary human bronchial epithelial (HBE) cells glucocorticoids promote robust RGS2 mRNA expression (Chivers *et al.*, 2006; Holden *et al.*, 2014). Likewise, the patterns of synergy may also differ. In BEAS-2B and ASM cells, LABA-induced expression of RGS2 is enhanced and prolonged by glucocorticoids (Holden *et al.*, 2011; Holden *et al.*, 2014), whereas in primary HBE cells, LABAs only modestly increased RGS2 expression, yet significantly enhanced the maximum response achieved by a glucocorticoid (Holden *et al.*, 2014).

Repression of gene expression by glucocorticoids and LABAs

The effects of LABAs and glucocorticoids may also extend, in an apparently additive manner, to the repression of inflammatory genes. These include adhesion molecules, cytokines and chemokines induced by multiple inflammatory stimuli from structural cells of the airways (Korn *et al.*, 2001; Pang and Knox, 2001; Silvestri *et al.*, 2001; Spoelstra *et al.*, 2002; Newton *et al.*, 2010b). In contrast to ASM and epithelial cells (above), apparently additive repression can also occur for CXCL8 release from cigarette smoke-treated macrophage and neutrophils (Sarir *et al.*, 2007; Mortaz *et al.*, 2008). Furthermore, additive effects of formoterol and budesonide were reported on the repression of ICAM-1 expression as well as cytokine and chemokine expression measured following human rhinovirus (HRV) infection of epithelial cells (Yamaya *et al.*, 2014). While the mechanistic basis for these effects remains to be established, it is equally clear that opposing effects, particularly of the LABA, for example on CXCL8 expression, are observed in a cell type- and potentially stimulus-dependent manner.

Evidence for synergistic, repressive effects is also available. Glucocorticoids and LABAs combine to produce more than an additive repression of TNF-induced CXCL8 release from human ASM cells (Pang and Knox, 2000), and of various mediators from airway epithelial cells in response to HRV infection (Edwards *et al.*, 2006; Volonaki *et al.*, 2006; Sarir *et al.*, 2007; Skevaki *et al.*, 2009). Although glucocorticoids and LABAs share the ability to recruit histone deacetylases to inflammatory gene promoters (Ito *et al.*, 2001; Nie *et al.*, 2005), the molecular mechanism(s) involved in any synergistic repression is unclear. Nevertheless, the glucocorticoid-dependent repression of many inflammatory genes shows a requirement for ongoing gene expression, which implies a role for GR-mediated transactivation (Newton and Holden, 2007; Clark and Belvisi, 2012). Furthermore, genes that appear to be repressed via mechanisms involving GR-mediated transactivation show a greater level of repression and a greater sensitivity (i.e. lower EC_{50}) to glucocorticoid compared with those inflammatory genes where direct GR-mediated transrepression is implicated (King *et al.*, 2013). In this context, it is salient that glucocorticoid-mediated repression can show marked differences in potency and overall repression depending on the inflammatory stimulus and/or the response measured (Fernandes *et al.*, 1999; Tran *et al.*, 2005). Such data are consistent with the concept of multiple mechanisms of glucocorticoid action involving different GR

effectors that could show differential dependence on GR transactivation and transrepression. Notwithstanding such complexity, many glucocorticoid-induced genes, including DUSP1 and glucocorticoid-induced leucine zipper (gene TSC22D3), which reduce NF-κB and activator protein 1 (AP-1)-dependent transcription, are implicated in the repression of inflammatory gene expression (Newton and Holden, 2007; Ayroldi and Riccardi, 2009; Clark and Belvisi, 2012). As increased expression of glucocorticoid-inducible genes, for example TSC22D3, occurs in the airways of normal individuals and in asthmatic subjects taking ICS monotherapy (Kelly *et al.*, 2012; Leigh *et al.*, 2016), clinically relevant roles for GR transactivation appear to be likely. Furthermore, as these effects may be enhanced by LABAs, this review focuses on transcriptional activation as a mechanism of synergy between glucocorticoids and LABAs that relates to asthma therapy.

LABA-induced responses and modulation by glucocorticoids

Promotion of bronchoprotection by glucocorticoids

Chronic β_2-adrenoceptor agonist exposure can impair β_2-adrenoceptor signalling and functional responsiveness via mechanisms that are collectively referred to as desensitization (Lefkowitz, 2007; Vasudevan *et al.*, 2011). However, it is well established that glucocorticoids offset such effects by increasing β_2-adrenoceptor number, G_s expression and/or coupling to adenylyl cyclase (Giembycz *et al.*, 2008; Newton *et al.*, 2010b).

An unanticipated effect of glucocorticoids and LABAs alone, and in combination, is their ability to protect against agonist-induced bronchoconstriction via mechanisms that involve gene expression. For example, the pro-contractile activity of MAPKs in ASM (Gerthoffer *et al.*, 1997; Hedges *et al.*, 2000; Dowell *et al.*, 2010; Sakai *et al.*, 2010) should be countered by a glucocorticoid via the induction of DUSP1 expression (Issa *et al.*, 2007; Kang *et al.*, 2008; Quante *et al.*, 2008). Indeed, *DUSP1*$^{-/-}$ mice exhibit enhanced airway contractile responses to ozone and a loss of glucocorticoid protection (Li *et al.*, 2011). Despite there being no apparent protection by DUSP1 using chronic ozone exposure in a mouse model of glucocorticoid resistance (Pinart *et al.*, 2014), it is possible that, by further enhancing DUSP1 expression with a LABA to increase glucocorticoid-induced DUSP1 expression (Kaur *et al.*, 2008; Manetsch *et al.*, 2012), protection could have been re-established. However, such effects remain to be tested.

Similarly, LABAs induce Rgs2 expression in murine airways to protect against agonist-induced bronchoconstriction (Holden *et al.*, 2011). Furthermore, analysis of cytosolic free calcium concentration ($[Ca^{2+}]_c$) as a surrogate of contraction in human ASM cells shows RGS2 can account for the ability of a glucocorticoid to prolong LABA-induced bronchoprotection (Holden *et al.*, 2011). The additional finding that the ICS, budesonide, induces DUSP1 and RGS2 mRNA *in vivo* in human airways (Leigh *et al.*, 2016) provides strong evidence that expression of these genes could attenuate bronchoconstriction in asthma and COPD. Clearly, this would be most pronounced with ICS/LABA combination therapies, where the LABA would not only promote direct smooth muscle relaxation, but also interact additively and synergistically with the glucocorticoid on DUSP1 and RGS2 expression, respectively (Kaur *et al.*, 2008; Holden *et al.*, 2011; Manetsch *et al.*, 2012). Such data are consistent with enhanced gene expression contributing to the clinical superiority of ICS/LABA combination therapies in moderate-to-severe asthma.

Transcriptional activation by LABAs and modulation by glucocorticoids

Studies from the late 1980s document PKA-dependent phosphorylation of cAMP-activated transcription factors, such as cAMP response element (CRE) binding protein, CREB1 (Mayr and Montminy, 2001; Sands and Palmer, 2008). At the simplest level, CREB1 binds CRE sites in the promoters of target genes and, when phosphorylated at Ser^{133}, recruits the co-activator gene CREB-binding protein to confer transcriptional competency (Chrivia *et al.*, 1993; Mayr and Montminy, 2001). However, glucocorticoids often antagonize cAMP-dependent transcription (Diaz-Gallardo *et al.*, 2010). Indeed, in bronchial epithelial cells, activation of CRE-dependent transcription by LABAs was modestly repressed by glucocorticoids (Kaur *et al.*, 2008). Thus, the enhancement of CRE-dependent transcription, *per se*, is unlikely to explain how LABAs and glucocorticoids synergistically induce gene transcription. Indeed, CREB1, acting at conventional CRE sites, may account for the actions of LABAs on IL6 and CXCL8 expression and is therefore consistent with repressive effects of glucocorticoids (Ammit *et al.*, 2002; Yin *et al.*, 2006; Tan *et al.*, 2007). Conversely, while the proximal promoter region for RGS2 harbours a simple CRE that binds CREB1 and responds to cAMP (Song *et al.*, 2010; Xie *et al.*, 2011), this may not explain synergy with a glucocorticoid. Rather, a putative GR binding site has been localized to a region within 10 kb of the mouse *Rgs2* transcription start site (So *et al.*, 2008). Thus, notwithstanding the involvement of other cAMP- and/or glucocorticoid-activated transcription factors (Mayr and Montminy, 2001; Chinenov *et al.*, 2014), a simple, but untested, hypothesis is that CREB1/GR interactions at the RGS2 promoter lead to transcriptional synergy.

CCAAT-enhancer binding factors are cAMP and glucocorticoid regulated

Many members of the CCAAT-enhancer binding protein (C/EBP) transcription factor family, including C/EBPα (gene CEBPA), C/EBPβ (gene CEBPB) and C/EBPδ (gene CEBPD), are activated and/or induced by cAMP-elevating stimuli, glucocorticoids and by pro-inflammatory stimuli (Cardinaux and Magistretti, 1996; Croniger *et al.*, 1998; Poli, 1998; Vogel *et al.*, 2004; Tsukada *et al.*, 2011). Thus, CEBPB, a PKA target, is implicated in the transcriptional activation of genes, such as IL6 or CXCL8, by inflammatory stimuli, and this could account for the induction and/or enhancement of their expression by β_2-adrenoceptor agonists (Akira *et al.*, 1990; Mukaida *et al.*, 1990; Matsusaka *et al.*, 1993; Kunsch *et al.*, 1994; Trautwein *et al.*, 1994). However, CEBPB expression is also induced by cAMP-dependent activation of CREB1, and this is partly responsible for up-regulating metabolic genes, such as PEPCK (Park *et al.*, 1993; Niehof *et al.*, 1997). C/EBPs are also glucocorticoid-inducible proteins (Park *et al.*, 1990; Baumann *et al.*, 1991; Cao *et al.*, 1991). For example, CEBPB and CEBPD

expression is induced by glucocorticoids in bronchial epithelial cells, and up-regulation of CEBPD occurs in the airways of human subjects following budesonide inhalation (Berg *et al.*, 2005; Zhang *et al.*, 2007a; Leigh *et al.*, 2016). Thus, while C/EBPs are implicated in metabolic and inflammatory gene expression, and controlling proliferation (Nerlov, 2007; Tsukada *et al.*, 2011), they are also involved in the maintenance of inflammation-induced innate immune genes in the context of glucocorticoids (Zhang *et al.*, 2007a; Didon *et al.*, 2011). While such effects could be enhanced by LABAs, it is unclear whether this would be a desirable outcome in the context of chronic inflammatory diseases, such as asthma or COPD. Furthermore, CREB1 and CEBPB can synergistically promote gene transcription (Park *et al.*, 1993; Niehof *et al.*, 1997; Niehof *et al.*, 2001), an effect that could explain the ability of glucocorticoids to enhance cAMP-mediated responses from promoters driven by CREs that act in conjunction with C/EBP sites. Finally, as C/EBPs can act as pioneer factors to promote GR-dependent transcription (Grontved *et al.*, 2013), key roles are likely in respect of the regulation of glucocorticoid/LABA co-regulated genes. However, the complex relationship between pro-inflammatory, metabolic and potentially other more desirable effects of CEBP transcription factors requires careful examination.

Modulation of gene expression by glucocorticoids and the effect of LABAs

Transrepression and transactivation in the anti-inflammatory effects of glucocorticoids

To understand how LABAs enhance the therapeutic activity of glucocorticoids, it is pertinent to consider mechanisms by which glucocorticoids act in diseases such as asthma. Glucocorticoids suppress various indices of inflammation, and their clinical efficacy in mild-to-moderate asthma relies on the ability to reduce inflammatory gene expression (Rhen and Cidlowski, 2005; Barnes, 2008). However, how glucocorticoids operate at a molecular level to exert repression of inflammatory gene expression remains controversial, and there are currently three main possibilities (Clark and Belvisi, 2012). One mechanism, often referred to as 'transrepression', is where activated GR undergoes nuclear translocation to interact with and inhibit those transcription factors (NF-κB, AP-1) responsible for driving inflammatory gene transcription (De Bosscher *et al.*, 2003; Sundahl *et al.*, 2015). In this scenario, GR may not directly bind DNA, but is tethered via the inflammatory transcription factor to recruit repressive factors, including histone deacetylases, which reduce gene transcription (Ito *et al.*, 2000; Ito *et al.*, 2006; Hua *et al.*, 2016a). In a second mechanism of transrepression, GR binds DNA at negative GRE sites to inhibit gene transcription of inflammatory and other genes that are potentially associated with side effects (Surjit *et al.*, 2011; Hua *et al.*, 2016b). This effect appears to involve monomeric GR, which binds DNA sites with a lower affinity than GR dimers at conventional positive GREs (Hudson *et al.*, 2013).

A third mechanism of action involves the repression of gene expression via GR-dependent transactivation. Initial evidence for this comes from long-standing observations that the repressive effects of glucocorticoids on the expression of inflammatory genes, including cyclooxygenase 2 (gene PTGS2), CXCL8, inducible gene NOS2 and GM-CSF (gene CSF2) (Ristimaki *et al.*, 1996; Newton *et al.*, 1998; Chang *et al.*, 2001; Korhonen *et al.*, 2002; Chivers *et al.*, 2006; Newton *et al.*, 2010a), are largely prevented by inhibitors of transcription and translation (Newton, 2000; Stellato, 2004; Newton and Holden, 2007; Clark and Belvisi, 2012). In this model, ligand-bound GR behaves as a positive transcriptional activator to induce expression of anti-inflammatory genes. Indeed, direct GR-mediated induction of the NF-κB inhibitor, IκBα (gene NFKBIA), has been suggested to mediate glucocorticoid-induced gene repression (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). Similarly, DUSP1 is glucocorticoid-induced and inhibits MAPK activity (Kassel *et al.*, 2001; Lasa *et al.*, 2002). This may affect multiple MAPK-dependent processes, including activation of inflammatory transcription factors, such as AP-1 and NF-κB, mRNA stability and protein expression of inflammatory genes (Lasa *et al.*, 2001; Abraham *et al.*, 2006; Furst *et al.*, 2007; Issa *et al.*, 2007; Diefenbacher *et al.*, 2008; Kang *et al.*, 2008; King *et al.*, 2009a; Shah *et al.*, 2014). Likewise, TSC22D3 expression is increased by glucocorticoids in HBE cells, ASM, mast cells and macrophage, as well as in asthmatic patients taking ICS (Berrebi *et al.*, 2003; Godot *et al.*, 2006; Eddleston *et al.*, 2007; Kelly *et al.*, 2012). As TSC22D3 represses NF-κB and AP-1-dependent transcription, roles in limiting inflammatory gene expression and inflammation are also implied (Mittelstadt and Ashwell, 2001; Di Marco *et al.*, 2007; Eddleston *et al.*, 2007; Ayroldi and Riccardi, 2009).

The relevance of GR-mediated transactivation is increasingly suggested by the range of glucocorticoid-induced effector genes [e.g. CD200, CRISPLD2, DUSP1, NFKBIA, RGS2 and TSC22D3; (Newton *et al.*, 2010b)] that have now been identified. Importantly, many of these effector genes are up-regulated by LABA and/or glucocorticoids in primary human ASM cells (Misior *et al.*, 2009). Furthermore, the fact that the expression of many such genes is increased in the human airways following budesonide inhalation, as well as in COPD patients taking inhaled fluticasone propionate/salmeterol, supports the concept that such effects are therapeutically relevant and may be enhanced in the presence of a LABA (Kelly *et al.*, 2012; Lee *et al.*, 2016; Leigh *et al.*, 2016).

Co-operation between GR and inflammatory transcription factors to maintain repression

Many glucocorticoid-induced genes are also involved in the normal regulatory processes that control inflammatory signalling and gene expression (Newton, 2014). In this regard, A20 (gene TNFAIP3) (Altonsy *et al.*, 2014) and IRAK-M (gene IRAK3) (Miyata *et al.*, 2015) are informative. These genes are NF-κB-dependent. This allows their expression to be rapidly induced by inflammatory stimuli and, thereby, provides negative feedback control of NF-κB-dependent gene expression. However, rather than agonist-bound GR causing repression of these genes, as is routinely established for other NF-κB-dependent inflammatory genes (De Bosscher *et al.*, 2003), there is a robust, positive, transcriptional cooperativity between NF-κB and GR that leads to enhanced TNFAIP3 and IRAK3 expression (Altonsy *et al.*, 2014; Miyata *et al.*, 2015). Such positive NF-κB/GR interactions, which have been

previously demonstrated (Hofmann and Schmitz, 2002), provide a mechanism by which glucocorticoids enhance gene expression in the context of an inflammatory stimulus and are remarkably common (Kadiyala *et al.*, 2016). Thus, by increasing (or at least maintaining) feedback control by TNFAIP3 and IRAK3, positive interactions between GR and NF-κB may play important anti-inflammatory roles (Altonsy *et al.*, 2014; Newton, 2014; Miyata *et al.*, 2015). Similarly, expression of the NF-κB inhibitor, NFKBIA, is glucocorticoid-induced and is up-regulated by inflammatory stimuli acting via NF-κB to reduce NF-κB signalling (Le Bail *et al.*, 1993; Auphan *et al.*, 1995; Scheinman *et al.*, 1995). Likewise, while DUSP1 and the mRNA destabilizing protein, tristetraprolin (gene ZFP36), are rapidly and highly induced by pro-inflammatory stimuli to provide negative regulation of MAPK pathways, or AUUUA-containing mRNAs, such as IL6, CXCL8 or CSF2, they are also up-regulated by glucocorticoids (Abraham *et al.*, 2006; Smoak and Cidlowski, 2006; King *et al.*, 2009b; Prabhala and Ammit, 2015). Such data are consistent with AP-1 also co-operating with GR at composite elements containing both AP-1 and GR binding sites (So *et al.*, 2007). Furthermore, transcription factors, including AP-1, may modify the local chromatin structure, for example by co-operating with GR to displace nucleosomes, and thereby play key roles in enhancing GR access and action at responsive promoters (Biddie *et al.*, 2011; He *et al.*, 2013). Thus, an emerging principle is that glucocorticoid-bound GR co-operates with core inflammatory transcription factors to promote expression of regulatory genes and maintain, or enhance, inhibition of inflammatory signalling and gene expression.

GR-dependent transactivation is enhanced by LABAs

The exact contribution of direct, GR-mediated transrepression in the anti-inflammatory effects of glucocorticoids is currently unclear. While some studies fail to show the presence of GR at repressed genes and others report the widespread presence of negative GREs (So *et al.*, 2008; Reddy *et al.*, 2009; Surjit *et al.*, 2011; Kadiyala *et al.*, 2016), it is also plausible that both transrepression and transactivation play important roles in the glucocorticoid-dependent repression of inflammatory gene expression (King *et al.*, 2013). However, while we are unaware of data reporting enhancement of GR-mediated transrepression, whether via tethering or nGRE mechanisms, by LABAs, there is unequivocal evidence that LABAs can augment glucocorticoid-dependent transcription (Giembycz *et al.*, 2008; Newton *et al.*, 2010b) (Figure 2).

In HBE BEAS-2B cells and primary human ASM cells, simple GRE-dependent transcription is synergistically enhanced by the co-administration of agonists that increase cAMP (Kaur *et al.*, 2008; Wilson *et al.*, 2009; Greer *et al.*, 2013; Moodley *et al.*, 2013; Joshi *et al.*, 2015b). Thus, LABAs, such as salmeterol or formoterol, which alone have no obvious effect on a simple 2 × GRE-driven luciferase reporter, increase by 2–3 fold the maximum effect (E_{max}; Box 1) of a glucocorticoid (Figure 2B). Similarly, any given level of response to glucocorticoid is achieved at a lower concentration in the presence of a LABA, which is therefore glucocorticoid-sparing (Figure 2B) (Giembycz *et al.*, 2008). Significantly, this effect is reproduced with real genes, including CDKN1C (Figure 2B) (Joshi *et al.*, 2015b), and occurs in primary cells (Kaur *et al.*, 2008; Wilson *et al.*, 2009; Moodley *et al.*, 2013). Given roles for CDKN1C in cell cycle control (Samuelsson *et al.*, 1999), and possibly the regulation of c-Jun *N*-terminal kinases (Chang *et al.*, 2003), these synergistic and steroid-sparing interactions may translate into enhanced therapeutic effects.

Box 1 – Glossary of Pharmacodynamic Terms

Affinity. (*aka* the equilibrium association constant): The tenacity to which a ligand binds to a receptor. Arithmetically, it is the molar concentration of ligand that at equilibrium binds to 50% of the receptor population and is equal to the reciprocal of the equilibrium dissociation constant, K_A.

Biased Agonism. Receptors can couple to multiple signalling pathways. Biased agonism is a term that describes ligand-dependent selectivity whereby a given ligand stabilizes a particular receptor conformation to preferentially activate certain downstream signalling pathways relative to others.

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.

Law of Mass Action. A law that states 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.

Maximum Response (E_{max}). The response produced by a maximally effective concentration of an agonist. For a full agonist $E_{max} = E_m$ whereas $E_{max} < E_m$ for a partial agonist.

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. 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 the maximum response. The remaining receptors will produce stimulus that is surplus to requirement and constitute a 'reserve' or are said to be 'spare'. The K_A/EC_{50} ratio is a measure of receptor reserve; the greater this ratio, the larger is the receptor reserve and the occupation of fewer receptors is required to elicit a particular level of response.

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

Mechanisms underlying enhanced GR-mediated transactivation by LABAs

How LABAs enhance glucocorticoid-dependent transcription is subject to considerable debate. Data from the 1990s revealed that the cAMP-PKA pathway could enhance GR-mediated transcription (Rangarajan *et al.*, 1992; Gruol and Altschmied, 1993; Nordeen *et al.*, 1993; Zhang *et al.*, 1993). At that time, there was a lack of clarity as to the mechanism(s) of action. Some studies suggested enhanced GR DNA binding, increased GR expression and/or the ability of LABA to prevent the loss of GR that occurs following glucocorticoid exposure (Dong *et al.*, 1989; Rangarajan *et al.*, 1992; Korn *et al.*, 1998). Conversely, other investigators showed no effect on GR expression, ligand binding, nuclear translocation or GR DNA binding (Gruol and Altschmied, 1993; Moyer *et al.*, 1993; Zhang *et al.*, 1993). More recently, such controversy has been perpetuated with claims that LABAs may enhance GR translocation to the nucleus (Eickelberg *et al.*, 1999; Roth *et al.*, 2002; Usmani *et al.*, 2005; Mortaz *et al.*, 2008), along with reports that this does not occur (Loven *et al.*, 2007; Rider *et al.*, 2015a). Intriguingly, the LABA, formoterol, was reported to increase GR DNA binding by low-dose budesonide in sputum macrophages from mild asthmatics and COPD patients (Essilfie-Quaye *et al.*, 2011; Haque *et al.*, 2013). Furthermore, the ability of formoterol to restore impaired GR activity in U937 cells treated with combined IL2/IL4 was explained by an effect on GR phosphorylation that was mediated by the protein phosphatase, PP2A (Kobayashi *et al.*, 2012). However, this was neither blocked by the β_2-adrenoceptor antagonist, ICI 118551, nor mimicked by forskolin, which raises questions over mechanism. Conversely, enhancement of glucocorticoid action by a PDE4 inhibitor was shown not to involve altered GR translocation (Grundy *et al.*, 2016).

In considering mechanisms underlying the ability of LABAs, or the cAMP pathway, to enhance GR activity, the possibility of cell- and system-dependent effects cannot be ignored (Rider *et al.*, 2015b). Nevertheless, it is clear that, within a given system, the expression of some glucocorticoid-induced genes are not enhanced by LABAs, whereas others show either additivity or synergy (Kaur *et al.*, 2008; BinMahfouz *et al.*, 2015; Rider *et al.*, 2015b). Such observations correspondingly require explanations that allow for gene-specific enhancements, rather than generic mechanisms that should produce global changes in GR-mediated gene expression (Giembycz *et al.*, 2008; Newton *et al.*, 2010b) (Table 1). Using a simple GRE reporter in BEAS-2B cells, LABAs enhance transcription in the absence of any change in the potency, affinity or efficacy of the GR agonist (Joshi

Table 1

LABA-dependent mechanisms of enhancing GR-mediated gene transcription

Possible mechanism of LABA action	Effect on GR-mediated gene expression		Predicted nature of the expected effect on GR-mediated gene expression
	General	Gene-specific	
Enhanced ligand affinity for GR	Yes	No	Leftward-shift in the concentration–response curve for all responses.
Enhanced GR expression	Yes	No	Leftward shift in the concentration–response curves for all responses that were occurring at the system maximum (E_m; Box 1). For responses below the system maximum, E_{max} would increase until the system maximum was reached.
Enhanced GR translocation	Yes	No	Leftward shift in the concentration–response curves for all responses that were occurring at the system maximum. For responses below the system maximum, E_{max} would increase until the system maximum was reached.
Enhanced DNA binding of GR	Yes	Yes	Enhanced DNA binding by GR could result in global effects on gene expression. Alternatively, LABA-induced enhancements in GR binding could be gene specific.
Phosphorylation/other modification of GR	Yes	Yes	Changes in GR phosphorylation, or other modification, have the capacity to affect all, or just a specific group, of GR responsive genes.
Phosphorylation/modification of transcriptional apparatus	Yes	Yes	Changes in phosphorylation, or other modification, of any part of the transcriptional apparatus have the capacity to affect all, or just a specific group, of GR responses genes.
Interaction with cAMP-activated TF	No	Yes	Genes with binding sites for other transcriptional activators will have the potential for interaction with GR, if GR is also recruited to this same promoter.
Activation/induction of a co-activator or repression/ inactivation/degradation of a co-repressor	Yes	Yes	Activation or repression of coactivator, or co-repressors may have effects on all or a population of GR regulated genes. Alternatively, gene-dependent requirement for a co-activator or repressor could allow gene-specific regulation by a LABA

et al., 2015b) (Box 1). As this effect is mimicked by forskolin and other cAMP-elevating agents, and is blocked by a selective inhibitor of PKA (Kaur *et al.*, 2008; Wilson *et al.*, 2009; Moodley *et al.*, 2013; BinMahfouz *et al.*, 2015), these data suggest that activation of the canonical cAMP signalling cascade augments the transcriptional competency of liganded GR and, therefore, the magnitude of GR-mediated gene expression. This concept is consistent with findings that transcriptional co-activators involved in GR-mediated transcriptional responses are PKA substrates and provides a mechanism for gene-dependent enhancements (Rowan *et al.*, 2000; Constantinescu *et al.*, 2004; Hoang *et al.*, 2004; Fenne *et al.*, 2008). As noted above, CEBP family members are glucocorticoid-induced and up-regulated by PKA-dependent processes (Yeagley and Quinn, 2005). Thus, the presence of CEBP sites or, alternatively, co-localization with CREs or other factors including co-activators could all produce transcriptional synergies at target genes.

Optimizing ICS/LABA combination therapy by exploiting pharmacodynamics

Potential for biased agonism

Early concepts of drug action assumed an agonist-bound receptor existed in a single conformation and that the magnitude of response was due to the strength of signal. However, we now know that a GPCR does not exist in a single, static conformation, and, consequently, the intrinsic efficacy (Box 1) of a ligand can be response-specific depending on the particular coupling, or transducer, involved (Figure 4). Indeed, GPCRs adopt multiple, interchangeable conformations (or activation states) that are differentially stabilized in an agonist-dependent manner (Spengler *et al.*, 1993; Pantaloni *et al.*, 1996; Onaran and Costa, 1997; Liu *et al.*, 2012). This leads to the concept of biased agonism (also known as

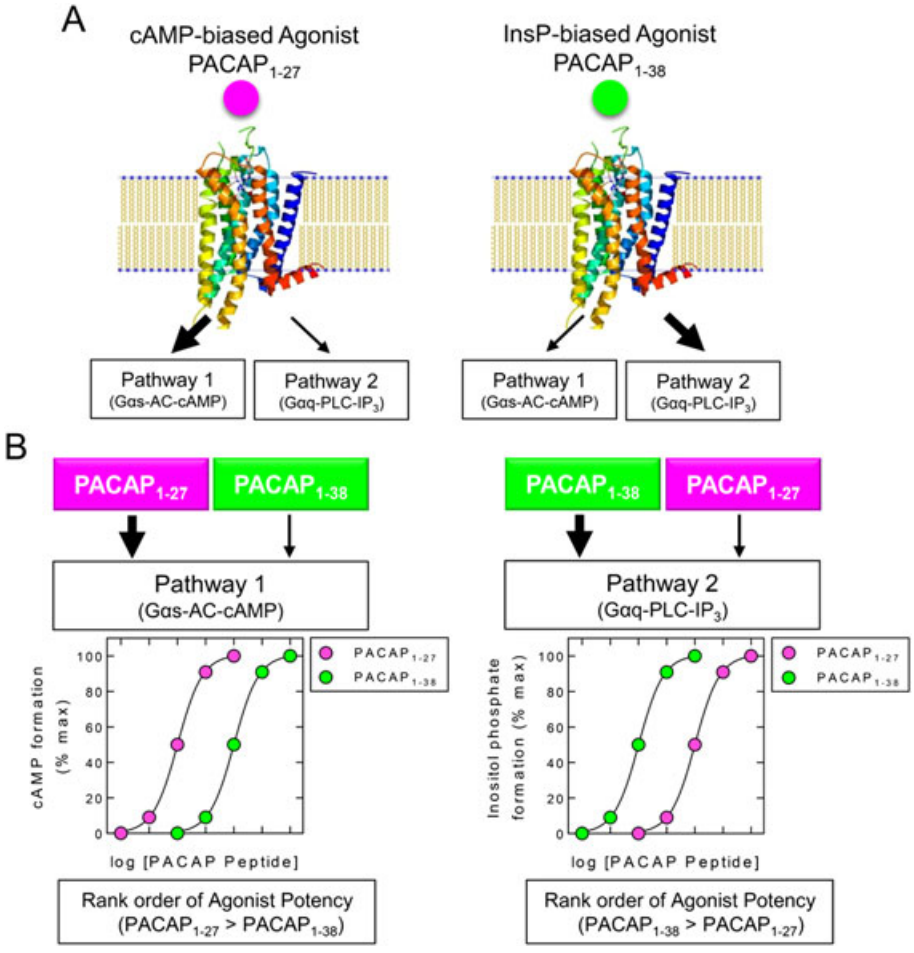

Figure 4

Biased agonism at the pituitary adenylate cyclase-activating polypeptide type I (PAC_1) receptor. Schematic representation of data taken from Spengler *et al.* (1993) where the PAC_1 receptor was over-expressed in LLC PK1 porcine kidney epithelial cells. A. $PACAP_{1-27}$ preferentially promotes cAMP formation (Pathway 1) via a $G\alpha_s$/adenylyl cyclase-dependent mechanism. $PACAP_{1-38}$ preferentially promotes inositol phosphate production (Pathway 2) through a $G\alpha_q$/phospholipase Cβ activation pathway. Large and small arrows indicate the major and minor pathways activated by each PACAP peptide. B. Activation of each pathway is shown by $PACAP_{1-27}$ and $PACAP_{1-38}$, and the effect on the main downstream second messenger (cAMP or inositol phosphates) is depicted. Figure generated from data reported by Spengler *et al.* (1993) using the ribbon diagram found at https://commons.wikimedia.org/wiki/File:A2A_receptor_bilayer.png

stimulus trafficking, functional selectivity and ligand-directed signalling) whereby a ligand stabilizes a particular receptor conformation to preferentially activate certain downstream signalling pathways relative to others (Kenakin, 1995; Kenakin, 2009; Evans *et al.*, 2010; Seifert, 2013; Kenakin, 2015). For example, biased agonism was first shown for the pituitary adenylyl cyclase-activating polypeptide 1 receptor 1 (gene ADCYAP1R1) at which the agonists $PACAP_{1–27}$ and $PACAP_{1–38}$ elevate cAMP (via G_s) and inositol phosphates (via G_q), respectively, with opposite orders of potency (Spengler *et al.*, 1993) (Figure 4). In the sections below, we discuss biased agonism in the context of β_2-adrenoceptor agonists and consider whether ligands that bind GR could produce biased gene expression.

Pharmacodynamics of GR-mediated gene expression

Cooperative interactions of ligand-bound GR with tissue-dependent factors, including sequence-specific transcription factors, co-repressors and co-activators (Szapary *et al.*, 1999; Simons, Jr., 2006), combined with the linear arrangement of transcription factor binding sites and their actual DNA sequence will, along with the specific chromatin structure and any epigenetic modifications, result in a very specific three-dimensional architecture for each individual gene promoter (Keenan *et al.*, 2016). As a consequence, the ability of any given ligand-induced conformation of GR to interact with each gene promoter will be unique and may, therefore, give rise to gene-specific, patterns of transactivation, as is now reported (Tanigawa *et al.*, 2002; Reddy *et al.*, 2009; Simons, Jr., 2010; Uings *et al.*, 2013; Joshi *et al.*, 2015a; Rider *et al.*, 2015b). For example, an analysis of dexamethasone-induced genes in A549 cells revealed an average EC_{50} of ~3 nM, with the potency of the most and least sensitive genes varying by a factor of ~20-fold (Reddy *et al.*, 2009).

Extending this concept to different ligands, which are known to stabilize GR in distinct conformations (Allan *et al.*, 1992; Wagner *et al.*, 1996; Biggadike *et al.*, 2009a; Biggadike *et al.*, 2009b; Edman *et al.*, 2014), raises the

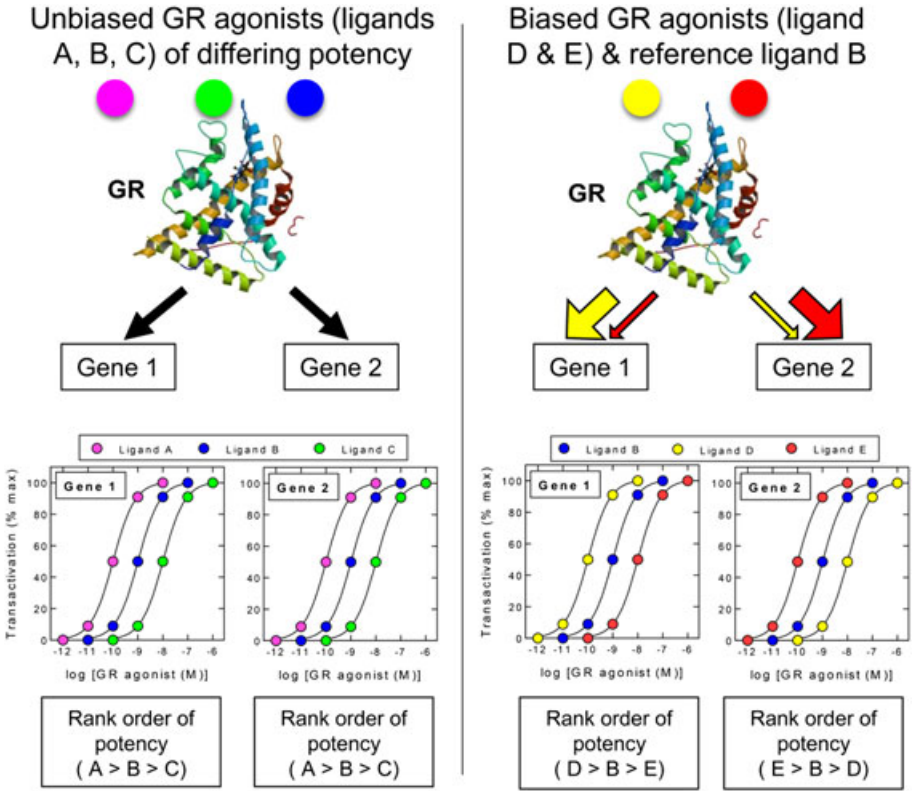

Figure 5

Biased agonism at GR. The possibility that nuclear hormone receptors such as GR can adopt different active conformations in a ligand-dependent manner could provide a means to promote gene expression bias in a given tissue. According to this hypothetical model, unbiased ligands (**A, B** and **C**) generate a GR conformation(s) that promotes the expression of the GC transcriptome (shown here as Gene 1 and Gene 2) with the same rank order of potency where, in the figure, ligand **A** is more potent than ligand **B**, which is more potent that ligand **C** on all genes. In contrast, biased agonists (**D** and **E**) lead to the stabilization of different GR conformers that more favourably promote the transcription of one gene (or gene population) over another relative to the reference ligand **B**. Gene expression bias could result from either a change in the way GR interacts with essential co-factors and/or its ability to bind DNA and effect transcription. In each case, this will be dictated by the promoter context of each target gene. The rank order of agonist potency can therefore vary in a gene-dependent manner as depicted by the concentration–response curves describing ligands **D** and **E** relative to the reference ligand **B**. Figure generated using the GR ribbon diagram found at http://www.rcsb.org/pdb/explore/explore.do?pdbId=1P93

possibility of agonist-directed gene expression (Figure 5). Such agonists, by stabilizing different GR conformations, may differentially affect GR's ability to interact productively at different gene promoters and may thereby lead to agonist-specific programmes of gene expression (Keenan *et al.*, 2016). Accordingly, gene expression changes within the glucocorticoid-induced transcriptome will be described by a family of concentration–response curves that are unique to the agonist of interest. Central to this concept is that the intrinsic efficacy for an agonist is governed by the relationship between the specific agonist-induced conformation of GR and differences in the promoter context for each gene (Mercier *et al.*, 1983; Newton *et al.*, 2010b; Joshi *et al.*, 2015a). Thus, in A549 cells, dexamethasone and the dissociated steroidal ligand, RU24858 (Vayssiere *et al.*, 1997), showed differential abilities to induce the expression of glucocorticoid-inducible genes (Chivers *et al.*, 2006). Moreover, the abundance and stoichiometry of GR relative to other factors necessary for gene expression are not invariant across cell types. Therefore, tissue-dependent heterogeneity in agonist-induced, gene expression signatures is predicted (Simons, Jr., 2010). Although evidence for such differences in gene expression is sparse, Tanigawa and colleagues reported that dexamethasone was a robust activator of a mouse mammary tumour virus (MMTV)-based reporter transfected into HeLa cells under conditions where RU24858 was a very weak agonist (Tanigawa *et al.*, 2002). In contrast, these ligands were almost equi-effective in driving the same reporter transfected into CV-1 cells (Tanigawa *et al.*, 2002). Thus, a cell-dependent factor, or factors, is responsible for this discrepant behaviour. Consistent with this idea, the rank order of potency of three glucocorticoids for activating a different (3 × GRE) reporter transfected into both HeLa cells and CV-1 cells was distinct (dexamethasone > prednisolone > RU24858 versus dexamethasone > RU24858 > prednisolone respectively). Tanigawa and colleagues also reported that substituting the MMTV-based construct in HeLa cells with the 3 × GRE reporter transformed RU24858 from a very partial agonist into one that was as effective as dexamethasone (Tanigawa *et al.*, 2002). Thus, the conformation adopted by the RU24858:GR complex was more favourable for driving the 3 × GRE reporter than for the MMTV-based construct, and this establishes the principle that differential signalling from GR can occur in different cells (Figure 5). Further evidence for this proposal is that the potency of dexamethasone differed by ~10-fold in driving transcription of either the MMTV- and 3 × GRE reporters in both HeLa and CV-1 cells, and the genes encoding glutamine synthetase (glutamate-ammonia ligase; gene: Glul) and tyrosine aminotransferase (gene Tat) in FU5-5 rat hepatoma cells (Mercier *et al.*, 1983; Tanigawa *et al.*, 2002).

Recent studies using BEAS-2B bronchial epithelial cells also show striking differences in the ability of a panel of GR ligands to induce expression of CDKN1C, CRISPLD2, PDK4 and TSC22D3 (Joshi *et al.*, 2015a; Joshi *et al.*, 2015b). In those studies, the GR agonists tested were equi-effective at inducing TSC22D3 and CRISPLD2, whereas marked differences were apparent for CDKN1C and PDK4. Notably, GW870086X and desisobutyryl-ciclesonide behaved as weak and very weak partial agonists on CDKN1C and PDK4, respectively, relative to a reference agonist, dexamethasone. Similar data were also reported for GW870086X in A549 cells, where intrinsic activity values (Box 1) varied from 0.1 to 0.9 across eight genes with EC_{50} values that differed by 20-fold (Uings *et al.*, 2013). Collectively, these data are consistent with agonist intrinsic efficacy varying in a gene-dependent manner that is dictated by sequence and structural differences between promoters in a given cell (Figure 5). This finding has significant implications for drug development as it may be possible to design GR agonists that selectively enhance the expression of some genes, while leaving others relatively unaffected.

GR number as a determinant of efficacy

The Law of Mass Action (Box 1) predicts that increases or decreases in the density of a given receptor will increase or decrease, respectively, agonist potency in that cell type. This is an important concept because GR expression varies considerably across structural and immune cells in the lungs, which are the intended sites of action for ICS, to off-target tissues responsible for side-effects (Bourgeois and Newby, 1979; Vanderbilt *et al.*, 1987; Lowy, 1989; Spencer *et al.*, 1991; Miller *et al.*, 1998). This effect is shown in over-expression studies where the maximal response is enhanced and/or the agonist concentration–response curves are displaced to higher potency (Robertson *et al.*, 2013). For example, mifepristone (also known as RU486) appears to function as a full agonist in promoting the translocation of GR to the nucleus (Schaaf and Cidlowski, 2003; Chivers *et al.*, 2004; Lewis-Tuffin *et al.*, 2007). However, mifepristone is generally inactive, or poorly active, in glucocorticoid-sensitive systems that rely on endogenous levels of GR (i.e. no over-expression), where it behaves as a GR antagonist. However, with GR over-expression, mifepristone displays partial agonist activity in reporter assays of transcriptional activation and repression (Zhao *et al.*, 2003; Zhang *et al.*, 2007b). Thus, increasing GR expression allows mifepristone to show some agonist activity. This finding suggests that the conformation of GR stabilized by mifepristone, while capable of binding DNA, does so in a manner that is only poorly favourable for activating gene transcription. In other words, mifepristone is a very weak partial agonist. Likewise, RU24858-bound GR adopts a conformation that is suboptimal for the recruitment of steroid receptor co-activator 1 (gene NCOA1) (Dezitter *et al.*, 2014). As this is necessary for GR-mediated transactivation, the failure to recruit sufficient NCOA1 results in transactivation becoming highly dependent on GR number (Dezitter *et al.*, 2014). Collectively, such data show that GR density, the specific-conformation of ligand-bound GR and the promoter context of a gene, determines 'transcriptional competency'. In pharmacological terms, this defines a given ligand as a full agonist, partial agonist or antagonist at any particular gene promoter.

As individual genes interpret equivalent degrees of GR occupancy differently, the concept described above suggests that the relationship between the ligand occupancy of GR and transcription is not uniform. Pharmacologically, the relationship between receptor occupancy and measured response is described by the receptor reserve, of which the affinity (K_A)/EC_{50} ratio (Box 1) is a typical measure. The greater this ratio (i.e. where $K_A > EC_{50}$), the larger is the receptor reserve, and the occupation of fewer receptors is required to elicit a particular level of response Conversely, as the EC_{50} approaches the K_A, the reserve declines toward zero where all receptors must

be occupied to achieve the measured response. The concept of receptor reserve is traditionally associated with agonists that act at GPCRs. However, recently, this was applied to GR-mediated gene expression. In BEAS-2B cells, the potency of the glucocorticoid fluticasone furoate was found to vary in a gene-dependent manner (Joshi *et al.*, 2015a). Receptor occupancies necessary for half-maximal gene induction were calculated as 21, 24, 29 and 39% for the glucocorticoid-induced genes, TSC22D3, CRISPLD2, CDKN1C and PDK4 respectively (Joshi *et al.*, 2015a) (Figure 6A). Thus, engagement of an equivalent fraction of GR by fluticasone furoate produced different degrees of gene activation with TCS22D32 and PDK4 being the most and least sensitive genes respectively. These results were reproduced in cells subjected to fractional GR inactivation, confirming that agonist intrinsic efficacy varies in a gene-dependent manner (Joshi *et al.*, 2015a). Likewise, GW870086X, which has lower intrinsic efficacy relative to fluticasone furoate, revealed increasing partial agonist behaviour that correlated inversely with the receptor reserve for each gene (Figure 6B).

Differences in receptor reserve have particular significance in tissues in which GR number is limiting as two agonists, one with low and the other with a relatively high intrinsic efficacy, will not generate the same gene expression signature. In tissues with low GR expression, agonists with low intrinsic efficacy, such as RU24858 or GW870086X, fail to appreciably transactivate certain populations of genes, and this may contribute to their improved therapeutic ratio in some models (Vayssiere *et al.*, 1997; Uings *et al.*, 2013). However, whether tissue-dependent variation in GR number affects the anti-inflammatory activity of glucocorticoids used in clinical practice is unknown. Furthermore, there is no published precedent to suggest that GR number in target tissues is considered when selecting glucocorticoids for development. Nevertheless, it is noteworthy that the active metabolite of the GR agonist, ciclesonide, behaves in BEAS-2B cells similarly to GW870086X (Joshi *et al.*, 2015a). Collectively, these data suggest that the exploitation of partial GR agonists for enhanced therapeutic benefit requires an assessment of their ability to induce therapeutically desirable and undesirable response genes in target and off-target tissues.

Biased agonism and GR-mediated gene expression

While differential binding of agonists to nuclear hormone receptors is established (Allan *et al.*, 1992; Wagner *et al.*, 1996; Biggadike *et al.*, 2009a; Biggadike *et al.*, 2009b; Edman *et al.*, 2014), it is unclear whether this can lead to biased gene expression (Figure 5) or merely different degrees of partial agonism. Certainly, GR effector functions and sub-cellular localization of GR can occur in a ligand-specific manner (Croxtall *et al.*, 2002; Schaaf *et al.*, 2005). Furthermore, unique patterns of ligand-directed GR stabilization, differences in cofactor recruitment and agonist-dependent heterogeneity of GR-mediated gene expression all support the possibility of selectively inducing one gene (or gene population) over another (Bledsoe *et al.*, 2002; Coghlan *et al.*, 2003; Kauppi *et al.*, 2003; Miner *et al.*, 2007; Ronacher *et al.*, 2009; Edman *et al.*, 2014). This idea is supported by data derived with non-steroidal GR ligands, such AL-438 and LGD5552, which retain anti-inflammatory activity with reduced induction of, at least some, side-effect genes (Coghlan *et al.*, 2003; Miner *et al.*, 2007). Similarly, mifepristone preferentially recruits the co-repressor, NCoR1 (Schulz *et al.*, 2002; Ronacher *et al.*, 2009), thereby establishing the principle that GR ligands can differentially modify gene expression by affecting co-activator/co-repressor recruitment. However, one

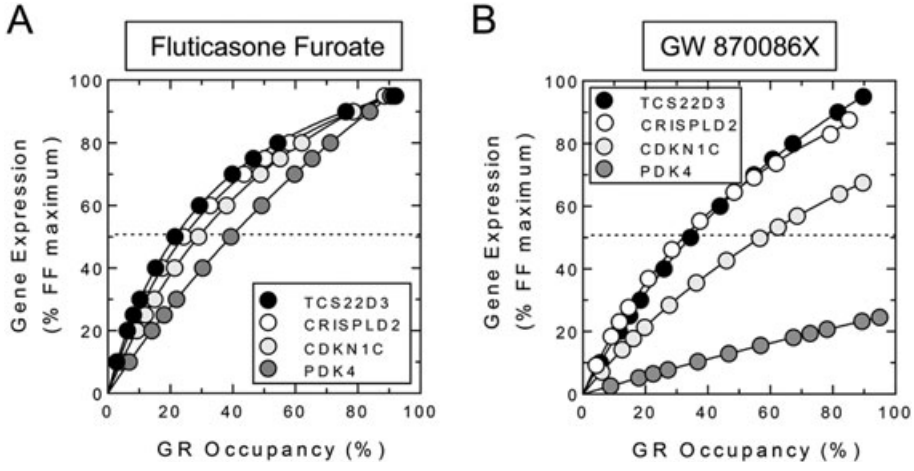

Figure 6

Relationship between GR occupancy and gene expression in human airway epithelial, BEAS-2B, cells. The data show that gene induction expressed as a function of GR occupancy is not uniform across genes. This indicates that a given cell type interprets the same degree of GR occupancy differently for each gene. In panel A, half maximal TSC22D3, CRISPLD2, CDKN1C and PDK4 induction was achieved with a concentration of fluticasone furoate that produced 21, 24, 29 and 39% GR occupancy respectively. In panel B, a more extreme profile is shown with a different GC, GW 870086X. Relative to fluticasone furoate, half maximal expression of GILZ and CRISPLD2 required ~23% GR occupancy. However, GW 870086X was a partial agonist on both CDKN1C and PDK4 such that 100% GR occupancy induced these genes to a level that was only 70 and 30% of the same responses produced by fluticasone furoate. Data re-drawn from Joshi *et al.* (2015a).

attempt to identify biased agonism showed that fluticasone furoate and GW870086X induced TSC22D3, CRISPLD2, CDKN1C and PDK4 in BEAS-2B cells, but there was no evidence of agonist potency reversal; fluticasone furoate was always 5–7-times more potent than GW870086X for each gene (Joshi *et al.*, 2015a). Nevertheless, that study was limited by the number of ligands and genes examined, as well as the structural similarity of the agonists. Clearly, comprehensive testing will be necessary to determine if different conformations of GR are associated with selective transcriptional signatures and whether these result from partial or, possibly, biased agonism. Furthermore, the relationship between any such biased agonism and the enhancement by LABA will need to be carefully quantified and assessed functionally.

Pharmacodynamics of β_2-adrenoceptor signalling

Tissue-dependent variation in β_2-adrenoceptor number, coupling efficiency to adenylyl cyclase and differences in agonist intrinsic efficacy also have the potential to influence the magnitude of cAMP-induced responses. This applies to the enhancement of GR-mediated gene transcription and the direct induction of cAMP-inducible genes by a LABA. In the latter case, the promoter context of the gene of interest will dictate whether PKA-phosphorylated transcription factors, such as CREB1 or ATF1, bind to DNA consensus sequences and effect transcription. In humans, pulmonary β_2-adrenoceptor number varies by approximately 50-fold. For example, ASM cells express a high number of β_2-adrenoceptors (30 000–40 000/cell), whereas T-lymphocytes express relatively few (~750/cell) (Johnson, 2002). Therefore, the ability of LABAs to promote cAMP-dependent transcription could be compromised in cell types where β_2-adrenoceptor number is limiting and/or when coupling to adenylyl cyclase is weak. Likewise, the LABA enhancement of GR-mediated gene expression and resultant anti-inflammatory activity could also be impaired. This concept is illustrated by studies with eosinophils, where salmeterol failed to suppress granule protein secretion and activation of the NADPH oxidase under conditions where the higher intrinsic activity agonist, formoterol, was effective (Rabe *et al.*, 1993; Munoz *et al.*, 1995).

A number of mutually inclusive approaches could improve the therapeutic activity of a LABA in cells that express low β_2-adrenoceptor number: (i) combine a GR agonist with a LABA that has very high intrinsic efficacy, (ii) add-on a PDE4 inhibitor to an ICS/LABA combination therapy, and (iii) add-on, or replace with, a ligand for another Gs-coupled receptor that is either more highly expressed or couples to adenylyl cyclase with higher efficiency than the β_2-adrenoceptor. With the exception of salmeterol, most LABAs described to date already have high intrinsic efficacy, and it is unclear to what extent this property can be improved. In contrast, attenuating cAMP hydrolysis with a PDE4 inhibitor might transform a pro-inflammatory or immune cell that is normally insensitive to a LABA into one that now generates a cAMP signal of sufficient magnitude to promote cAMP-dependent transcriptional responses, and/or enhance GR-mediated gene transcription. Likewise, agonists of GPCRs, other than the β_2-adrenoceptor, which also increase cAMP, will synergize with glucocorticoids. For example, activation of the prostacyclin receptor (gene PTGIR) or the adenosine A_{2B} receptor (gene ADORA2B) enhanced simple GRE-dependent transcription and synergized with glucocorticoids to induce gene expression (Wilson *et al.*, 2009; Greer *et al.*, 2013).

Adverse effects of β_2-adrenoceptor agonists, non-canonical signalling and response to glucocorticoids

In asthma, β_2-adrenoceptor agonists alleviate bronchoconstriction, but fail to combat the underlying inflammation. For this reason, chronic use of β_2-adrenoceptor agonists, in the 1970s, was identified as a risk factor contributing to asthma deaths (Tattersfield, 2006). Consequently, asthma treatment guidelines now recommend that β_2-adrenoceptor agonists, as a class, should only be used on an as-needed basis or, in the case of LABAs, in combination with an ICS (Cates and Cates, 2008).

Mechanistically, β_2-adrenoceptor agonists may be harmful in asthma by promoting or enhancing pro-inflammatory gene expression through direct, cAMP-dependent signalling mechanisms. The β_2-adrenoceptor may also mediate G-protein-independent signalling that involves the recruitment of β-arrestin-2 and the activation of ERK (Shenoy *et al.*, 2006; Billington *et al.*, 2013; Walker and DeFea, 2014; Pera and Penn, 2016). In murine models of asthma, this pathway is linked to pro-inflammatory effects of certain β_2-adrenoceptor agonists in the airways (Nguyen *et al.*, 2009; Walker *et al.*, 2011; Thanawala *et al.*, 2013; Penn *et al.*, 2014; Zhou *et al.*, 2014). For example, in allergen-sensitized β-arrestin-2 knockout mice, the typical asthma-like phenotype (AHR, pulmonary leukocyte burden, histological features of inflammation and mucus hyper-secretion) resulting from allergen challenge was decreased relative to effects in wild-type animals (Walker *et al.*, 2003; Hollingsworth *et al.*, 2010; Nichols *et al.*, 2012; Chen *et al.*, 2015). Similar results were obtained in sensitized and challenged mice deficient in either the β_2-adrenoceptor (Nguyen *et al.*, 2009) or phenylethanolamine *N*-methyltransferase (Gene PNMT; an enzyme central to adrenaline biosynthesis) (Thanawala *et al.*, 2013). Collectively, these data suggest that, in mice, the cardinal features of asthma are dependent on the release of adrenaline and its ability to activate β_2-adrenoceptors. Significantly, chronic administration of β_2-adrenoceptor agonist to *PNMT*$^{-/-}$ and wild-type mice reconstitutes or exacerbates, respectively, these features of asthma (Lin *et al.*, 2012; Thanawala *et al.*, 2013). Thus, β_2-adrenoceptor agonists may exert pro-inflammatory effects in the mouse lung, via a mechanism that is *independent* of cAMP generation.

If clinical evidence for such β_2-adrenoceptor-mediated biased agonism is established, it would raise important questions about existing LABAs and how the next generation should be designed. For example, to what extent are currently marketed LABAs biased toward β-arrestin recruitment and signalling? Can the effect of LABA bias towards β-arrestin-dependent effectors be reduced or mitigated? Currently, there is some evidence that formoterol and salmeterol display a

degree of bias for β-arrestin (Rajagopal *et al.*, 2011; van der Westhuizen *et al.*, 2014). If confirmed, this highlights the need to understand the structural elements in these ligands that preferentially stabilize the β_2-adrenoceptor in this unwanted conformation. One means to compensate for adverse-effects mediated by β-arrestin-dependent ERK activation is to offset bias by enhancing LABA-induced, cAMP signalling with a PDE4 inhibitor (Forkuo *et al.*, 2016; Pera and Penn, 2016). Indeed, in sensitized and challenged *PNMT*-deficient mice, roflumilast and rolipram prevented formoterol and salmeterol from restoring the asthma-like phenotype when compared with wild-type animals. Similarly, the ability of glucocorticoids, and LABAs, to induce DUSP1 expression, for example in primary human ASM or bronchial epithelial cells (Quante *et al.*, 2008; Manetsch *et al.*, 2012; Rider *et al.*, 2015b; Shah *et al.*, 2016), would independently inactivate ERK. This effect may not only protect against MAPK-dependent desensitization (Nino *et al.*, 2010) but should also reduce unwanted signalling due to biased agonism from the β_2-adrenoceptor. Thus, reducing the effect of β_2-adrenoceptor-mediated, β-arrestin signalling with a PDE4 inhibitor and/or a glucocorticoid could tip the balance away from 'pro-asthma' effects towards more favourable 'therapeutic' outcomes (Forkuo *et al.*, 2016; Pera and Penn, 2016).

These concepts of biased agonism are relevant to a recent reappraisal of the PDE4 inhibitor, roflumilast, in mild-to-moderate asthma (Bardin *et al.*, 2015; Bateman *et al.*, 2015; Meltzer *et al.*, 2015). Clinical efficacy (measured by improvement in forced expiratory volume in 1 s, time to first exacerbation, rescue medication consumption and symptoms) was found, perhaps surprisingly, not to be inferior to an ICS (Bateman *et al.*, 2015). If these findings are confirmed, a strong case for a β_2-adrenoceptor agonist/PDE4 inhibitor combination therapy can be made based on their ability to increase airway calibre, suppress inflammation and offset unwanted effects that may be associated with signal bias. Moreover, this approach would necessarily require combination with an ICS (Meltzer *et al.*, 2015), which should further mitigate adverse effects of LABAs that occur via canonical and non-canonical β_2-adrenoceptor-mediated signalling. The possibility that LABAs and PDE4 inhibitors interact synergistically on target cells and tissues with an attendant improvement in clinical outcomes is also predicted. Logic dictates that this would be most effective in cells and tissues where β_2-adrenoceptor density is limiting and/or coupling efficiency to adenylyl cyclase is weak (Giembycz and Newton, 2014). It is noteworthy, that this approach has been adopted for the treatment of COPD with the development of the novel, bifunctional ligand, GS-5759, which is formulated for inhaled delivery (Salmon *et al.*, 2014; Tannheimer *et al.*, 2014). GS-5759 is a single chemical entity composed of a PDE4 inhibitor linked via a spacer to a β_2-adrenoceptor agonist. This provides greater lung retention, low oral bioavailability, reduced systemic exposure and an improved therapeutic ratio that is typical of inhaled, high molecular weight molecules (Phillips and Salmon, 2012; Robinson *et al.*, 2013). Furthermore, the identical deposition characteristics of the two pharmacophores maximize the opportunity for synergistic interaction at the same target tissues.

Outlook and opportunity for drug discovery

The clinical superiority of ICS/LABA combination therapy is well established and, for some time, has been the standard-of-care where ICS monotherapy fails to provide adequate disease control. While this enhanced effect seems likely to have arisen as a consequence of the natural interactions between endogenous stress response mechanisms, for example between glucocorticoids and catecholamines, the molecular basis remains unclear. Indeed, although several explanations have been proposed to explain how LABAs enhance ICS action, their ability to account for gene-specific effects is questionable (Table 1). It is clear that glucocorticoids and LABAs independently and in combination induce gene transcription. This is important given the increasing appreciation that GR-mediated transactivation contributes to the mechanism of action of ICS. The ability of LABAs to enhance glucocorticoid-dependent transcription and for LABAs and glucocorticoids to synergistically enhance the expression of genes, such as RGS2, provides a compelling explanation for the improved asthma control seen in clinical practice. Therefore, understanding the consequences of LABA/glucocorticoid-induced gene expression is central to the development of improved combination therapies. Knowing which genes are desirable, and which are not, is necessary to comprehend integrated biological function in complex disease networks. Equally, knowing that the enhancement of GR-mediated gene expression is cAMP-dependent provides additional opportunities to improve anti-inflammatory glucocorticoid therapies. For example, adding-on a PDE4 inhibitor may prove useful by augmenting the effects of a LABA in target cells where β_2-adrenoceptor density is low. This approach has the additional advantage of reducing the potential undesirable consequences of biased agonism from the β_2-adrenoceptor. Alternatively, the identification of non-biased, or perhaps G_s-biased, β_2-adrenoceptor agonists or ligands for other GPCRs that are more highly expressed and/or better coupled to adenylyl cyclase on target cells may be highly effective in combination with an ICS. In each case, such effects may be further optimized by combining multiple drugs into single molecules that display unique polypharmacological characteristics.

A further, and currently underexplored, avenue for improving therapeutic efficacy lies with the realization that every GR responsive gene, each with its own unique promoter environment, may be maximally activated only by specific GR conformations (Keenan *et al.*, 2016). Thus, each gene is effectively an independent signal transducer that responds to ligand-activated GR. Furthermore, as each different GR ligand has the potential to preferentially activate gene expression in manner that is dictated by the ligand-induced conformation that is adopted by GR, there is the potential for biased GR-dependent gene expression. However, approaches to improve glucocorticoid effectiveness have generally rested on the concept that GR transactivation is undesirable, whereas transrepression, for example of NF-κB, is beneficial. This may have unwittingly resulted in the identification of partial agonists of GR. Thus, the repression of NF-κB-dependent transcription or genes, such as IL6 and CXCL8, that are NF-κB-dependent versus the ability to activate simple GR-

responsive reporters have been used to screen for candidate ligands. However, the repression of NF-κB-dependent reporters and inflammatory gene expression is routinely observed at lower glucocorticoid concentrations compared with transactivation of simple GRE reporters (Jonat *et al.*, 1990; Chivers *et al.*, 2004; Chivers *et al.*, 2006; King *et al.*, 2013). Thus, partial agonists of GR will inevitably produce separation of repression from transactivation. Indeed, ligands such as RU24858, GW870086X and ciclesonide can behave as partial GR agonists (Joshi *et al.*, 2015a). While this may produce some improvement in therapeutic benefit, the details and functions of genes showing differential expression to agonists with high and low intrinsic efficacy remain to be fully explored. Nevertheless, we submit that the possibility of biased GR agonism offers greater therapeutic potential compared with partial GR agonists.

In conclusion, GR-mediated transactivation of the numerous response genes that contribute to the therapeutic activity of ICS in asthma offers new opportunity for the identification and selection of ligands that may transactivate more desirable groups of effector genes. At present, this area of GR biology is relatively underdeveloped, but could contribute substantially to the discovery of novel therapeutics. Exploiting gene expression bias to design improved anti-inflammatory GR ligands will require changes in gene expression to be correlated with an extensive understanding of their function in on- and off-target tissues. While this is necessarily an empirical approach, modern high-throughput technologies now make the initial part of this objective an achievable goal. Probably more challenging will be to accurately assign gene function as beneficial or undesirable in asthma management. Finally, the identification of biased GR agonists will need to be considered in the context of combination therapies. While this substantially increases the level of complexity, there are correspondingly more opportunities for drug optimization and discovery.

Conflict of interest

The authors declare no conflicts of interest.

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