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RESEARCH PAPER

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

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BACKGROUND AND PURPOSE

Inhaled glucocorticoid (ICS)/long-acting β_2-adrenoceptor agonist (LABA) combination therapy is a recommended treatment option for patients with moderate/severe asthma in whom adequate control cannot be achieved by an ICS alone. Previously, we discovered that LABAs can augment dexamethasone-inducible gene expression and proposed that this effect may explain how these two drugs interact to deliver superior clinical benefit. Herein, we extended that observation by analysing, pharmacodynamically, the effect of the LABA, indacaterol, on glucocorticoid receptor (GR)-mediated gene transcription induced by seven ligands with intrinsic activity values that span the spectrum of full agonism to antagonism.

EXPERIMENTAL APPROACH

BEAS-2B human airway epithelial cells stably transfected with a 2× glucocorticoid response element luciferase reporter were used to model gene transcription together with an analysis of several glucocorticoid-inducible genes.

KEY RESULTS

Indacaterol augmented glucocorticoid-induced reporter activation in a manner that was positively related to the intrinsic activity of the GR agonist. This effect was demonstrated by an increase in response maxima without a change in GR agonist affinity or efficacy. Indacaterol also enhanced glucocorticoid-inducible gene expression. However, the magnitude of this effect was dependent on both the GR agonist and the gene of interest.

CONCLUSIONS AND IMPLICATIONS

These data suggest that indacaterol activates a molecular rheostat, which increases the transcriptional competency of GR in an agonist- and gene-dependent manner without apparently changing the relationship between fractional GR occupancy and

response. These findings provide a platform to rationally design ICS/LABA combination therapy that is based on the generation of agonist-dependent gene expression profiles in target and off-target tissues.

Abbreviations

BDP, beclomethasone-17,21-dipropionate; C5AR1, complement component 5 receptor 1; CRISPLD2, cysteine-rich secretory protein LCCL (Limulus clotting factor C, Cochlin, Lgl1) domain-containing 2; DC, des-isobutyrylciclesonide; Dex, dexamethasone; FF, fluticasone furoate; GILZ, glucocorticoid-inducible leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK 9027, *N*-[4-[1-(4-fluorophenyl)-1*H*-indazol-5-yl-3-(trifluoromethyl)phenyl]benzene sulphonamide]; GW, GW 870086X (6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(2,2,3,3-tetramethylcyclo propylcarbonyl)-oxoandrosta-1,4-diene-17β-carboxylic acid cyanomethyl ester); Mif, mifepristone; Org, Org 34517 (11β-(1,3-benzodioxolo)-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one); $p57^{kip2}$, kinase inhibitor protein 2 of 57 kDa; PDK, pyruvate dehydrogenase kinase; RGS2, regulator of G-protein signalling

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
β2-adrenoceptor	PDK4
C5AR1 ($C5a_1$ receptor)	PKA
Nuclear hormone receptors[b]	**Other protein targets**
Glucocorticoid receptor (GR; NR3C1)	RGS2
Progesterone receptor (NR3C3)	

LIGANDS	
Budesonide	ICI 118551
Dexamethasone	Indacaterol
Formoterol	Mifepristone
Forskolin	Salmeterol
	Vilanterol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

International asthma guidelines recommend that patients with moderate-to-severe disease who are not well controlled by low-dose inhaled glucocorticoid (ICS) be given an ICS/long-acting β_2-adrenoceptor agonist (LABA) combination therapy (Bateman *et al.*, 2008). Currently, four such drug combinations are widely available in single inhaler devices: *Symbicort®* (formoterol fumarate dihydrate plus budesonide, Astrazeneca, London, UK), *Seretide®/Advair®* (salmeterol xinafoate plus fluticasone propionate, GlaxoSmithKline, Brentford, UK), *Foster®/Fostair®* (formoterol fumarate dihydrate plus beclomethasone dipropionate, Chiesi, Parma, Italy) and, more recently, *Zenhale®/Dulera®* (formoterol fumarate dihydrate plus mometasone furoate, Merck, Kenilworth, NJ, USA). The rationale for developing this multicomponent therapeutic stemmed from a study conducted by Greening and colleagues that was published in 1994. In a trial of 6 month duration, these investigators found that the addition of the LABA, salmeterol, to asthmatic subjects who were symptomatic despite maintenance therapy with a standard dose of the ICS, beclomethasone dipropionate (BDP), improved lung function to a significantly greater degree than that achieved by merely increasing the dose of BDP (Greening *et al.*, 1994). The superior clinical benefit of this combination therapy was subsequently corroborated in subjects with more severe asthma in whom symptoms were not controlled by BDP or equivalent drugs (Woolcock *et al.*, 1996). Since the publication of those seminal studies, many asthma trials have confirmed the clinical superiority of ICS/LABA combination therapy relative to an ICS alone using a variety of outcome measures including lung function, symptoms, the need for rescue medication and frequency of exacerbations (Pauwels *et al.*, 1997; Shrewsbury *et al.*, 2000; O'Byrne *et al.*, 2001; Frois *et al.*, 2009; Ducharme *et al.*, 2010; Sears, 2011). Testament to the success of *Symbicort* and *Seretide/Advair* in asthma management is the emergence of second-generation, ICS/LABA combination therapy. An example is *Breo®/Relvar®* [vilanterol trifenatate plus fluticasone furoate (FF), GlaxoSmithKline], which has the advantage of once-a-day dosing (Cazzola *et al.*, 2011) and was recently granted marketing authorization by the European Commission. Additional, so-called, ultra-LABAs are likely to become available within the next few years including indacaterol, which will probably be combined with long-acting GCs such as mometasone furoate (Tamm *et al.*, 2012).

The molecular mechanism(s) that accounts for the enhanced clinical efficacy of ICS/LABA combination therapy is unclear. It is believed that ICS exert their beneficial effects in asthma by attenuating the expression of pro-inflammatory genes. Two general mechanisms have been proposed. In the classical repressive mode of glucocorticoid action, termed *trans*repression, the activity of pro-inflammatory transcription factors, such as NF-κB and AP-1, is inhibited via direct

interactions with the agonist-bound glucocorticoid receptor (NR3C1; here on referred to as GR) (De Bosscher *et al.*, 2003). *Trans*repression via a direct interaction of the agonist-bound GR to negative glucocorticoid response elements (GREs) in the promoter regions of target genes has also been defined (Surjit *et al.*, 2011). However, in simple model systems, glucocorticoids are often only partial inhibitors of pro-inflammatory gene transcription, implying that processes in addition to *trans*repression must be operative to account for their anti-inflammatory effects in *bona fide* models of inflammation (Clark, 2003; 2007; Newton and Holden, 2007; Newton *et al.*, 2010). To accommodate these findings, compelling evidence has emerged that the induction (*trans*activation) of genes, many encoding proteins with anti-inflammatory potential, also constitutes a major mechanism of glucocorticoid action (Clark, 2003; 2007; Newton and Holden, 2007; Newton *et al.*, 2010). In this respect, Kaur *et al.* (2008) found that the ability of dexamethasone (Dex) to induce a variety of genes in human airway myocytes and bronchial epithelial cells was significantly enhanced by a LABA above the level achieved by the glucocorticoid alone irrespective of its concentration. We hypothesized that this mechanism may help explain why ICS/LABA combination therapy often controls moderate-to-severe asthma whereas high-dose ICS monotherapy does not (Giembycz *et al.*, 2008; Kaur *et al.*, 2008; Newton *et al.*, 2010; Giembycz and Newton, 2011). Moreover, to our knowledge, there is no evidence that LABAs enhance GR-mediated *trans*repression.

Despite the success of ICS/LABA combination therapy, the beneficial clinical outcomes reported in the original study of Greening *et al.* (1994) were serendipitous. Clearly, therefore, the rational design of new ICS/LABA combinations based on gene expression profiles (Newton *et al.*, 2010; Joshi *et al.*, 2015) could offer opportunities to improve safety and efficacy. To aid this objective, we have extended the observations of Kaur *et al.* (2008) by examining the effect of a LABA on the pharmacodynamics of GR-mediated gene expression in BEAS-2B human airway epithelial cells using indacaterol as a representative example. A simple GRE luciferase reporter was used for this purpose together with an analysis of a panel of glucocorticoid-inducible genes encoding proteins with both anti-inflammatory and adverse effect potential. Seven ligands that bind GR were compared including the clinically relevant compounds Dex, FF and des-isobutyrylciclesonide (DC), which were assumed to be full agonists, GW870086X (GW); a novel ligand that displays partial agonism on a number of functional outputs (Uings *et al.*, 2013); and a non-steroidal agonist, GSK 9027 (Yates *et al.*, 2010). For completeness, two purported GR receptor antagonists, mifepristone (Mif; Gagne *et al.*, 1985) and Org 34517 (Org; Peeters *et al.*, 2004), were also included in the analysis.

Methods

Measurement of GRE-dependent transcription

BEAS-2B cells stably transfected with a 2× GRE luciferase reporter (Chivers *et al.*, 2004) were cultured with GR agonist alone or in combination with indacaterol or forskolin as indicated. In some experiments, cells were pretreated with the GR antagonist, Org (60 min; Peeters *et al.*, 2004), or the alkylating agent, dexamethasone 21-mesylate (Dex-Mes; 30 min; Simons and Thompson, 1981) before being exposed to a GR agonist. At 6 h, cells were lysed and luciferase activity was measured by luminometry. Alternatively, total RNA was extracted and gene expression was assessed by real-time PCR.

RNA isolation, reverse transcription and real-time PCR

Real-time PCR was performed as described previously (Joshi *et al.*, 2015). In brief, total RNA was extracted from BEAS-2B cells, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer sequences (Table 1) designed to amplify glucocorticoid-induced leucine zipper (GILZ), kinase inhibitor protein 2 of 57 kDa ($p57^{kip2}$), cysteine-rich secretory protein LCCL domain-containing 2 (CRISPLD2), pyruvate dehydrogenase kinase 4 (PDK4) and complement component 5 receptor 1 (C5AR1).

Curve fitting

Monophasic agonist *E*/[A] curves were fitted by least-squares, non-linear iterative regression to the following form of the Hill equation (Prism 4®, GraphPad, San Diego, CA, USA) (Motulsky and Christopoulos, 2003):

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

where E is the effect, $E_{\min}$ and $E_{\max}$ are the lower and upper asymptote, $p[A]$ is the negative log molar concentration of agonist, $p[A]_{50}$ is the negative log molar concentration of agonist producing $(E_{\max} - E_{\min})/2$ and n is the gradient of the E/[A] curve at the $p[A]_{50}$ level.

Determination of antagonist equilibrium dissociation constants

The affinity (K_B) of Org was determined by global, least-squares, non-linear regression using a modification of the Hill and Gaddum/Schild equations (Waud *et al.*, 1978) according to equation 2. Thus,

$$E = E_{\min} + \left(\frac{(E_{\max} - E_{\min})}{1 + \left(\frac{10^{p[A]50}\left[1 + \left(\frac{[B]}{10^{-pA2}}\right)^{S}\right]}{[A]} \right)^{n}} \right) \quad (2)$$

where $[A]$ and $[B]$ are the molar concentration of full agonist and partial agonist/antagonist, respectively; S is the Schild slope factor and pA_2 is the affinity of the partial agonist/antagonist when $S = 1$, which is equivalent to the pK_B.

Determination of agonist equilibrium dissociation constants

Agonist affinity (K_A) 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). *E*/[A] curves were generated in cells treated (30 min) with vehicle or Dex-Mes at the concentrations indicated. Each set of *E*/[A] curves was then fitted

Table 1
Primer pairs for real-time PCR

Gene	Oligonucleotide	Accession number(s)
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′	
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′	
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′	
C5AR1		NM_003955.3
Forward	5′-GAT TCT CCT TCA ATT CCT CAG CTT-3′	
Reverse	5′-ATT AGT TCA GCA TTC CCG AAG TGT-3′	
GAPDH		NM_002046.5, NM_001256799.2
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 gene names are shown and, where appropriate, official HUGO gene symbols are given in brackets. Generic primers were used for genes encoding multiple isoforms.

simultaneously to the operational model of agonism (equation 3; Black and Leff, 1983). Algebraically,

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

where E_m is the theoretical maximum response of the tissue, $[A]$ is the agonist concentration, n is the gradient of the relationship between the concentration of agonist–receptor complexes (AR) and response, and τ is the efficacy of the agonist, which is defined as $[R_t]$ (the total functional receptor concentration)/K_E (the concentration of 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. Only τ, which at submaximal responses decreases proportionally with the remaining fraction of non-inactivated receptors, was allowed to vary between individual E/[A] 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 agonist before (τ) and after (τ') receptor inactivation. The fraction of functionally active GR (q) remaining after treatment of cells with Dex-Mes is given by τ'/τ.

Determination of partial agonist equilibrium dissociation constants

The K_A value of a partial agonist was determined by operational model fitting (Black and Leff, 1983) using the comparative method (Barlow *et al.*, 1967) as described previously (Joshi *et al.*, 2015).

Drugs and analytical reagents

Des-isobutyrylciclesonide, Org 34517, GSK 9027 and indacaterol were from Nycomed (Konstanz, Germany), Organon Laboratories (Oss, The Netherlands), Tocris Bioscience (Bristol, UK) and Gilead Sciences (Seattle, WA, USA) respectively. GW870086X and fluticasone furoate were from GlaxoSmithKline (Stevenage, Hertfordshire, UK). Dexamethasone, dexamethasone 21-mesylate and mifepristone were purchased from Steraloids (Newport, RI, USA). Forskolin was purchased from Calbiochem/EMD Technologies/Merck KGaA (Damstadt, Germany). All drugs were dissolved in MSO and diluted to the required working concentrations in culture medium. We confirm that the nomenclature of GPCRs and nuclear hormone receptor conforms to BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013a,b).

Statistics

Data are presented as the mean ± SEM of n independent determinations and analysed by Student's two-tailed t-test or repeated measures one-way ANOVA followed, when appropriate, by Tukey's multiple comparison test. To establish whether an association existed between the transcriptional responses produced by a panel of GR agonists in the absence and presence of indacaterol, linear (r^2) and rank-order correlations (Spearman's ρ) were performed. The null hypothesis was rejected when $P < 0.05$.

Results

Effect of indacaterol on GRE-dependent transcription

Concentration–effect curves were constructed to six steroidal GR ligands for their ability to drive GRE-dependent transcription in 2× GRE BEAS-2B reporter cells in the absence and presence of a maximally effective concentration (100 nM) of the LABA, indacaterol (Figure 1A–F). Five of these ligands were active with a rank order of potency of FF > DC > Dex = GW > Mif (Table 2). However, these ligands displayed varying degrees of agonism (Figure 2A). Relative to FF, which was the strongest agonist and assigned an intrinsic activity (α) value of 1, Dex, DC, GW and Mif were partial agonists with α values that ranged from 0.96 for Dex to 0.02 for Mif (Figure 2A; Table 2). The sixth steroidal ligand studied, Org, was inactive (Figures 1F and 2A; Table 2).

Indacaterol (100 nM) did not activate 2× GRE BEAS-2B reporter cells *per se* (Supporting Information Fig. S1) but significantly enhanced the expression of luciferase activity in a GR agonist-dependent manner. Pretreatment of cells with ICI 118551 (10 nM for 60 min) produced a 186-fold dextral displacement of the indacaterol concentration–response curve from which a pA_2 value of 9.49 was derived, which is consistent with a β_2-adrenoceptor-mediated mechanism (see Alexander *et al.*, 2013a). In all cases, the enhancement of reporter activation by indacaterol occurred in the absence of any change in glucocorticoid potency (Figure 1A–F; Table 2). Indacaterol did not markedly alter the intrinsic activity values of Dex, DC, GW and Mif relative to the reference agonist, FF (Figure 2B; Table 2), although Org now displayed extremely weak but, nevertheless, detectable agonism (Figure 1F). Accordingly, the rank-order correlation (Spearman's ρ) between GR agonist intrinsic activity values in the absence and presence of indacaterol was 1 (Figure 2C).

Indacaterol, added concurrently with a fixed, maximally effective concentration of each of the five active glucocorticoids, augmented luciferase expression in a concentration-dependent manner with identical potency ($[A]_{50}$s ~700 pM; Figure 1G–K; Table 3). Org (1 μM) also promoted GRE-dependent transcription in the presence but not in the absence of indacaterol (Figure 1L), although the maximum degree of agonism (α = 0.005) relative to FF was very modest (Table 3).

Effect of indacaterol on GSK 9027-induced GRE-dependent transcription

GSK 9027, a non-steroidal GR agonist (Yates *et al.*, 2010), induced luciferase activity in a concentration-dependent manner with a $p[A]_{50}$ of 6.77 and intrinsic activity values of 0.77 and 0.85 (relative to FF and Dex, respectively; Figures 2A and 3A; Table 2). Indacaterol (100 nM) enhanced (approximately threefold) the magnitude of GSK 9027-induced, GRE-dependent transcription without changing its potency or intrinsic activity (Figures 2B, 2C and 3A; Table 2). In 2× GRE BEAS-2B reporter cells treated with a fixed, maximally effective concentration of GSK 9027 (3 μM), indacaterol augmented luciferase activity in a concentration-dependent manner with a potency ($p[A]_{50}$ = 9.13) similar to that found for the enhancement of luciferase activity by steroidal GR agonists (Figure 3B; Table 3). Pretreatment of these cells with Org (1 μM) antagonized (>90%) GSK 9027-induced GRE-dependent transcription in the absence and presence of indacaterol, suggesting that this was a GR-mediated effect (Supporting Information Fig. S2).

Relationship between intrinsic activity and the enhancement of GRE-dependent transcription by indacaterol

The relationship between the fold induction of luciferase activity by the seven GR ligands examined in the absence and presence of indacaterol (100 nM) was linear (Figure 4A); the stronger the agonism at GR, the greater was the fold induction produced by the LABA (ρ = 1). Replotting the *y*-axis as fold *enhancement* of GRE-dependent transcription produced by indacaterol for each ligand indicated that this was a curvilinear, saturable response (Figure 4B). It is noteworthy that ligands with little (Mif) or no (Org) measurable agonist activity were rendered very partial agonists in the presence of indacaterol.

Effect of indacaterol on the pharmacodynamics of GR-mediated reporter activation

The pharmacodynamics of ligands that signal through nuclear hormone receptors is poorly studied. Accordingly, we applied the operational model of agonism to assess the effect of indacaterol on GRE-dependent transcription. Concentration–effect curves were constructed to Dex and FF in the absence and presence of indacaterol (100 nM) before and after controlled, fractional GR inactivation with Dex-Mes under conditions (10 nM for 30 min followed by washout) that suppressed the upper asymptote of the control agonist concentration–response curve. As shown in Figure 5 (panels A and B), the upper asymptote of the Dex and FF E/[A] curves were reduced by 61 and 53%, respectively, by Dex-Mes in the absence of any significant change in agonist potency. Similar data were obtained in the presence of indacaterol (100 nM) (Figure 5C and D). Operational model fitting showed that indacaterol (100 nM) had no effect on the affinity (K_A) or operational efficacy (τ) of FF or Dex for GR (Table 4). Thus, $K_A/[A]_{50}$ ratios were unchanged and approached values of 1 indicating a lack of GR reserve where response is a linear function of occupancy. Indeed, the fraction ($1 - q$) of GR inactivated by Dex-Mes (0.58), defined as $1 - (\tau'/\tau)$ in the operational model, mirrored the percentage loss in maximum luciferase activity (Figure 5). However, model estimates of the tissue maximum response parameter, E_m (dashed lines in Figure 5), were considerably greater than the respective upper asymptotes of the Dex and FF E/[A] curves. Thus, despite the significant increase in transcriptional competency of ligand-bound GR imparted by indacaterol, there was still extra capacity in the system suggesting that these glucocorticoids behaved as partial agonists in driving GRE-dependent transcription in BEAS-2B cells in the absence and presence of indacaterol.

Operational model fitting was also used to determine if the enhancement of GRE-dependent transcription by indacaterol (100 nM) 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, GSK9027 and DC for GR were not changed by indacaterol (Supporting Information Fig. S4; Table 4).

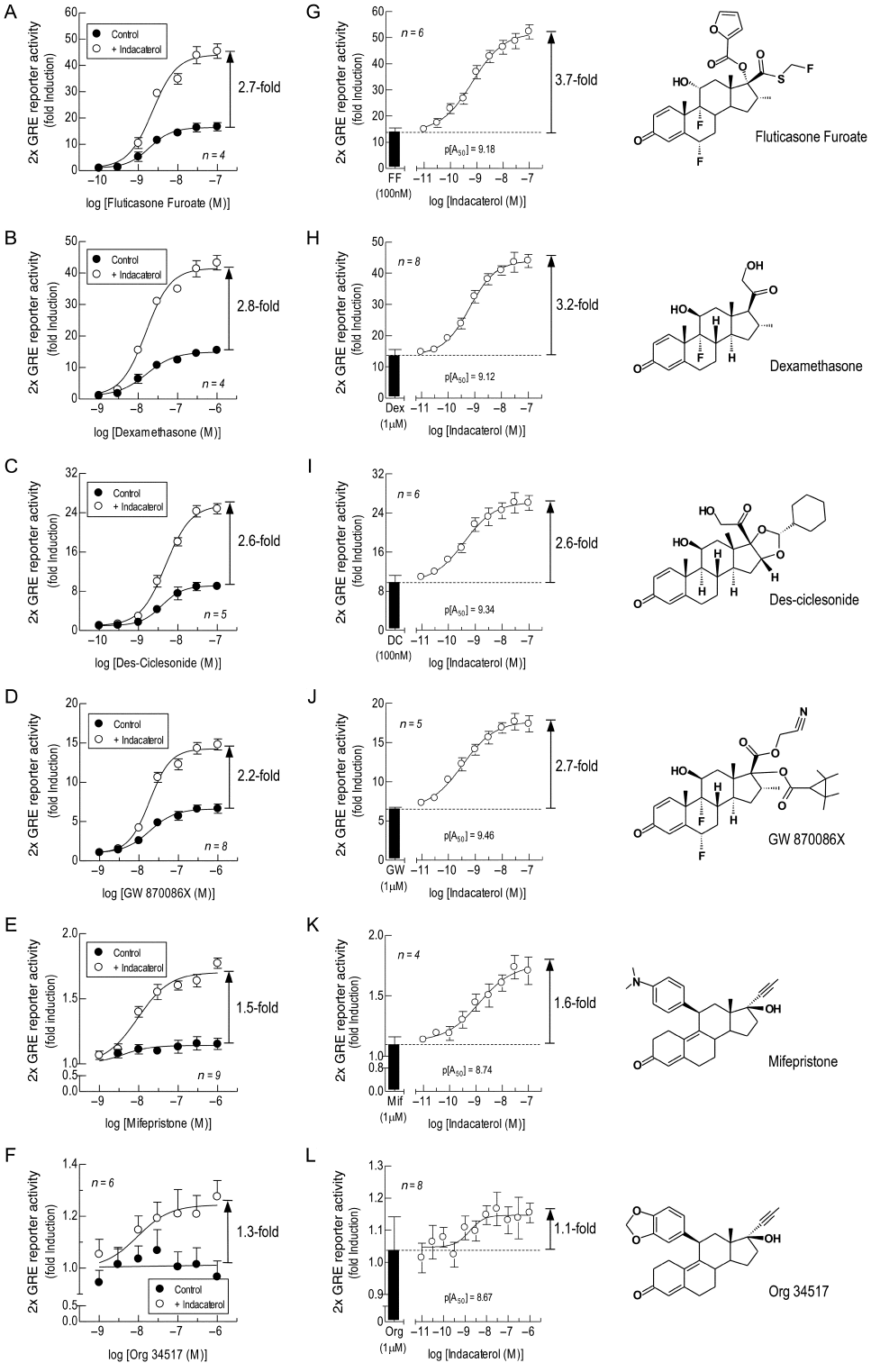

Figure 1

Effect of indacaterol on GRE-dependent transcription in 2× GRE BEAS-2B reporter cells. Cells were treated with FF (A), Dex (B), DC (C), GW (D), Mif (E) or Org (F) at the concentrations indicated in the absence and presence of indacaterol (100 nM), which was added concurrently. In panels G–L, cells were treated with indacaterol over the concentration ranges indicated in the presence of a maximally effective concentration of each glucocorticoid that was determined from the data shown in panels A–F. At 6 h cells were harvested, luciferase activity was determined and *E*/[A] curves were constructed. Data points represent the mean ± SEM of *n* independent determinations. The fold values in each panel indicate the maximal fold enhancement of GRE-dependent transcription produced by indacaterol (100 nM). The dashed lines in panels G–L indicate luciferase activity produced by glucocorticoid alone.

Table 2

Effect of a maximally effective concentration of indacaterol on the potency and intrinsic activity values of a panel of glucocorticoid receptor ligands to promote GRE-dependent transcription in BEAS-2B reporter cells

Glucocorticoid	*n*	p[A]$_{50}$ –Indacaterol	Fold induction –Indacaterol	α[a]	p[A]$_{50}$ +Indacaterol	Fold Induction +Indacaterol[b]	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 1A–F and 3A using indacaterol at a concentration of 100 nM.
[a]Intrinsic activity (fold induction by GR ligand – 1)/(fold induction by FF – 1).
[b]Values determined from pooled data.

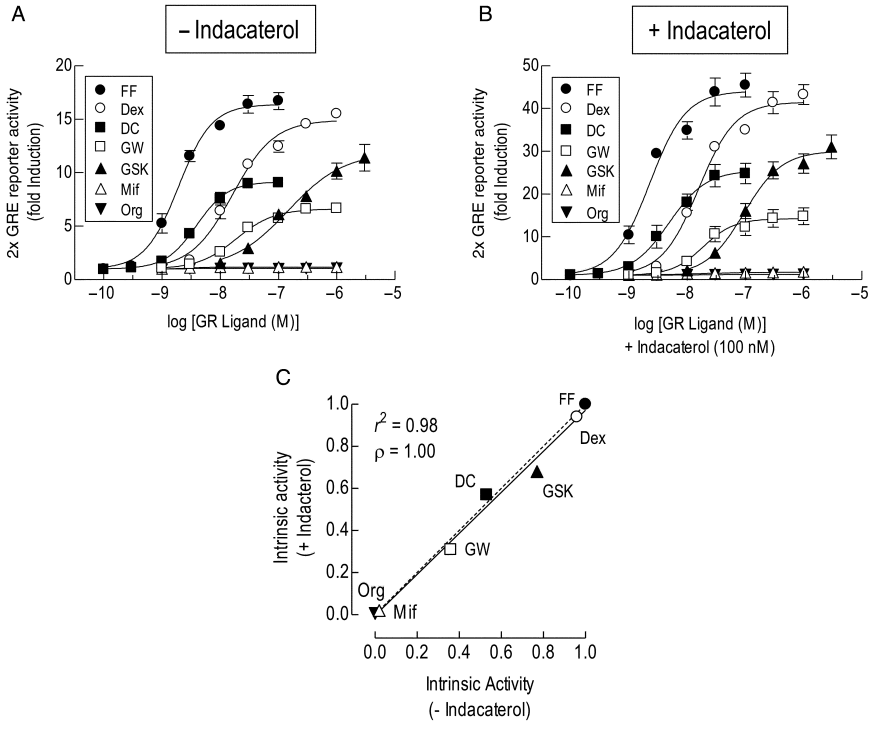

Figure 2

Effect of indacaterol on the intrinsic activity values of a panel of GR ligands in promoting GRE-dependent transcription in 2× GRE BEAS-2B reporter cells. Cells were treated with GR ligands at the concentrations indicated in the absence (panel A) and presence (panel B) of indacaterol (100 nM) and *E*/[A] curves were then constructed. Panel C shows the relationship between the intrinsic activity values of each GR ligand 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 ± SEM of four to seven independent determinations (see Table 2).

Effect of indacaterol and forskolin on antagonist affinity for GR

Pretreatment (60 min) of 2× GRE BEAS-2B reporter cells with Org (10 and 100 nM) produced graded, parallel, dextral displacements of the *E*/[A] curves that described Dex-induced GRE-dependent transcription. Analyses of these data using a modification of 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 indacaterol (100 nM) when added concurrently with Dex (Supporting Information Fig. S4A and B). The affinity of Org was also unchanged when forskolin (10 μM) was substituted

Table 3

Concentration-dependent enhancement by indacaterol of GRE-dependent transcription in BEAS-2B reporter cells

Glucocorticoid	Concentration (μM)	*n*	2× GRE BEAS-2B reporter activity (fold induction)		Fold enhancement by indacaterol[a]	Indacaterol potency (p[A]$_{50}$ (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 1G–L and 3B.
[a]Maximum fold induction induced by GR ligand in the presence of indacaterol/maximum fold induction by GR ligand alone.
[b]Values are the mean of eight experiments.

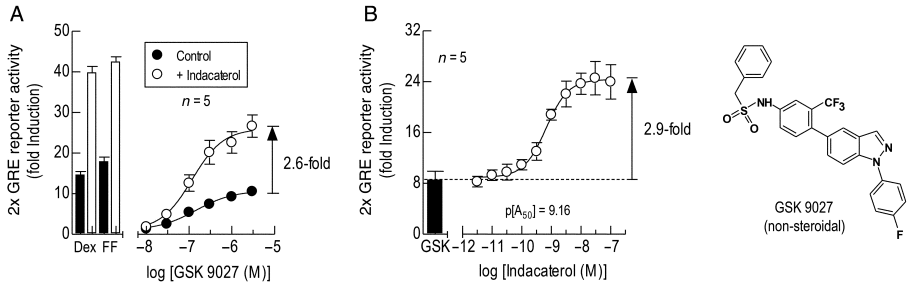

Figure 3

Effect of indacaterol on GSK 9027-induced, GRE-dependent transcription in BEAS-2B reporter cells. In panel A, cells were treated with GSK 9027 at the concentrations indicated in the absence and presence of indacaterol (100 nM), which was added concurrently. Dex (1 μM) and FF (100 nM) in the absence and presence of indacaterol were included as comparators. In panel B, cells were treated with indacaterol (3 pM to 100 nM) in the presence of a maximally effective concentration of GSK 9027 (3 μM) determined from the data shown in panel A. At 6 h cells were harvested, luciferase activity was determined and *E*/[A] curves were constructed. Data points represent the mean ± SEM of *n* independent determinations. The fold values in each panel indicate the maximal fold enhancement of GRE-dependent transcription produced by indacaterol (100 nM). The dashed line in panel B indicates luciferase activity produced by GSK 9027 alone.

for indacaterol using a larger panel of glucocorticoids (FF, Dex, DC and GW; Supporting Information Fig. S4C–J; Table 5).

Effect of indacaterol on GR-mediated gene expression

The data presented in the preceding sections indicated that the enhancement of GRE-dependent transcription in BEAS-2B reporter cells by indacaterol was positively related to the intrinsic activity of the GR agonist. To determine the extent to which this relationship held for the expression of *bona fide* genes, which are under considerably more complex regulation than a simple 2× GRE reporter, we took advantage of data derived from two previous microarray analyses in which glucocorticoid-inducible genes were identified in pulmonary type II A549 cells and BEAS-2B cells respectively. Several of these genes have anti-inflammatory (*GILZ*, *p57*^{kip2}, *CRISPLD2*, *C5AR1*) or adverse effect (*PDK4*) potential and were selected for that reason to examine the interaction between GR agonists and indacaterol (Samuelsson *et al.*, 1999; Sugden and Holness, 2002; Köhl *et al.*, 2006; Eddleston *et al.*, 2007; Wang *et al.*, 2009; Giembycz and Newton, 2014; Vasarhelyi *et al.*, 2014). These genes were also chosen to illustrate that the ability of indacaterol to enhance GR-mediated transcription was gene and GR agonist dependent (*vide infra*).

As shown in Figure 6, the interaction between indacaterol and GR agonists varied in a gene-dependent manner. The expression of *PDK4* was similar to that found on the 2× GRE reporter with Dex, GSK 9027, DC and GW demonstrating increasing partial agonism relative to FF (Figure 6; Table 6A). Indacaterol (100 nM) significantly augmented the GR-mediated expression of *PDK4* preserving the same relative degrees of agonism (Figure 6A; Table 6). Mif had no effect on the expression of *PDK4*, but produced significant, albeit weak, agonism in cells exposed concurrently to indacaterol (Figure 6A). In contrast, Org did not induce *PDK4* in the absence or presence of indacaterol (Figure 6A). Similar data

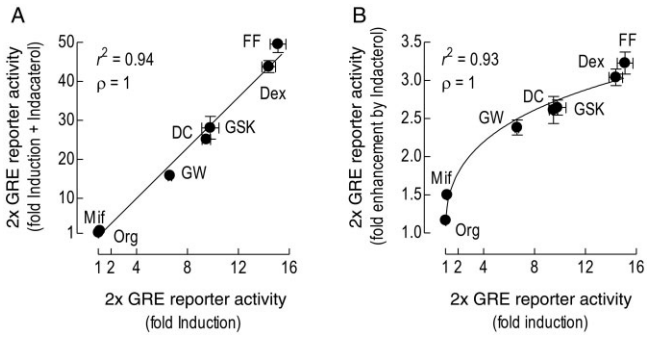

Figure 4

Influence of agonist intrinsic activity on the ability of indacaterol to enhance GRE-dependent transcription in 2× GRE BEAS-2B reporter cells. Panel A shows the relationship between GRE-dependent luciferase activity produced by seven GR ligands in the absence and presence of indacaterol (100 nM), which was linear. Panel B shows that the fold enhancement by indacaterol of the maximal transcription produced by each GR ligand as a saturable function of agonist intrinsic activity.

were obtained for *p57*^{kip2} although there were noticeable differences (Figure 6B; Table 6). In particular, FF was significantly more effective at inducing *PDK4* than Dex in the absence and presence of indacaterol, whereas with *p57*^{kip2} there was no discrepancy (Figure 6B). Furthermore, indacaterol rendered Org a very weak partial agonist on *p57*^{kip2} (cf. *PDK4*; Figure 6B).

A different agonist-dependent expression profile was seen with *CRISPLD2*. Thus, maximally effective concentrations of GSK 9027, GW and DC were as effective as FF and Dex in driving the expression of this gene (Figure 6C; Table 6). Indacaterol (100 nM) alone produced a robust induction (~10-fold) of *CRISPLD2* and, in addition, augmented the activity of these five GR agonists to a similar degree that was greater than the sum of their individual effects (Figure 6C). Indacaterol also significantly increased the very modest agonist activity of Mif and Org (Figure 6C).

The final glucocorticoid-inducible gene examined, *GILZ*, was induced to a similar degree by all GR agonists tested except Mif and Org (Figure 6D; Table 6). However, in contrast to *CRISPLD2*, indacaterol (100 nM) had no effect *per se* on this gene and failed to significantly enhance *GILZ* expression produced by FF, Dex, GSK 9027, DC and GW (Figure 6D). Conversely, *C5AR1*, whose expression may suppress allergic sensitization (Köhl *et al.*, 2006), was significantly ($P < 0.05$) increased by indacaterol but not by FF (9.8 ± 0.9-fold and 1.9 ± 0.3-fold induction, respectively; 6 h exposure, $n = 6$).

The relationship between the ability of each GR ligand to induce *PDK4* and *p57*^{kip2} (expressed as fold induction) in the absence and presence of indacaterol was linear (Figure 7A and B). Re-plotting these data as fold *enhancement* of the maximum GR agonist-induced response showed that the effect of indacaterol was saturable with a maximal fourfold (*PDK4*) and 2.7-fold (*p57*^{kip2}) enhancement produced with GR agonists of high intrinsic efficacy (Figure 7C and D).

Effect of indacaterol on the E/[A] relationship that describes FF- and GW-induced gene expression

FF and GW were selected to further investigate the effect of indacaterol on GR-mediated gene expression because they were the strongest and weakest agonists identified that could be studied in a quantitative manner. Both of these GR agonists increased the expression of *GILZ*, *PDK4*, *CRISPLD2* and *p57*^{kip2} in a concentration-dependent manner (Figure 8). Consistent with the 2× GRE reporter data, FF was more potent (3.3- to 6.9-fold) than GW on each of the four genes studied (sensitivity: *GILZ* = *CRISPLD2* > *p57*^{kip2} > *PDK4*; Table 7). Moreover, although both glucocorticoids were equi-effective at inducing *CRISPLD2* and *GILZ*, GW 870086X was a partial agonist on *p57*^{kip2} and *PDK4* with intrinsic activity values of 0.76 and 0.30, respectively (relative to FF; Figure 8A–D), which is in broad agreement with the results displayed in Figure 7 (see Table 6). Indacaterol (100 nM) significantly augmented FF- and GW-induced *p57*^{kip2}, *PDK4* and *CRISPLD2* expression. Notably, the increases in gene expression maxima were not associated with any changes in agonist potency. Therefore, because indacaterol did not modify the affinity of either FF or Dex for GR (Supporting Information Fig. S4), K_A/A_{50} ratios were preserved. Although these data mimicked the effect of indacaterol on the 2× GRE BEAS-2B reporter (cf. Figures 1 and 8), their interpretation is more complex because *CRISPLD2* and, to a much lesser extent, *p57*^{kip2} were induced by indacaterol alone (Figure 8; Table 7). Nevertheless, inspection of the *E*/[A] curves showed that the combination of indacaterol (100 nM) 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, indacaterol did not significantly affect glucocorticoid-induced *GILZ* expression under identical experimental conditions (Figure 8A and E; Table 7).

Indacaterol was 'steroid sparing' in a gene-dependent manner

The data in Figure 8 show that indacaterol exerted a steroid-sparing effect on transcription that was gene dependent. This was clearly evident on the expression of *p57*^{kip2} and *PDK4* but not on *GILZ*. Figure 9 illustrates the interaction between indacaterol and FF in more detail using *p57*^{kip2} as a representative gene. Thus, at the $p[A]_{95}$, FF increased the gene expression by 11.5-fold. Indacaterol (100 nM), which was a weak activator of *p57*^{kip2} (2.6-fold), produced two main effects. First, the same degree of induction was achieved at a concentration of FF that was 10-fold lower (Figure 9; green line). Second, the same concentration of FF significantly enhanced maximum *p57*^{kip2} mRNA levels by 270% to 31.5-fold (Figure 9; pink line). With regard to *CRISPLD2*, indacaterol (100 nM), by itself, produced a robust response that was equal to or greater than a maximally effective concentration of both FF and GW.

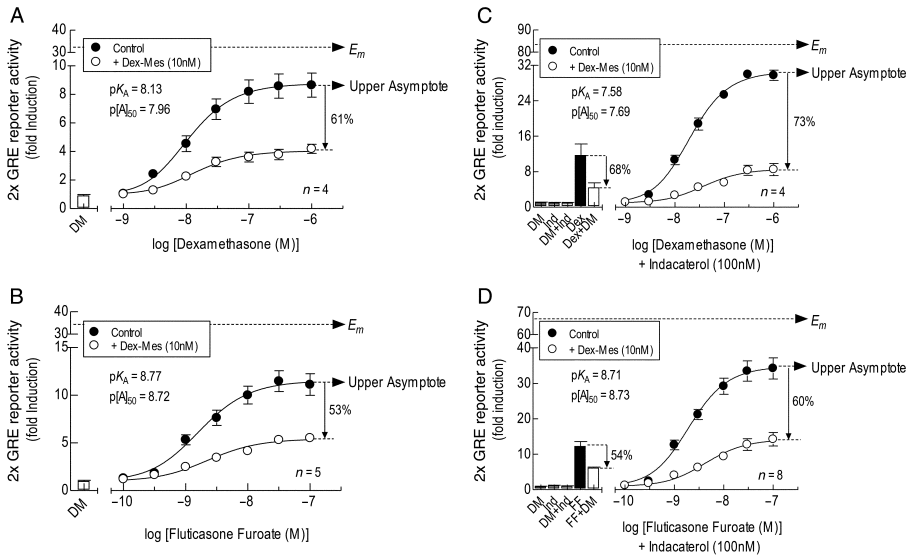

Figure 5

Analysis of Dex and FF *E*/[A] curve data in the absence and presence of Dex-Mes by operational model fitting. 2× GRE BEAS-2B reporter cells were treated with Dex-Mes (10 nM or 30 min) or vehicle. The cells were washed in Dex-Mes-free medium and *E*/[A] curves constructed to Dex (panels A and C) or FF (panels B and D) in the absence and presence of indacaterol (100 nM) as described in the legend to Figure 1. The model parameter estimates for Dex and FF are provided in Table 4. The bars in each panel show the effect of Dex-Mes, indacaterol, Dex-Mes/indacaterol, GR agonist and GR agonist after treatment of cells with Dex-Mes respectively. The downward arrows in each panel reflect the percentage inhibition of luciferase activity produced by Dex-Mes. The system maximum parameter, E_m, and upper asymptote of control *E*/[A] curves are shown in each panel. Data points represent the mean ± SEM of *n* independent determinations.

Discussion

ICS/LABA combination therapy was first launched in Europe in 1998 and has become firmly established worldwide as an effective treatment option for many patients with moderate-to-severe asthma. Indeed, the Global Initiative for Asthma guidelines recommend that an ICS/LABA combination therapy be used in patients in whom adequate control cannot be achieved by an ICS alone (http://www.ginasthma.org). Despite the success of ICS/LABA combination therapy, it remains unclear how these two drug classes interact at a molecular level to produce superior clinical benefit over ICS monotherapy (Giembycz *et al.*, 2008). We have reported previously that a LABA can augment glucocorticoid-inducible gene expression beyond the maximum effect produced by a glucocorticoid alone (Kaur *et al.*, 2008; Rider *et al.*, 2011; 2013; Moodley *et al.*, 2013; Holden *et al.*, 2014) and have provided empirical evidence that this could contribute to the mechanism of action of these medicines (Holden *et al.*, 2011). The present study extends those observations by examining the effect of the LABA, indacaterol, on the pharmacodynamics of gene expression produced by a panel of GR ligands that span the spectrum from full agonism to antagonism. Herein we show that in the human airway epithelial cell line, BEAS-2B, indacaterol (i) augmented GR-mediated transcription in a gene- and GR agonist-dependent manner by a mechanism that was associated with an increase in the system maximum parameter, E_m, in the absence of any change in agonist potency, efficacy or affinity; (ii) enhanced gene expression produced by a *non-steroidal* GR agonist; (iii) was steroid sparing in a gene-dependent manner; and (iv) rendered ligands that behaved as GR antagonists very weak agonists.

Indacaterol enhanced GRE-dependent transcription in an agonist-dependent manner

As shown in Figure 2, the GR ligands examined were not equivalent in their ability to promote GRE-dependent transcription. The intrinsic activity of these ligands varied from a value of 1 for FF (the strongest agonist tested) to a value of 0 for Org with the remaining compounds displaying intermediate partial agonist behaviour. In the presence of a maximally effective concentration of indacaterol, which enhanced GR-mediated reporter activation, these intrinsic activity values were preserved. Thus, a plot of α values in the absence and presence of indacaterol was linear with a rank-order correlation value of 1 indicating that the degree of augmentation was proportional to the intrinsic efficacy of the ligand.

Indacaterol also enhanced GRE-dependent transcription produced by the indazole-based GR agonist, GSK 9027. This is an important observation because non-steroidal ligands such AZD 5423, a structural analogue of GSK 9027 in phase II clinical development for respiratory diseases (Gauvreau *et al.*, 2015), may bind GR in a manner that is distinct from classical glucocorticoids and could logically form part of a novel combination therapy.

Effect of indacaterol on the pharmacodynamics of GR-mediated luciferase expression

We have shown previously that LABAs augment the activity of 2× GRE BEAS-2B reporter cells by a mechanism that

Table 4

Effect of indacaterol 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 S* +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

Operational parameters were derived from the data shown in Figures 5 and Supporting Information Fig. S4. Indacaterol was used at a concentration of 100 nM.

[a]E_m values for GSK 9027, DC and GW were derived by the comparative method using FF as a reference agonist and, by definition, are equivalent to the E_{max} values determined directly from the FF *E*/[A] curve by logistic curve fitting. Accordingly, values of τ assume that FF generates a response that is equivalent to the E_m. However, GR inactivation studies (Figure 5) indicate that this assumption is incorrect. Thus, in the comparative method, E_m and τ values are significantly underestimated and overestimated, respectively, and are italicized for that reason.

[b]Values determined from mean data.

involves the activation of cAMP-dependent PKA (Kaur *et al.*, 2008). Although the identity of the PKA substrate(s) is unknown, it is possible that the increase in gene transcription is dependent on the phosphorylation of GR and a consequent change in agonist pharmacodynamics. The precedent for this idea derives from several studies in which phosphorylation of GR at Ser^{211} induces a conformational change and an attendant increase in gene transcription (Miller *et al.*, 2005; 2007; Chen *et al.*, 2008; Garza *et al.*, 2010; Trevino and Weigel, 2013). However, although this possibility cannot be excluded, indacaterol (and forskolin) did not affect the potency or affinity of any GR agonist tested. The affinity of the GR antagonist, Org, was also preserved. An analysis of Dex and FF *E*/[A] curve data by operational model fitting, before and after fractional GR inactivation, demonstrated that indacaterol had no effect on the efficacy parameter, τ (Black and Leff, 1983), which is also consistent with these findings. 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, which increases the transcriptional competency of liganded GR and, therefore, the magnitude of gene expression, without changing the relationship between fractional GR occupancy and response (*vide infra*). This conclusion is consistent with the observation that indacaterol enhanced GRE-dependent transcription by resetting the system maximum parameter, E_m.

Effect of indacaterol on GR-mediated gene expression

The preceding discussion centres on the pharmacodynamics of GR-mediated reporter activation and its enhancement by a LABA. However, 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 indacaterol. Strikingly, the behaviour of these agonists varied in a gene-dependent manner as we have reported previously (Joshi *et al.*, 2015). Thus, on *PDK4* and, to a lesser degree, $p57^{kip2}$, FF, Dex, GSK 9027, DC and GW displayed increasing partial agonism and this was reproduced in the presence of indacaterol, 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 indacaterol 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 *E*/[A] analysis focusing on FF and GW, which displayed marked, gene-dependent differences in agonist activity. On *PDK4*, indacaterol 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

Table 5

Effect of indacaterol and forskolin on the affinity of Org 34517 for GR

GR agonist	pK_B	pK_B + Forskolin	pK_B + Indacaterol
Fluticasone furoate	8.34[a]	8.36	ND
Dexamethasone	8.38[a]	8.35	8.39[b]
Des-ciclesonide	8.12[a]	8.39	ND
GW 870086X	8.46[a]	8.38	ND

The affinity of the GR antagonist, Org 34517, was calculated by Schild analysis from the data shown in Supporting Information Fig. S4. Indacaterol and forskolin were used at concentrations of 100 nM and 10 μM respectively.
[a]Data taken from Joshi *et al.* (2015).
[b]pA_2 value determined using Org 34517 at 10 and 100 nM.
ND, not determined.

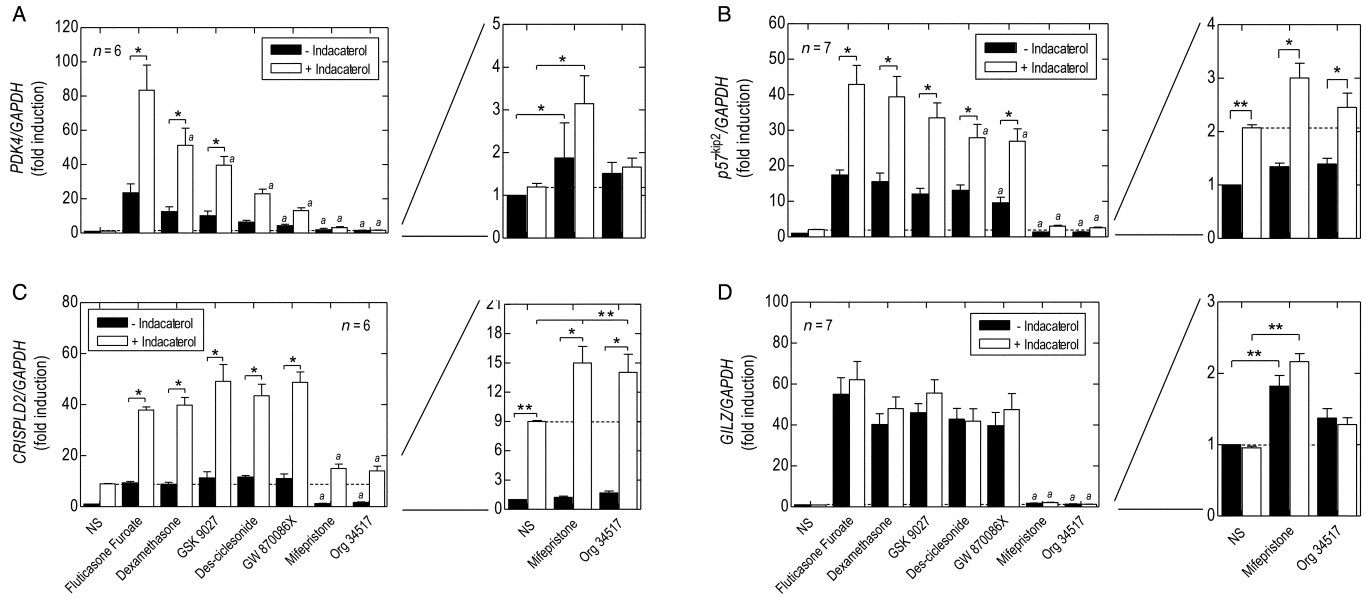

Figure 6

Comparative effects of indacaterol on gene expression produced by a panel of GR ligands. 2× GRE BEAS-2B reporter cells were treated with FF (100 nM), Dex (1 μM), GSK 9027 (3 μM), DC (100 nM), GW (1 μM), Mif (1 μM) or Org (1 μM) in the absence and presence of indacaterol (100 nM). At 6 h, total RNA was extracted, reverse transcribed and the resulting cDNA subjected to real-time PCR using primer pairs specific for *PDK4*, *p57*^{kip2}, *CRISPLD2* and *GILZ*. Data are the mean ± SEM of *n* independent determinations and are expressed a ratio to *GAPDH*. **P* < 0.05, significant enhancement of gene expression produced by indacaterol; ***P* < 0.05, significant induction of gene expression relative to untreated (NS) or indacaterol-treated cells. Data were analysed by repeated measures, one-way ANOVA/Tukey's multiple comparisons test. ^{a}P < 0.05, significant difference in gene expression relative to that produced by fluticasone furoate.

reporter. However, the interpretation of these results is not straightforward. A previous study (Jeong *et al.*, 2012) allied with transcription factor binding site mining using MatInspector® (Genomatix, Munich, Germany) identified putative GREs and CREs in the *PDK4* promoter. Therefore, the mechanistic basis of positive cooperativity between indacaterol and GR agonist on *PDK4* and the GRE reporter may not be shared. With *p57*^{kip2} and *CRISPLD2* more complex regulation is clear. Indeed, these genes were directly up-regulated by indacaterol in the absence of GR agonist presumably following the phosphorylation and binding of CREB family transcription factors to putative CREs in their promoters. Thus, several processes could account for the supra-additive effect produced by indacaterol and GR agonists on *p57*^{kip2} and *CRISPLD2* that are not mutually exclusive. These could include the operative mechanism in GRE reporter cells and/or cooperativity between GR and CREB as has been described for the somatostatin gene (Liu *et al.*, 1994). Equally, there is the possibility of independent, but concurrent, activation of gene expression through GRE-, CRE- and/or composite sites, which may vary in number and arrangement in different gene promoters. The final gene studied, *GILZ*, was strongly induced by FF and GW. However, *GILZ* expression was not significantly affected by indacaterol at any GR agonist concentration tested

Table 6

Intrinsic activity values of GR agonists for the induction of *p57*^{kip2}, *GILZ*, *PDK4* and *CRISPLD2* in 2× GRE BEAS-2B reporter cells and the effect of indacaterol

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

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 FF, which was assigned a value of 1. Indacaterol was used at a concentration of 100 nM and added concurrently with the GR ligand.

[a]Intrinsic activity values calculated from the data shown in Figure 6. Effects produced by indacaterol alone have been subtracted.

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

Collectively, the findings discussed earlier are relevant to understanding how these two drug classes interact at a molecular level to provide asthma control. It has been reported that LABAs enhance agonist-dependent translocation of GR to the nucleus and that this effect may lead to superior clinical outcomes (Eickelberg *et al.*, 1999; Roth *et al.*, 2002; Profita *et al.*, 2005; Usmani *et al.*, 2005; Haque *et al.*, 2013). However, it is difficult to reconcile this 'translocation' hypothesis with the data reported herein. For example, it cannot explain how indacaterol augmented GR-mediated gene expression produced by a maximally effective concentration of FF that would presumably promote the translocation of all GR to the nucleus. Moreover, the ability of a LABA to enhance GR translocation would be expected to up-regulate all glucocorticoid-inducible genes rather than selected populations. The fact that evidence against this hypothesis has also been reported (Loven *et al.*, 2007) suggests that alternative explanations should be considered. One possibility is that cAMP modulates the behaviour of obligatory co-activators and/or co-repressors. Literature precedent for this idea derives from studies of the progesterone receptor, where 8-Br-cAMP is reported to augment gene expression by a mechanism that involves the dissociation of the co-repressors, NCoR and SMRT, rendering the ligand-bound receptor more transcriptionally competent (Wagner *et al.*, 1998; Chen *et al.*, 2014). Significantly, this effect extended to partial agonists, and also 'antagonists', which now demonstrated very weak agonism (Wagner *et al.*, 1998). Such a mechanism is attractive because those proteins targeted by PKA may only regulate a subset of glucocorticoid-inducible genes, which accommodates the observation that indacaterol did not significantly augment *GILZ* expression.

Clinical implications

The agonist-dependent differences in GR-mediated *trans*activation reported herein and the enhancement by indacaterol raise an important question. How much GR agonism is required in a clinically effective ICS/LABA combination therapy? The answer to this question is unclear although one could speculate that high-efficacy GR agonists like FF are necessary as they will ensure the robust induction of all desirable genes that are also susceptible to further up-regulation by a LABA. Clearly, a potential disadvantage could be the unwanted co-expression of adverse effect genes, especially if there is significant systemic exposure (although this could be mitigated by enhancing pulmonary retention, first-pass hepatic metabolism and plasma protein binding). Alternatively, drugs like GW, an agonist of moderate intrinsic efficacy, may be preferable. Such agonists should *trans*activate many therapeutically relevant genes that can be further enhanced by LABA, but with a more favourable adverse effect profile providing a 'safer' medicine. As we have commented previously (Newton *et al.*, 2010), an understanding of gene function is essential if ICS/LABA combination therapy is to be rationally designed based on gene expression fingerprinting.

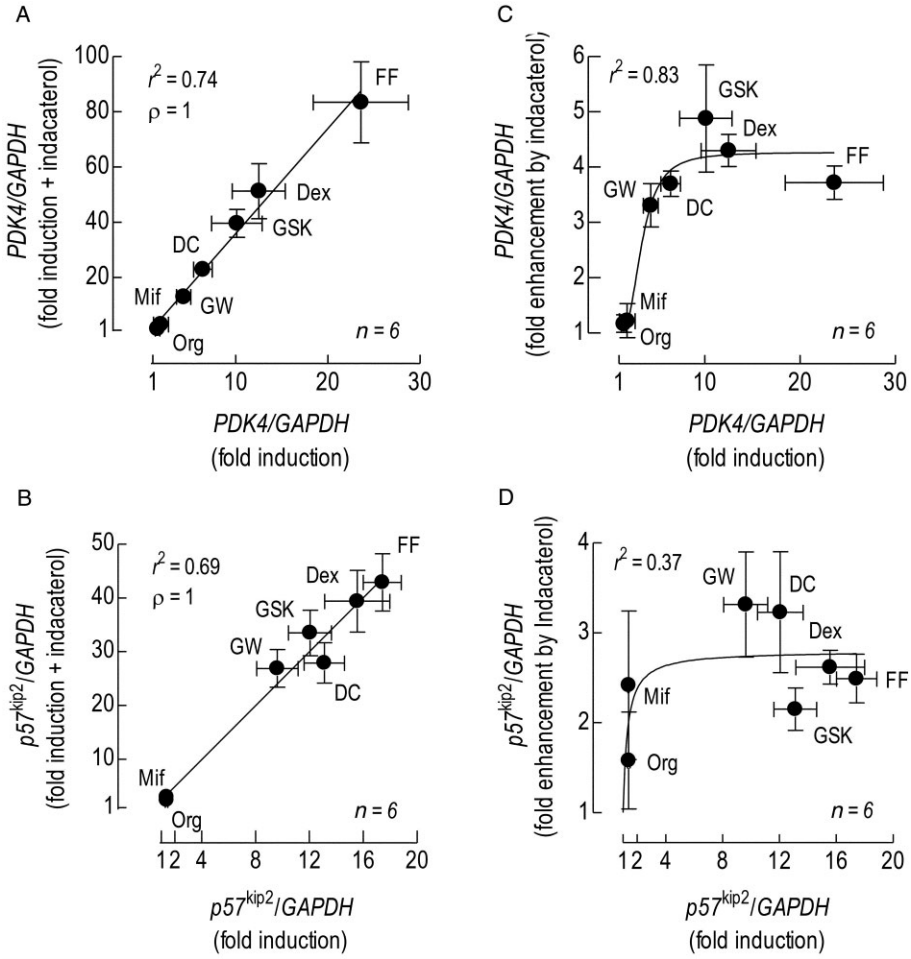

Figure 7

Effect of GR ligand intrinsic activity on the ability of indacaterol to enhance the expression of *PDK4* and *p57*^{kip2}. Panels A and B show the relationship between the expression of *PDK4* and *p57*^{kip2} produced by seven GR agonists in the absence and presence of indacaterol (100 nM), which was linear. Panels C and D show that the fold enhancement by indacaterol of the maximal transcription produced by each GR agonist produced by indacaterol was a saturable function of agonist intrinsic activity.

Limitations of the study

Herein, we have used data garnered from the BEAS-2B human airway epithelial cell line to further develop the hypothesis that ICS/LABA combination therapy may provide effective asthma control by augmenting the expression of glucocorticoid-inducible genes (Giembycz and Newton, 2015). Although this cell line is not always predictive of the behaviour of primary cells in culture or of the airway epithelium *in vivo*, similar data have been reported in human primary bronchial epithelia and human airway myocytes in the absence and presence of pro-inflammatory stimuli (Kaur *et al.*, 2008; Rider *et al.*, 2011; 2013; Moodley *et al.*, 2013). Moreover, glucocorticoids given by the inhaled and oral routes up-regulate many genes in the airways of patients with asthma that have putative anti-inflammatory potential (Kelly *et al.*, 2012; Yick *et al.*, 2013). We propose that these clinical data support the possibility that LABAs may further enhance glucocorticoid-induced gene expression in asthmatic subjects as they do in cells in culture. However, whether such effects, if found, contribute to improved clinical outcomes will be difficult to investigate. Indeed, establishing a causal relationship between genes that are up-regulated by a glucocorticoid, either alone or in the presence of a LABA, and 'anti-asthma' outcomes in simple cell-based systems has been challenging possibly because of gene redundancy. Nevertheless, evidence for such causality is available with genes encoding RGS2 (Holden *et al.*, 2014), CRISPLD2 (Himes *et al.*, 2014) and dual specificity phosphatase-1 (Shah *et al.*, 2014) being representative examples.

Conclusions

The present study provides a plausible explanation that may contribute to the clinical efficacy of ICS/LABA combination therapy in asthma management. It derives from the premise that each drug works in a distinct and mutually cooperative way to up-regulate genes that have anti-inflammatory, bronchoprotective and, potentially, anti-remodelling properties. Thus, discrete populations of genes exist that are induced by

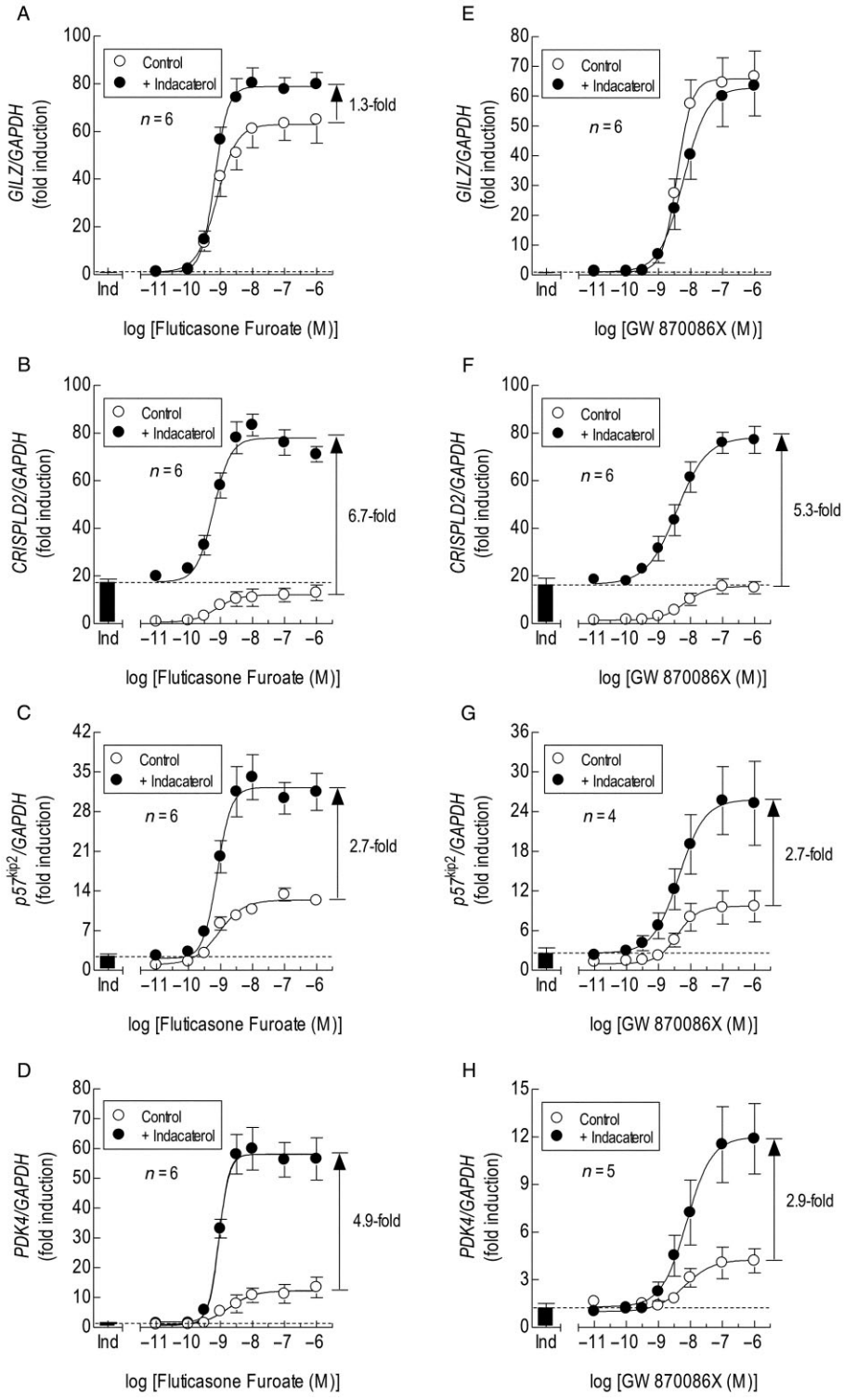

Figure 8

Effect of indacaterol on the ability of FF and GW to promote gene expression. 2× GRE BEAS-2B reporter cells were treated with FF (panels A–D) and GW (panels E–H) in the absence and presence of a maximally effective concentration of indacaterol (100 nM). At 6 h, 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 *E*/[A] curves. Each bar and data point represents the mean ± SEM of *n* independent determinations. The dashed line in each panel defines gene expression produced by indacaterol alone. Quantification and statistical analyses of these data are presented in Table 6. The upward arrows in each panel show the fold enhancement by indacaterol of gene expression produced by a maximally effective concentration of GR agonist.

Table 7
Effect of indacaterol on the potency and ability of fluticasone furoate and GW 870086X to promote gene expression in 2× GRE BEAS-2B reporter cells

Gene	*GILZ*		*CRISPLD2*		*p57*^{kip2}		*PDK4*	
Treatment	p[A]$_{50}$ (M)	Maximum induction (fold)	p[A]$_{50}$ (M)	Maximum induction (fold)	p[A]$_{50}$ (M)	Maximum induction (fold)	p[A]$_{50}$ (M)	Maximum induction (fold)
Indacaterol[a]	ND	0.88 ± 0.1	ND	17.5 ± 1.1**	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 +indacaterol	9.16 ± 0.07	80.0 ± 5.5	9.15 ± 0.10	78.6 ± 3.8*	9.20 ± 0.02	33.9 ± 1.3*	9.10 ± 0.07	58.0 ± 6.5*
Indacaterol[a]	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*	8.37 ± 0.10	25.9 ± 6.1*	8.08 ± 0.17	12.2 ± 2.3*

Data were calculated from the *E*/[A] curves shown in Figure 8 and represent the mean ± SEM of six independent determinations with the exception of GW 870086X on *p57*^{kip2} and *PDK4* where $n = 4$ and 5 respectively.
*$P < 0.05$, significant enhancement by indacaterol of FF- and/or GW-induced gene expression – Student's two-tailed, unpaired *t*-test.
**$P < 0.05$, significant induction of gene expression by indacaterol alone relative to untreated cells – Student's two-tailed, unpaired *t*-test.
[a]A fixed concentration of indacaterol (100 nM) was added concurrently with the glucocorticoid and gene expression measured at 6 h.
ND, not determined.

Figure 9

Effect of indacaterol on the expression of *p57*^{kip2} produced by FF. The graph in Figure 8C has been redrawn to illustrate that the addition of indacaterol (100 nM) to FF (10 pM to 1 μM) enhanced the expression of a representative glucocorticoid-inducible gene, *p57*^{kip2}, in a 'steroid-sparing' manner. In these cells, indacaterol had 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 p[A]$_{95}$ (a 270% increase; pink line). Indacaterol was also 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). Ind, indacaterol.

ICS alone, by LABA alone and by ICS and LABA in combination, with opportunities for additive and/or synergistic interactions (Figure 10). Implicit in this model is that an ICS and a LABA when combined, but *not* individually, generate a unique gene expression 'fingerprint' that helps control patients with moderate-to-severe asthma. Moreover, because transcriptional competency of GR varies markedly in an agonist- and gene-dependent manner (*vide supra*), it may be possible to rationally design an improved ICS/LABA combination therapy on the basis of agonist-dependent gene expression profiles in target and off-target tissues (Newton *et al.*, 2010). Clearly, if maximum therapeutic benefit is to be realized by this mechanism, coincident deposition of an ICS and a LABA in target tissues is vital. Logic dictates that this is more likely to occur when a patient inhales the combination in one breath from a single device as this would facilitate the ability of a LABA and an ICS to interact at a molecular level. Consistent with this prediction, Theophilus *et al.* (2006) found significantly greater co-deposition of fluticasone propionate and salmeterol from a combination meter dose inhaler than when each drug was given separately. More importantly, greater improvements in morning peak flow have been reported for fluticasone propionate and salmeterol fixed dose combination therapy when compared with both drugs given to subjects concurrently by separate inhaler devices (Nelson *et al.*, 2003; Huchon *et al.*, 2009). It is important to emphasize that the interaction between an ICS and a LABA could also lead to the up-regulation of adverse effect (typically metabolic) genes. However, currently, there is little evidence that this occurs at extra-pulmonary sites presumably because of low systemic exposure (Kirby *et al.*, 2001;

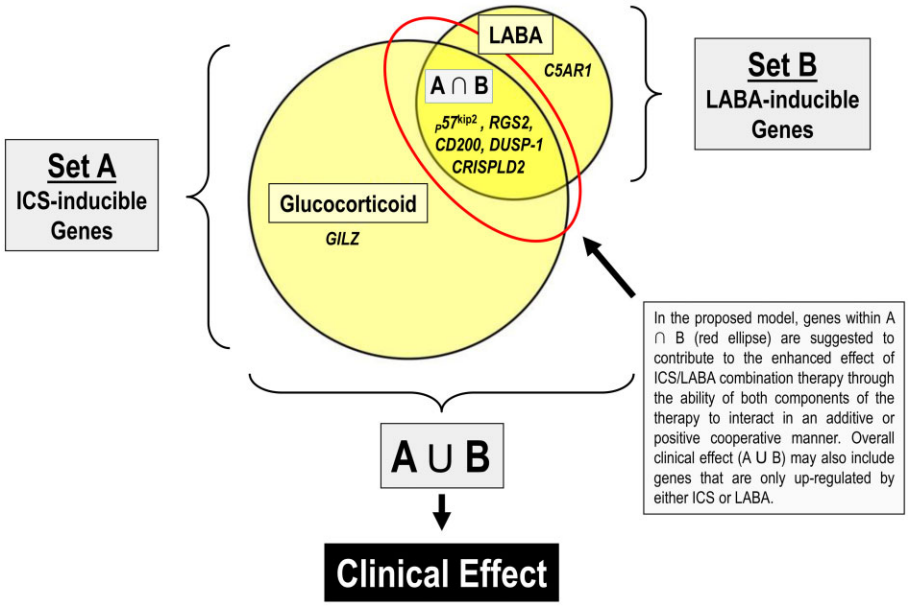

Figure 10

Hypothetical Venn diagram illustrating a mechanism that may contribute to the clinical superiority of ICS/LABA combination therapy. Two 'sets' of genes are shown that are induced by glucocorticoid (A) or LABA (B). Some of these genes are only induced by one component of the combination therapy, while others are induced by both glucocorticoid and LABA. According to the model, A ∩ B is suggested to be primarily responsible for the enhanced effect of ICS/LABA combination therapy through the ability of both components to interact in an additive or positive cooperative manner. However, disease-modifying genes that are induced by ICS alone (e.g. *GILZ*) and LABA alone (*C5AR1*) may also play an important role such that it is the 'union' of both gene populations (A U B) that delivers the clinical effect. The Venn diagram is populated with examples of genes that fall within set A, set B or A ∩ B. The size of each set and the area of intersection that defines the union are unknown. It is important to note that potential adverse effect genes (e.g. *PDK4*, not shown) will also constitute part of the Venn diagram and could, by the same mechanism, increase side effects. Similarly, there may be populations of LABA-inducible, anti-inflammatory genes that are inhibited by ICS. Currently, *in vivo* studies in humans to determine if a LABA can augment glucocorticoid-induced gene expression in the airways have not been performed. *C5AR1*, complement component 5 receptor 1 (Köhl *et al.*, 2006); *CD200*, cluster of differentiation 200 (Snelgrove *et al.*, 2008); *CRISPLD2*, cysteine-rich secretory protein LCCL (limulus clotting factor C, cochlin, lgl1) domain-containing 2 (Vasarhelyi *et al.*, 2014, Wang *et al.*, 2009; Himes *et al.*, 2014); *GILZ*, glucocorticoid leucine zipper (Eddleston *et al.*, 2007; Ayroldi and Riccardi, 2009); *DUSP-1*, MAPK phosphatase-1 (Korhonen and Moilanen, 2014); *p57*^{kip2}, kinase inhibitor protein 2 of 57 kDa (Samuelsson *et al.*, 1999); *RGS2*, regulator of G-protein signalling 2 (Holden *et al.*, 2011; 2014).

Barnes, 2002). In conclusion, the present study supports the working hypothesis that the clinical efficacy of an ICS and a LABA in combination may be attributable, in part, to their collective actions on gene transcription, which necessarily requires coincident drug deposition in target cells and tissues involved in asthma pathogenesis.

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Author contributions

M. A. G., T. J. and R. N. participated in research design. T. J. conducted the experiments. M. J. contributed novel reagents. M. A. G. and T. J. performed data analysis. M. A. G., M. J., T. J. and R. N. wrote or contributed to the writing of the manuscript.

Conflict of interest

T. J., M. J., R. N. and M. A. G. have nothing to declare. T. J. is a recipient of an Alberta Lung Association Graduate Studentship. R. N. is an Alberta Innovates–Health Solutions Senior Scholar. M. A. G. holds a Tier 1 Canada Research Chair in Pulmonary Pharmacology.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13087

Figure S1 Effect of the selective β2-adrenoceptor agonist, ICI 118551, on the enhancement of dexamethasone-induced, GRE-dependent transcription by indacaterol. In 2× GRE BEAS-2B reporter cells, *E*/[A] curves were constructed to indacaterol (Ind) alone and after pretreatment (60 min) with ICI 118551 (ICI; 10 nM) in the presence of dexamethasone (Dex; 1 μM). Each pair of *E*/[A] curves was then fitted simultaneously to equation 2 from which a pA2 of 9.49 was derived, which is consistent with a β2-adrenoceptor-mediated effect (Alexander *et al.*, 2013a). The effect of indacaterol in the absence of dexamethasone is also shown. Bars and data points represent the mean ± SEM of five independent determinations.

Figure S2 Effect of the GR antagonist, Org 34517, on GSK 9027-induced, GRE-dependent transcription. 2× GRE BEAS-2B reporter cells were pretreated with Org 34517 (Org; 1 μM; 60 min) or its vehicle and then exposed to GSK 9027 (GSK; 3 μM), or GSK 9027 and indacaterol (Ind; 100 nM) in combination. At 6 h cells were harvested and luciferase activity was determined. The effect of indacaterol and Org 34517 alone and in combination was also examined as controls. Bars represent the mean ± SEM of *n* independent determinations. Data were analysed by repeated measures, one-way ANOVA/Tukey's multiple comparisons test. **P* < 0.05, significant antagonism of GRE-dependent transcription by Org 34517.

Figure S3 Determination of the affinity of GW 870086X, des-ciclesonide and GSK 9027 for GR by operational model fitting using the comparative method. In 2× GRE BEAS-2B reporter cells, *E*/[sA] curves were constructed to fluticasone furoate (FF) and GW 870086X (panels A and B), fluticasone furoate and des-ciclesonide (panels C and D) and fluticasone furoate and GSK 9027 (panels E and F) in the absence and presence of indacaterol (100 nM). The resulting pairs of curves were fitted simultaneously to equations 1 and 3 from which estimates of *K*A were derived (see Table 4). Data points represent the mean ± SEM of *n* independent determinations. It should be noted that *E*m values for GSK 9027, DC and GW 870086X were derived by the comparative method using FF as a reference agonist and, by definition, are equivalent to the *E*max values determined directly from the FF *E*/[A] curve by logistic curve fitting. Accordingly, values of τ assume that FF generates a response that is equivalent to the *E*m. However, GR inactivation studies (Figure 5) indicate that this assumption is incorrect. Thus, in the comparative method, *E*m and τ values are significantly underestimated and overestimated respectively.

Figure S4 Schild analysis of the antagonism of GRE-dependent transcription by Org 34517. In 2× GRE BEAS-2B reporter cells, *E*/[A] 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 pretreatment (60 min) with Org 34517 (Org) in the absence and presence of forskolin (Forsk; 10 μM) or indacaterol (Ind; 100 nM) as indicated. Each family of *E*/[A] curves was then fitted simultaneously to equation 2 from which the pKB or, in panel B, pA2 of Org 34517 was derived. The bars in each panel show the effect on luciferase activity of each concentration of Org 34517 alone; the 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 Joshi *et al.* (2015).