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RESEARCH PAPER

Discovery of GW870086: a potent anti-inflammatory steroid with a unique pharmacological profile

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BACKGROUND AND PURPOSE

Glucocorticoids are highly effective therapies for a range of inflammatory diseases. Advances in the understanding of the diverse molecular mechanisms underpinning glucocorticoid action suggest that anti-inflammatory molecules with reduced side effect liabilities can be discovered. Here we set out to explore whether modification of the 17α position of the steroid nucleus could generate molecules with a unique pharmacological profile and to determine whether such molecules would retain anti-inflammatory activity.

EXPERIMENTAL APPROACH

The pharmacological properties of GW870086 were compared with fluticasone propionate (FP) using a range of cellular and *in vivo* model systems, including extensive gene expression profiling.

KEY RESULTS

GW870086 repressed inflammatory cytokine release from lung epithelial cells in a similar manner to FP but antagonized the effect of dexamethasone on MMTV-driven reporter gene transactivation. GW870086 had a strong effect on the expression of some glucocorticoid-regulated genes (such as *PTGS2*), while having minimal impact on the expression of other known target genes (such as *SGK*). GW870086 retained the ability to strengthen tight junctions in epithelial cell culture but, unlike FP, was unable to protect the culture from elastase-mediated damage. In murine models of irritant-induced contact dermatitis and ovalbumin-induced allergic inflammation, GW870086 showed comparable anti-inflammatory efficacy to FP.

CONCLUSION AND IMPLICATIONS

GW870086 is a potent anti-inflammatory compound with a unique ability to regulate only a subset of those genes that are normally affected by classical glucocorticoids. It has the potential to become a new topical steroid with a different safety profile to existing therapies.

Abbreviations

AHR, airway hyperresponsiveness; COPD, chronic obstructive pulmonary disease; FF, fluticasone furoate; FP, fluticasone propionate; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MMTV, mouse mammary tumour virus; SGK, serum- and glucocorticoid-induced PK

Introduction

Since the discovery of the remarkable anti-inflammatory activity of cortisol in 1948, glucocorticoids have continued to be highly effective agents for the control of a wide range of inflammatory diseases (Gifford, 1973; Barnes and Adcock, 1993; Weisman, 1996; Cohen and Conn, 1997; Falkenstein *et al.*, 2000; Allen *et al.*, 2003; Katz, 2004; Lundberg *et al.*, 2004). However prolonged systemic exposure results in a plethora of unwanted physiological changes, and these severely limit the therapeutic potential of glucocorticoids. These effects include diabetogenesis, myopathy and

osteoporosis (Reid, 2000; Schacke *et al.*, 2002; Lafage-Proust *et al.*, 2003; McIlwain, 2003; Uings and Farrow, 2005). Topical delivery is one approach that has been adopted to limit the systemic liabilities of treatment, and considerable effort has been made to optimize the physicochemical and pharmacokinetic properties of such molecules (Barnes, 2000; Lipworth and Jackson, 2000; Crim *et al.*, 2001; Allen *et al.*, 2003; Humbert, 2004; Uings and Farrow, 2005; Salter *et al.*, 2007; Farrow, 2008). This new generation of therapeutic agents [including budesonide, fluticasone propionate (FP) and mometasone furoate] have minimal systemic exposure, but are still very effective in the treatment of asthma, chronic obstructive pulmonary disease (COPD), rhinitis and dermatitis.

Despite the considerable advances made with topical therapy, the continuing absence of alternative therapeutic options with comparable efficacy for these diseases has led to a renewed focus on the molecular mechanisms of glucocorticoid function as a potential route to further improvements in therapeutic index (Belvisi *et al.*, 2001a; Uings and Farrow, 2005). The glucocorticoid receptor (GR) is a ligand-activated transcription factor that moves from the cytoplasm into the nucleus when activated, where it exerts its primary effects by regulating transcription of target genes (Cato and Wade, 1996; Barnes, 1998; Webster and Cidlowski, 1999; Herrlich, 2001; Gupta and Lalchhandama, 2002). GR contains a DNA-binding domain, which binds directly to glucocorticoid response elements (GREs) within regulatory regions of target genes, thereby either increasing or decreasing the transcription of such genes. However, not all glucocorticoid-responsive genes contain such GRE sequences, and it has long been recognized that GR also functions to repress the activity of transcription factors such as NF-kB and activator protein 1 in a manner that does not require DNA binding through the DNA-binding domain (Ray *et al.*, 1995). Over the last decade, a range of other mechanisms have been described for individual genes, including interactions with STAT proteins and Ets transcription factors (Mullick *et al.*, 2001; Goleva *et al.*, 2002). However it is clear that all the mechanisms described, and all the clinical effects observed with glucocorticoids, both the beneficial and the unwanted, are driven by activation of the same GR.

Experiments with mutated versions of GR began to suggest that, despite being mediated by a single receptor, some of these mechanisms were distinct and separable (Reichardt *et al.*, 2000a, b; Wintermantel *et al.*, 2004), and this sparked an extensive search for novel ligands that retained anti-inflammatory activity while lacking the unwanted side effect profile (Vanden Berghe *et al.*, 1999; Belvisi *et al.*, 2001b; Mohler *et al.*, 2007; Schacke *et al.*, 2007, 2008, 2009a; Reuter *et al.*, 2010). This effort rapidly moved away from the classical steroid chemical template in the search for more radical changes in pharmacology, and the most advanced of these non-steroidal ligands (ZK 245186) is now reported to be in early clinical development for atopic dermatitis (Schacke *et al.*, 2009a). However, in most cases, the pharmacology of these molecules has not been extensively described. Many were screened for potency and altered pharmacology using assays that rely on cellular measurement of GR-dependent reporter gene transactivation and transrepression, for example utilization of the mouse mammary tumour virus (MMTV) promoter and common NF-κB binding motifs coupled to readouts convenient for high-throughput screening. However, recent insights into GR pharmacology indicate that this simple transactivation-versus-transrepression approach may not reflect the real complexity of GR-mediated transcriptional responses (Clark and Belvisi, 2012; Joanny *et al.*, 2012). We therefore elected to additionally characterize novel GR ligands by utilizing specific gene expression profiling and by studying meaningful responses in established transformed cell lines.

Although the steroid template has been the subject of extensive manipulation by medicinal chemists over several decades, one area of the molecule that had until recently received relatively little attention was the 17α ester substituent. The most successful topical glucocorticoid, FP (1) bears a simple propionate ester (R' = ethyl) at this position. Replacement of this propionate ester with a 2-furoate ester gave fluticasone furoate (FF), showing enhanced affinity for the GR and improved tissue affinity (Salter *et al.*, 2007) This novel glucocorticoid was launched as a once-daily intranasal product for rhinitis (Veramyst) in 2007 and has recently completed Phase III clinical trials both alone and in combination with the long-acting β2 agonist vilanterol, showing once-daily efficacy in both asthma and COPD (Oliver *et al.*, 2012). An X-ray structure of FF in the ligand-binding domain of the GR shows the furoate ester to be accommodated in a lipophilic 17α pocket in the receptor (Biggadike *et al.*, 2008), and we undertook a detailed study of alternative 17α ester moieties to fully explore the effect of this substituent on GR pharmacology.

The data presented here show for the first time that it is possible to identify a novel steroidal GR ligand that can activate only a subset of those genes that are normally activated by classical glucocorticoids, but that retains the ability to repress key pro-inflammatory genes. This novel glucocorticoid also has pharmacokinetic properties that make it particularly suitable for topical use, and it is now in clinical development for the treatment of asthma.

Methods

Transrepression of TNF-stimulated NF-κB reporter gene activity

The transcriptional activity of NF-κB was assessed using a stably transfected A549 cell line containing a secreted placental alkaline phosphatase gene under the control of an E-selectin promoter exactly as described (Salter *et al.*, 2007). cells were seeded into 384-well plates at a density of 10 000 cells·per well and treated with glucocorticoid for 1 h prior to stimulation with 3.2 ng·mL^{-1} TNF-α. Alkaline phosphatase activity was determined spectrophotometrically 15 h later at 405 nm following addition of an equal volume of *p*-nitrophenylphosphate (2 mg·mL^{-1} in 1 M diethanolamine, pH 9.8, 0.5 mM $MgCl_2$ and 0.28 M NaCl). Results are normalized to the effect of 1 μM dexamethasone.

Transactivation of MMTV-dependent reporter gene activity

The transactivation capacity of compounds was assessed using stably transfected A549 or MG63 osteosarcoma cell

lines containing a *Renilla* luciferase reporter gene under the control of an MMTV long terminal repeat (LTR) promoter exactly as described (Austin *et al.*, 2002). cells were seeded into 96-well plates at a density of 10 000 cells·per well and treated with glucocorticoid for 16 h. *Renilla* luciferase activity was determined following the addition of 10 μM coelenterazine. Results are normalized to the effect of 1 μM dexamethasone.

Pro-inflammatory cytokine production

Cells were seeded into 96-well plates at a density of 10 000 cells·per well and treated with glucocorticoid for 1 h prior to stimulation with the indicated cytokines. cell supernatants were harvested 16 h later and the levels of IL-6 assessed by quantitative immunoassay using Meso Scale Discovery technology (Rockville, MD, USA). Results are normalized to the effect of 1 μM dexamethasone.

Gene expression

Analysis of glucocorticoid-regulated gene expression was carried out essentially as described previously (Kent *et al.*, 2008). A description of the Affymetrix (Santa Clara, CA, USA) data analysis process and the lists of identified genes are included in the Supporting Information. Quantitative PCR (Q-PCR) was used to assess glucocorticoid target gene expression, and details of the primers/probes are set out in Supporting Information Table S4.

Lung epithelial cell function

The effect of compounds of 16HBE lung epithelial cells was explored exactly as described previously (Salter *et al.*, 2007). Briefly, to monitor IL-8 release, cells were treated with glucocorticoid for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α. Supernatants were harvested 24 h later and assayed for IL-8 levels by ELISA (R&D Systems, Minneapolis, MN, USA). For other experiments, cells were cultured on Transwell membranes (Corning, Tewksbury, MA, USA) until they formed an electrically tight monolayer. The effect of glucocorticoids on transepithelial resistance was monitored after 24 h incubation using a Millicell electrical resistance system (Millipore, Billerica, MA, USA). In cultures that had been grown for 24 h in the presence of glucocorticoids, the basolateral medium was replaced with medium containing 5 μg mL^{-1} neutrophil elastase, and the morphology of the cell layer was examined 24 h later.

Assessment of compound activity

Concentration–response curves were fitted to a four-parameter logistic equation using non-linear regression. The potency of each compound is expressed as the negative log of the molar concentration representing half the maximal effect of each compound (pXC_{50}), and these values are combined to generate an arithmetic mean with standard error of the mean. The asymptotic maximum of each curve is expressed as a percentage of the effect of 1 μM dexamethasone in each assay value, and these are combined to generate an arithmetic mean with SEM.

In vivo *activity*

Bagg albino/c mice were supplied by Charles River (Margate, UK). All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

For delayed-type hypersensitivity assay, animals were sensitized by topical application of 2.5% oxazolone solution (25 mg·mL^{-1}) in 50 μL of vehicle (1 part olive oil to 4 parts acetone) onto a shaved flank. After 4 days, the thickness of the animals' ears was measured using an engineer's micrometer while animals were under isoflurane anaesthesia, and compounds were administered on the right ear only as an ethanol solution. One hour later, animals were challenged with 0.25% oxazolone solution on each ear (2.5 mg·mL^{-1} in 1 part olive oil to 4 parts acetone; total volume 20 μL), and a second dose of compound was applied 3 h post-challenge. Twenty-four hours after challenge, animals were anaesthetized, and the ears were remeasured and the thickness recorded.

For the ovalbumin-challenge model, animals were sensitized to a suspension of ovalbumin adjoined to aluminium hydroxide (10 μg ovalbumin; 2 mg alum, $Al(OH)_3$) by intraperitoneal administration on two occasions, 2 weeks apart. Ten days after sensitization, mice received intratracheal administration of compound or vehicle (0.2% Tween-80 in saline) prior to challenge with intranasal administration of ovalbumin (50 μg). Dosing and challenge continued for 3 days, with a final dose of compound the day after the final challenge.

Animals were assessed for airway hyperresponsiveness (AHR) to the spasmogen serotonin (5-HT) using a Buxco Inc. (Wilmington, NC, USA) whole-body plethysmography system. Animals were exposed to a nebulized solution of water or 5-HT at concentrations of 1, 3 or 10 mg·mL^{-1} for 2 min periods, and lung function was assessed for a further 18 min. AHR is expressed as units of enhanced pause (Penh), either as area under the curve or average Penh following each exposure.

On the day following AHR assessment, the animals were killed by an overdose of anaesthetic, and the lungs were lavaged *post mortem* using repeat instillations of fluid (10 mM EDTA, 0.1% BSA in PBS) that was pooled to form the bronchoalveolar lavage (BAL). BAL was assayed for inflammatory cell influx using an automated flow cytometry method based on cell size and granularity to separate different cell populations. Inflammatory cell infiltrate is expressed as cells·per mL total BAL.

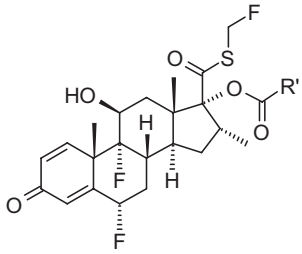
Results

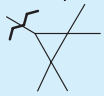
Modification of 17α position of fluticasone

To identify glucocorticoids with a primary pharmacology distinct from classical molecules, reporter gene assays were established in A549 cells to monitor repression of NFkB-dependent transcription, using an endothelial leukocyte adhesion molecule promoter, and direct transactivation of gene expression, using an MMTV-LTR driven promoter. FP is a very potent glucocorticoid that shows strong activity in

Table 1

Activity of compounds at NFkB and MMTV reporter genes in A549 cells



Compound	R′	NF-κB pIC_{50} (*n*)	NF-κB % maximal inhibition	MMTV pEC_{50} (*n*)	MMTV % maximal response
1 (FP)		10.4 ± 0.02 (324)	102 ± 0.3	9.7 ± 0.01 (217)	120 ± 0.8
2		10.1 ± 0.23 (4)	103 ± 3.6	9.7 (2)	113
3		10.3 ± 0.41 (3)	99 ± 1.0	9.6 ± 0.08 (3)	119 ± 9.8
4		10.2 ± 0.16 (16)	103 ± 0.9	9.8 ± 0.05 (21)	121 ± 2.8
5		10.1 ± 0.02 (291)	81 ± 0.6	9.7 ± 0.02 (163)	15 ± 0.4

Data are presented as mean ± SEM. Maximal responses are referenced to the effect of 1 μM dexamethasone in each assay.

both reporter gene systems (Table 1). Simple branched alkyl esters such as isopropyl (2) and the small cyclic cyclopropyl ester (3) showed similar profiles to FP. Introduction of a single methyl substituent at the α position of the cyclopropyl ester (4) again resulted in an FP-like profile, but more extensive substitution of the cyclopropyl ring in the form of the tetramethyl cyclopropyl ester (5) resulted in a dramatic change in the pharmacology. Thus, while compound (5) retained much of the desired transrepression efficacy (81%), it showed a greatly reduced maximum response (15%) in the MMTV transactivation assay.

Encouraging selectivity having been achieved with compound 5, further modification of the 17β substituent with the 17α tetramethylcyclopropyl ester in place was explored (Table 2). Replacement of the 17β fluoromethyl thioester of the fluticasone template with chloromethyl (6) and cyanomethyl (7) analogues reduced potency slightly, but was accompanied by a further reduction in transactivation efficacy (8 and 6% respectively). Finally, switching from the 17β thioester to the 17β carboxylate series gave derivatives 8 and 9, combining high potency with excellent transrepression/transactivation efficacy selectivity. From this series, the cyanomethyl carboxylate derivative 9 (GW870086) was selected for more extensive evaluation.

Transrepression and transactivation in multiple cell types

IL-6 is a pro-inflammatory cytokine whose expression is regulated by NF-κB. Pretreatment with either FP or GW870086 dose-dependently inhibited IL-6 release induced by TNF-α in A549 epithelial carcinoma cells and by IL-1 in MG63 osteosarcoma cells. In both cell types, the maximal levels of inhibition observed with GW870086 were comparable with those observed with FP, but GW870086 was around three times less potent [pIC_{50} 10.1 ± 0.02 vs 9.6 ± 0.16 in A549 (Figure 1A), and 10.6 ± 0.02 vs 10.2 ± 0.12 in MG63 (Figure 1B)].

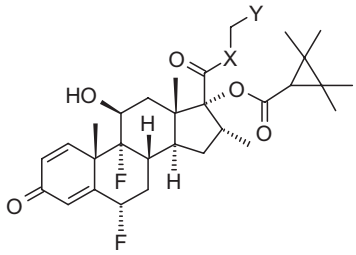
The MMTV-LTR contains a number of GREs, making the promoter highly inducible by glucocorticoids. In A549 and MG63 cells engineered to contain a stably integrated MMTV–luciferase reporter gene under the control of the MMTV-LTR, FP potently induced luciferase expression, with a pEC_{50} of 9.7 ± 0.01 and 9.6 ± 0.01 respectively. In contrast, GW870086 was completely unable to stimulate transactivation of the MMTV reporter in A549 (Figure 1C) and generated less than 25% of the reporter gene induction observed with FP in MG63 cells, even at very high concentrations (Figure 1D).

Partial agonist behaviour for MMTV transactivation

GW870086 displaced a fluorescently labelled glucocorticoid (Fluoromone GS red, Invitrogen, Life Technologies, Carlsbad, CA, USA) from isolated GR *in vitro* with a pIC_{50} of 8.2 ± 0.02 [*n* = 4, for method see Biggadike *et al.* (2009)]. This measured potency is the same as that observed for FP, but these values underestimate the affinity of both compounds because the measurement is limited by the high amount of GR protein required in the assay (tight binding limit). GW870086 showed potent activity at GR-dependent reporters but had very little activity at related steroid hormone receptors (Table 3). When tested across a panel of 50 receptors,

Table 2

Activity of compounds at NFkB and MMTV reporter genes in A549 cells



Compound	X	Y	NF-κB pIC_{50} (n)	NF-κB % maximal inhibition	MMTV pEC_{50} (n)	MMTV % maximal response
5	S	F	10.1 ± 0.02 (291)	81 ± 0.6	9.7 ± 0.02 (163)	15 ± 0.4
6	S	Cl	9.8 ± 0.03 (10)	71 ± 2.5	nd (8)	8 ± 1.7
7	S	CN	9.7 ± 0.13 (4)	68 ± 1.2	nd (6)	6 ± 0.5
8	O	Cl	10.0 ± 0.10 (16)	77 ± 1.1	nd (19)	4 ± 0.6
9 (GW870086)	O	CN	10.1 ± 0.05 (15)	68 ± 2.1	nd (29)	2 ± 0.3

Data are presented as mean ± SEM. Maximal responses are referenced to the effect of 1 μM dexamethasone in each assay.

Table 3

Activity of GW870086 at other steroid hormone receptors

Assay	FP	GW870086
Oestrogen receptor binding	<5	<5
Progesterone receptor reporter	8.5 ± 0.47	6.2 ± 0.3
Mineralocorticoid receptor reporter	7.7 ± 0.48	6.1 ± 0.4
Androgen receptor reporter	<5	<5
GR reporter (A549 NF-κB)	10.5 ± 0.18	10.1 ± 0.18

Data are presented as mean pIC_{50} with SEM of at least three separate determinations.

GW870086 showed no activity greater than 50% at a concentration of 1 μM (Supporting Information Table S5). Considering these data along with the chemical structure of the compound, it seems likely that GW870086 is acting specifically at the GR. If the effect of GW870086 on IL-6 release is being mediated by GR, then it must be binding efficiently to GR in the cell. If GW870086 is binding to GR, but not generating the transactivation activity of the receptor efficiently, then it should act as a competitive antagonist of other glucocorticoids in the MMTV assay. In A549 cells, dexamethasone stimulated a concentration-dependent increase in luciferase activity. In the presence of increasing concentrations of GW870086, the concentration–response curve for dexamethasone shifted to the right (Figure 2A). In MG63 cells, dexamethasone also stimulated reporter gene activity. At low concentrations of dexamethasone, co-incubation with GW870086 increased the measured response, consistent with the partial activation of the receptor described above. However, at higher concentrations of dexamethasone, GW870086 again shifted the concentration–response curve of dexamethasone to the right (Figure 2B). This is classical behaviour for a partial agonist and demonstrates that GW870086 is binding to GR in the cell.

The effect of corticosteroids on gene expression in A549 cells

In order to understand the effect of GW870086 on a range of endogenous GR target genes, we first set out to generate a full transcriptional profile for the effects of classical glucocorticoid drugs (dexamethasone, budesonide, FP) as well as the antagonist mifepristone (RU486). A549 cells were treated with pharmacologically equivalent concentrations of compounds for 1 h prior to stimulation with TNF-α (0.5 $ng \cdot mL^{-1}$), and RNA was extracted after 1, 3, 5, 8 and 24 h. Forty-seven genes were up-regulated by low-dose TNF-α by more than twofold (Supporting Information Table S1), and this induction was significantly reduced by glucocorticoid in 25 of these (53%) (Supporting Information Fig. S1). Expression of a further 49 genes was increased more than twofold following treatment by glucocorticoid, with 32 genes down-regulated by more than 1.5-fold (Supporting Information Tables S2 and S3). The effect of all three standard glucocorticoids was indistinguishable across the entire set of genes identified, while RU486 showed minimal activity, suggesting that we have identified a robust pharmacological fingerprint of glucocorticoid activity in A549 cells (Supporting Information Figs. S1–S3).

The effect of GW870086 on glucocorticoid target gene expression in A549 cells

A subset of the identified glucocorticoid-responsive genes that show a range of patterns of regulation was selected and Q-PCR assays established for each gene product to establish a gene expression panel to study the effect of compounds across a range of endogenous genes.

Expression of lymphotoxin-β (*LTB*) and COX-2 (*PTGS2*) was increased by TNF treatment, and pretreatment with any

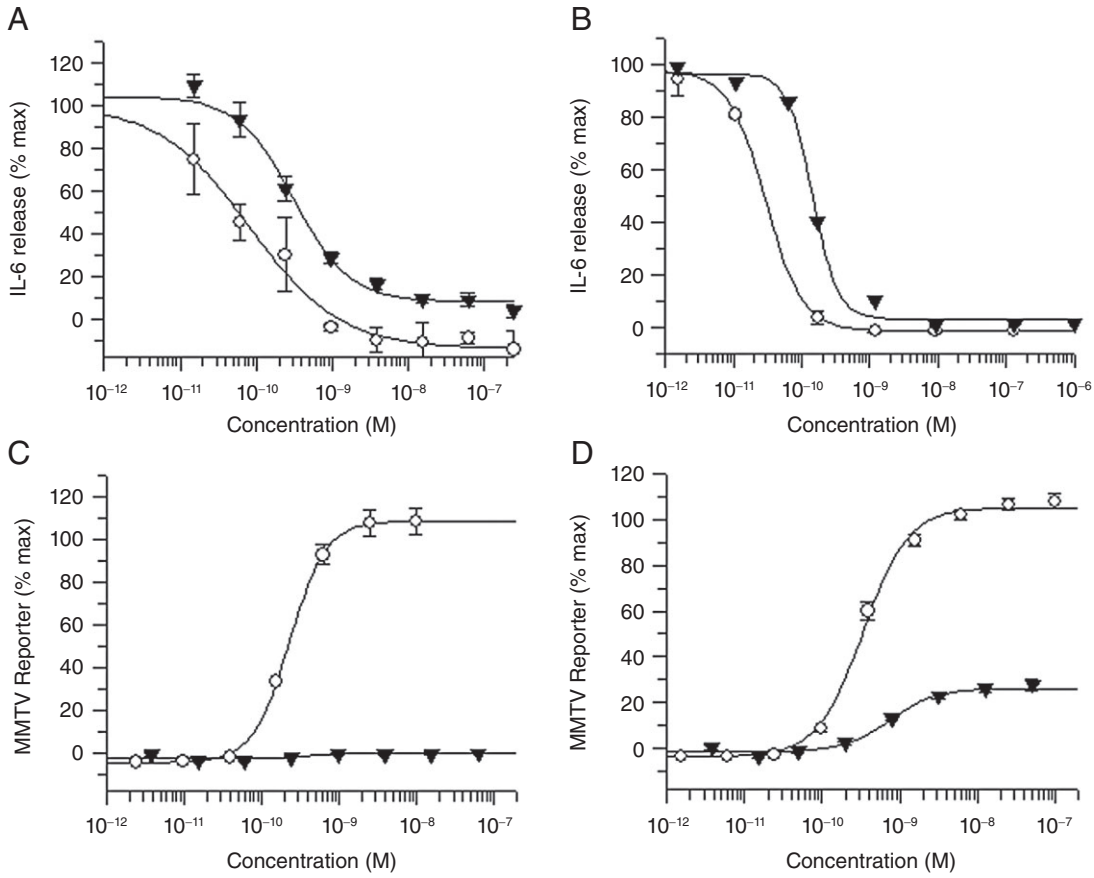


Figure 1

GW870086 represses cytokine production but does not stimulate MMTV reporter gene activity. A549 cells (A,C) or MG63 cells (B,D) containing an MMTV–luciferase reporter gene were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles). (A,B) After 1 h cells were stimulated with 2 ng·mL^{-1} TNF-α. Supernatants were collected 18 h later, and IL-6 levels were measured by ELISA. Data are normalized to the amount of cytokine released by TNF-α alone in each experiment. Data shown are means ± SEM of three separate experiments. (C,D) Luciferase activity in the cells was determined after 6 h of incubation with glucocorticoid. Data are normalized to the amount of luciferase stimulated by 1 μM dexamethasone in each experiment. Data shown are means ± SEM of 3 separate experiments.

of the standard glucocorticoids tested was able to prevent this up-regulation (Figure 3B,E). In contrast, RU486 did not inhibit TNF-α-induced expression of these genes and moreover acted as a competitive antagonist of dexamethasone, progressively shifting the dose–response curve to the right (Figure 3C,F), demonstrating that regulation of these genes by dexamethasone is mediated by GR. GW870086 was able to repress the TNF-driven expression of both these genes in a manner indistinguishable from the effects of dexamethasone (Figure 3A,D).

Expression of *Cyp24a1* and *MAP-7* was not induced by TNF, but treatment with any of the standard glucocorticoids decreased their expression level by more than fourfold (Figure 4B,E). Again, RU486 acted as a competitive antagonist (Figure 4C,F), and the effects of GW870086 were indistinguishable from those of dexamethasone (Figure 4A,D).

Eight directly up-regulated genes were studied (Figure 5), and in each case the effects of the standard compounds were very similar and RU486 acted as a competitive antagonist, confirming that these are all GR target genes. However, GW870086 showed a unique range of effects on the expression of these genes, inducing expression of *GPR64* in a similar manner to dexamethasone (Figure 5A), but having virtually no effect on serum- and glucocorticoid-induced PK (SGK) levels (Figure 5V).

A number of additional genes that were not found to be regulated by standard glucocorticoids were also studied as controls, including the TNF-responsive genes *MGC5618* and *SOD2*. Like FP, GW870086 also had no effect on the expression levels of these genes (Supporting Information Figure S4).

Thus, within a given cell, the overall pattern of gene expression changes induced by GW870086 is very different from that induced by other steroids, but consistent with its activity being mediated by GR.

Phenotypic responses in respiratory epithelial cells

Glucocorticoids are extremely effective medicines for respiratory disease and show very potent effects on cells of the respiratory epithelium. 16HBE cells maintain many characteristics of respiratory epithelial cells in culture and have been used previously to study a range of effects of glucocorticoids

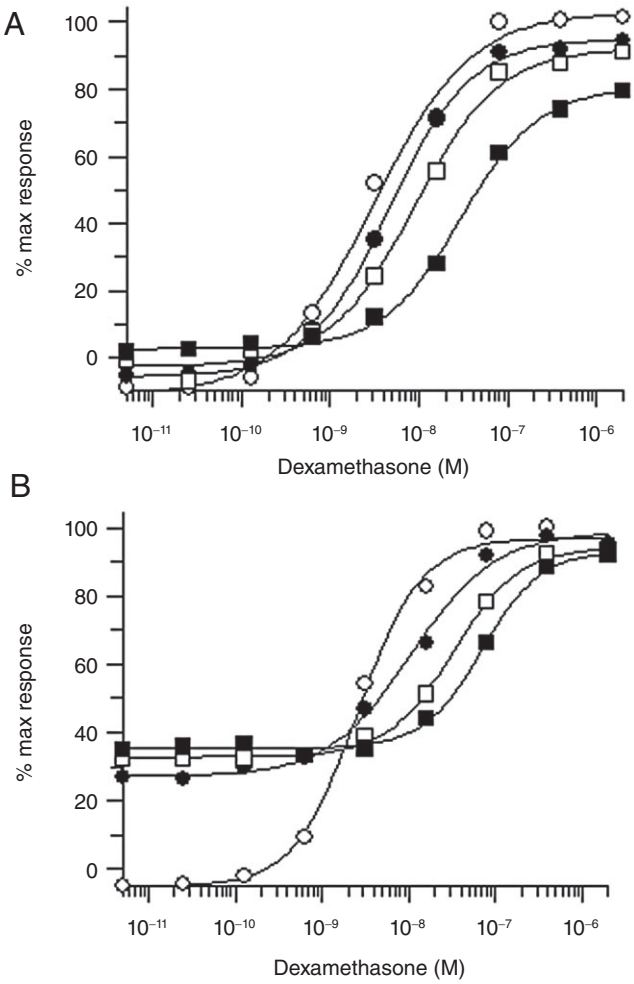


Figure 2

GW870086 antagonizes the effect of dexamethasone on the MMTV reporter gene. A549 cells (A) or MG63 cells (B) containing an MMTV–luciferase reporter gene were treated with 0.5% dimethyl sulphoxide (open circles) or with 1 (closed circles), 3 (open squares) or 10 nM (closed squares) GW870086 for 15 min prior to stimulation with indicated concentrations of dexamethasone for 6 h. Luciferase activity in the cells was determined, and the data are normalized to the amount of luciferase stimulated by 1 μM dexamethasone with no competitor. Results shown are from a single representative experiment that was repeated twice.

(Salter *et al.*, 2007). Treatment of the 16HBE cultures with TNF-α evokes the release of IL-8 into the medium. Pre-incubation of cells with either FP or GW870086 inhibited TNF-α-induced IL-8 secretion, although GW870086 was slightly less potent (Figure 6A).

In monolayer culture, 16HBE cells form tight junctions that provide an electrical resistance. Culture in the presence of FP results in an increase in the transepithelial resistance (TER) measured in the culture, indicating a strengthening of the intercellular interactions. GW870086 induced an increase in TER similar to that induced by FP, although it was slightly less potent (Figure 6B).

Treatment with elastase causes the culture to detach from the cell culture membrane, and glucocorticoids have previously been shown to protect epithelial cell cultures from this damage (Salter *et al.*, 2007). Indeed, pretreatment with FP completely protected the cells from elastase-induced damage. Curiously, despite demonstrating potent effects in these cells, GW870086 showed absolutely no protective effect at concentrations up to 1000× higher than those that inhibited cytokine release (Figure 6C). This dramatic difference within the same cell culture system underlines the unique pharmacological profile of the molecule.

Anti-inflammatory efficacy in vivo

As GW870086 offers a unique pharmacological profile, the best way of assessing the global anti-inflammatory potential of this compound is using *in vivo* models, where multiple pathways and cell types interact to generate the final measured responses. However, it was first necessary to demonstrate that GW870086 showed a similar pharmacology in murine cells and in human cells. A murine NIH-3T3 fibroblast cell line was constructed containing an integrated MMTV–luciferase reporter gene. Stimulation of these cells with IL-1β results in release of IL-6, and this could be maximally inhibited by both FP and GW870086 with similar potency (pIC_{50} 10.6 ± 0.18 and 10.8 ± 0.21 respectively; Figure 7A). Both FP and GW870086 were potent inducers of MMTV activity (pEC_{50} 9.9 ± 0.14 and 9.6 ± 0.18 respectively), but GW870086 displayed a reduced maximal response (26% compared with FP, Figure 7B). These data look very similar to those obtained in human MG63 cells (Figure 1B,D) and suggest that GW870086 is showing evidence of selective pharmacology in the mouse.

As a close relative of fluticasone, GW870086 has pharmaceutical properties well suited for topical administration onto the skin or into the lungs. The anti-inflammatory activity of GW870086 on the skin was demonstrated using a simple delayed-type hypersensitivity reaction. Administration of oxazolone topically to the ears of sensitized mice generates a T-cell-mediated inflammation that results in swelling of the ears. Direct topical application of either FP or GW870086 showed potent dose-dependent anti-inflammatory activity on the treated ear (Figure 7C). Inhibition of inflammation in the contralateral ear was also observed with both compounds at higher doses as a result of systemic exposure following topical dosing.

Administration of ovalbumin into the airways of sensitized mice results in an eosinophilic airway inflammation and a hyperresponsiveness to spasmogens such as 5-HT that is highly reminiscent of allergic asthma. Intratracheal administration of FP or GW870086 during the challenge period inhibited the ovalbumin-induced AHR and inflammatory cell influx in a dose-dependent manner (Figure 7D,E). In both responses GW870086 showed comparable efficacy to FP but was slightly less potent.

Discussion

Glucocorticoids are among the most effective anti-inflammatory drugs currently available, but their use at high doses is limited by a range of side effects. While the

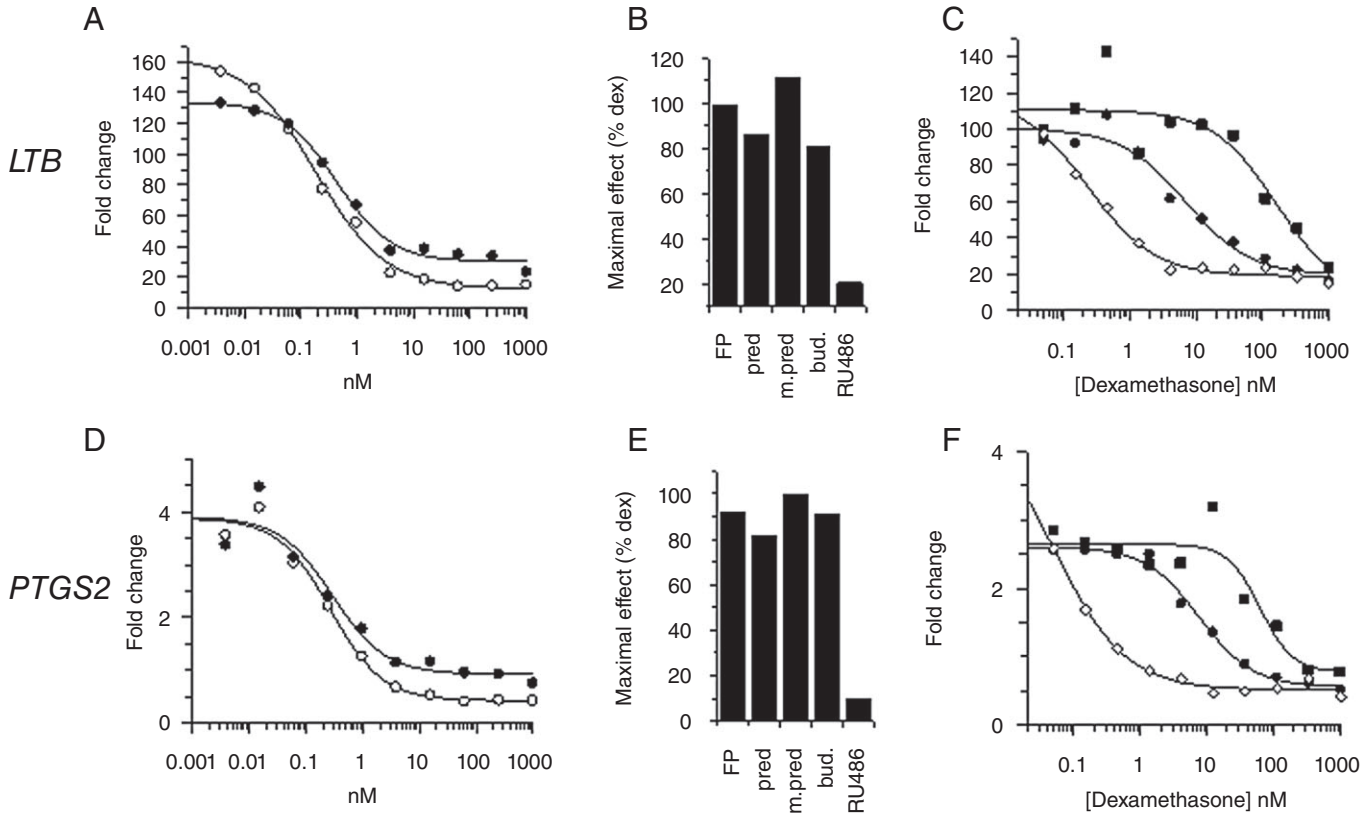


Figure 3

GW870086 retains transrepressive activity in A549 cells compared with classical glucocorticoids. Left panels: A549 cells were treated with indicated concentrations of dexamethasone (open circles) or GW870086 (closed circles) for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells. Middle panels: A549 cells were treated with 1 nM FP ($n = 2$), 1 μM prednisolone (pred, $n = 2$), 1 μM methylprednisolone (m.pred., $n = 1$), 50 nM budesonide (bud., $n = 2$), 1 μM RU486 ($n = 3$) or 1 μM dexamethasone (dex) for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as a percentage of the effect of 1 μM dexamethasone in each experiment. Right panels: A549 cells were treated with dimethyl sulphoxide (open diamonds) or RU486 [100 nM (closed circles) or 1 μM (closed squares)] for 15 min before addition of the indicated concentrations of dexamethasone for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells.

development of topically delivered compounds for treatment of specific diseases such as asthma, atopic dermatitis and COPD has allowed some of these effects to be avoided, the potential of discovering novel glucocorticoids that retain efficacy while lacking the dose-limiting side effects has generated considerable activity in many laboratories (Belvisi *et al.*, 2001a; Biggadike *et al.*, 2004; Uings and Farrow, 2005; Clark and Belvisi, 2012). Here we describe how further exploration of the 17α position of the steroid nucleus that previously led to the development of FF,, combined with the use of reporter gene assays in A549 cells, led to the discovery of GW870086, a molecule that shows potent anti-inflammatory activity *in vitro* and *in vivo* but that also demonstrates a robustly different and unique impact on cellular function.

There have been a considerable number of published reports describing novel glucocorticoid-like molecules (Belvisi *et al.*, 2001a; Clark and Belvisi, 2012), but in most little consideration has been given to the pharmacological implications of the data presented. Almost all the reported ligands bind orthosterically at the steroid-binding site of GR, and based on its chemical structure it is reasonable to assume that GW870086 binds in a similar fashion. Indeed, docking studies using the published X-ray crystal structure of GR (Biggadike *et al.*, 2008) indicate that the tetramethylcyclopropyl ester can be accommodated in the 17α pocket. We have demonstrated that in those situations where GW870086 is not able to elicit a full response, it nonetheless acts to antagonize the activity of dexamethasone, indicating that the lack of response is not due to any deficit in engaging the receptor within the cell. Moreover the data presented here show that GW870086 behaves exactly as expected for an agonist with low intrinsic efficacy for MMTV, showing greater activity in some cell systems than others, dependent on the 'system coupling' of each cell type.

The GR is intracellular and acts primarily as a transcriptional regulator, so gene expression changes can be considered as proximal events to receptor activation (Barnes, 1998; Webster and Cidlowski, 1999). Unlike studies aimed at uncovering signalling pathways or networks, our approach to transcriptional profiling was as a pharmacological tool, so we

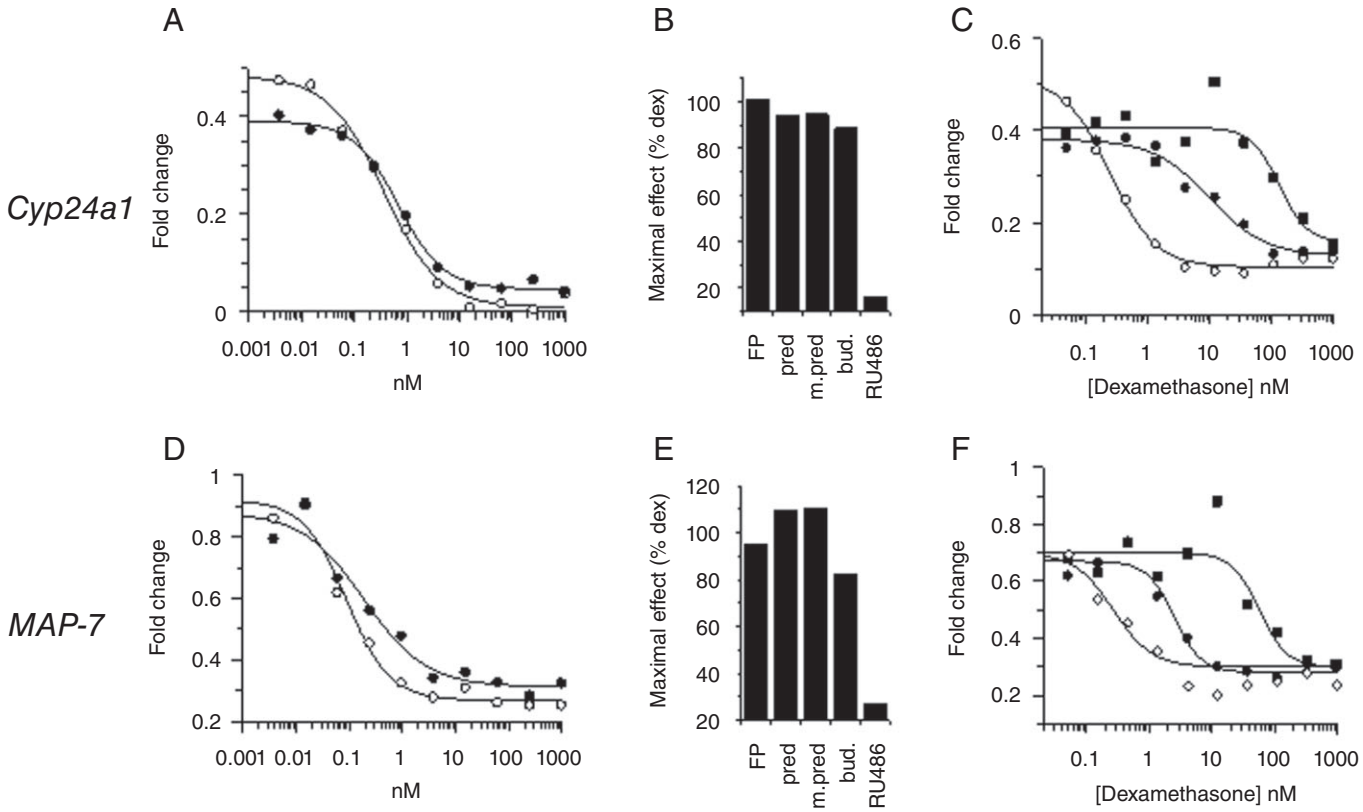


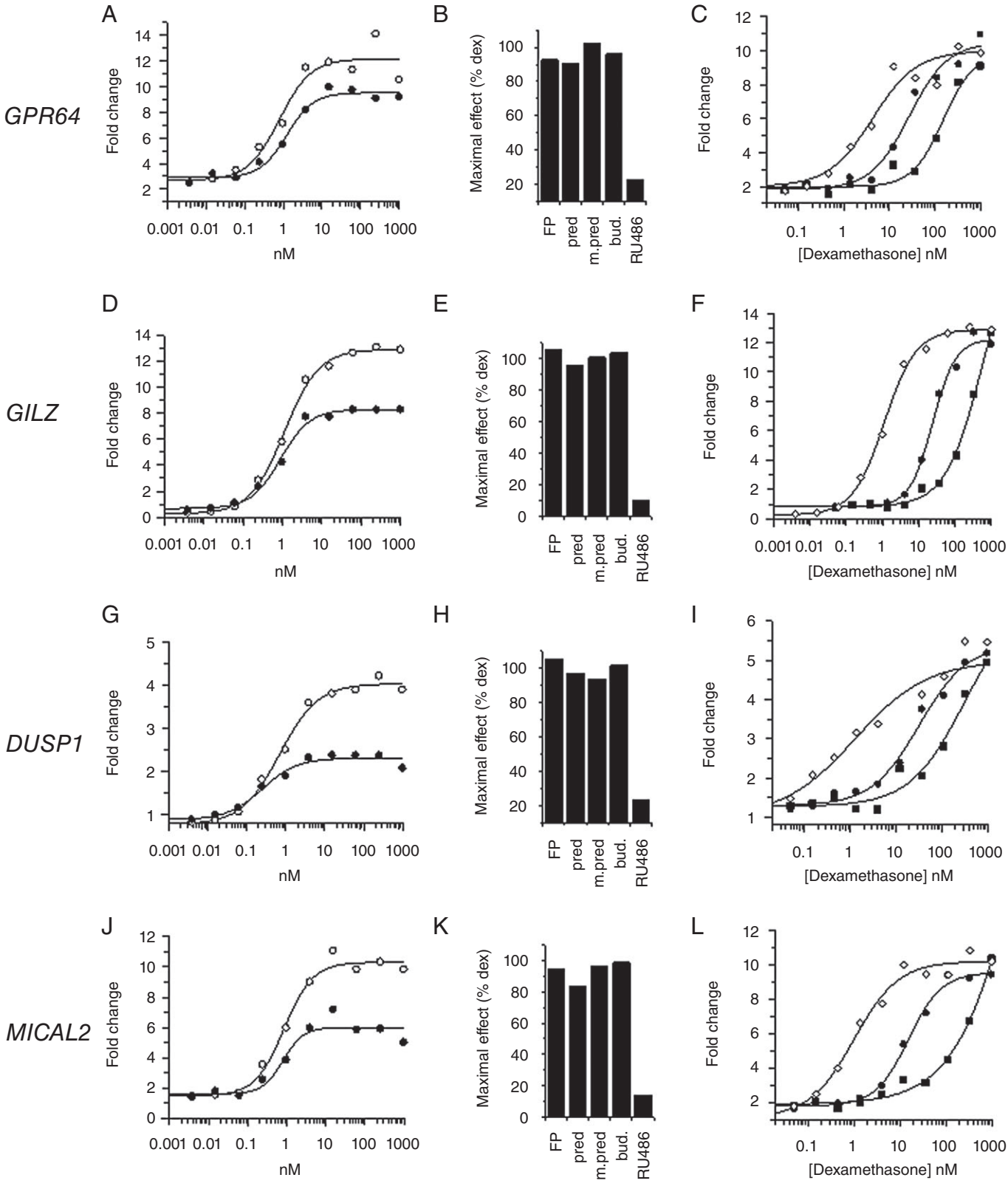
Figure 4

GW870086 retains direct down-modulation of steroid-responsive genes in A549 cells. Left panels: A549 cells were treated with indicated concentrations of dexamethasone (open circles) or GW870086 (closed circles) for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells. Middle panels: A549 cells were treated with 1 nM FP ($n = 2$), 1 μM prednisolone (pred, $n = 2$), 1 μM methylprednisolone (m.pred., $n = 1$), 50 nM budesonide (bud., $n = 2$), 1 μM RU486 ($n = 3$) or 1 μM dexamethasone (dex) for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as a percentage of the effect of 1 μM dexamethasone in each experiment. Right panels: A549 cells were treated with dimethyl sulphoxide (open diamonds) or RU486 [100 nM (closed circles) or 1 μM (closed squares)] for 15 min before addition of the indicated concentrations of dexamethasone for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells.

focussed on questions of robustness and reproducibility. We set out to compare the effects of the well-characterized glucocorticoids dexamethasone, budesonide and FP, which are known to behave in very similar ways. We were intrigued to discover that no substantive differences could be detected in the cell's transcriptional response to these agents across a range of doses and time points. We were thus able to define a pattern of gene expression changes consisting of both positively and negatively regulated genes that could serve as a fingerprint of GR activation in A549 cells. The effects of dexamethasone on all these genes could be antagonized in a competitive fashion by RU486, showing that the effects on each gene are mediated by the activity of GR. However, careful analysis reveals that the potency of dexamethasone varies up to 20-fold on different genes, with half-maximal potencies ranging from 150 nM to 3 μM. This suggests that the cell interprets the same degree of receptor activation differently for each gene product, that is to say, the 'system coupling' varies from gene to gene and not just from cell to cell.

The pattern of effects on gene expression observed with GW870086 was clearly unique and distinct from all other glucocorticoids tested. For some genes (e.g. *LTB*) the effects were indistinguishable from those of dexamethasone, while for others (e.g. *SGK*) they were profoundly different. While it is hard to translate the effects on these genes in A549 cells into clinically significant effects, it is curious to note that GW870086 retained nearly complete inhibition of COX-2 expression (*PTGS2*) and substantial up-regulation of dual-specificity protein phosphatase 1 (*MKP1*), both of which have been described as key components of the anti-inflammatory response (Clark *et al.*, 2008; Clark and Belvisi, 2012).

The dramatic difference in effect observed in 16HBE cells serves to underline the unpredictable results of this unique gene expression profile. Some aspects of glucocorticoid biology are completely preserved, and some are completely absent. To establish the physiological consequences of this profile it is therefore essential to examine responses in an *in vivo* animal model. Previous investigations of novel steroid-based ligands with altered pharmacology showed that the


GPR64
GILZ
DUSP1
MICAL2
A
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[Dexamethasone] nM

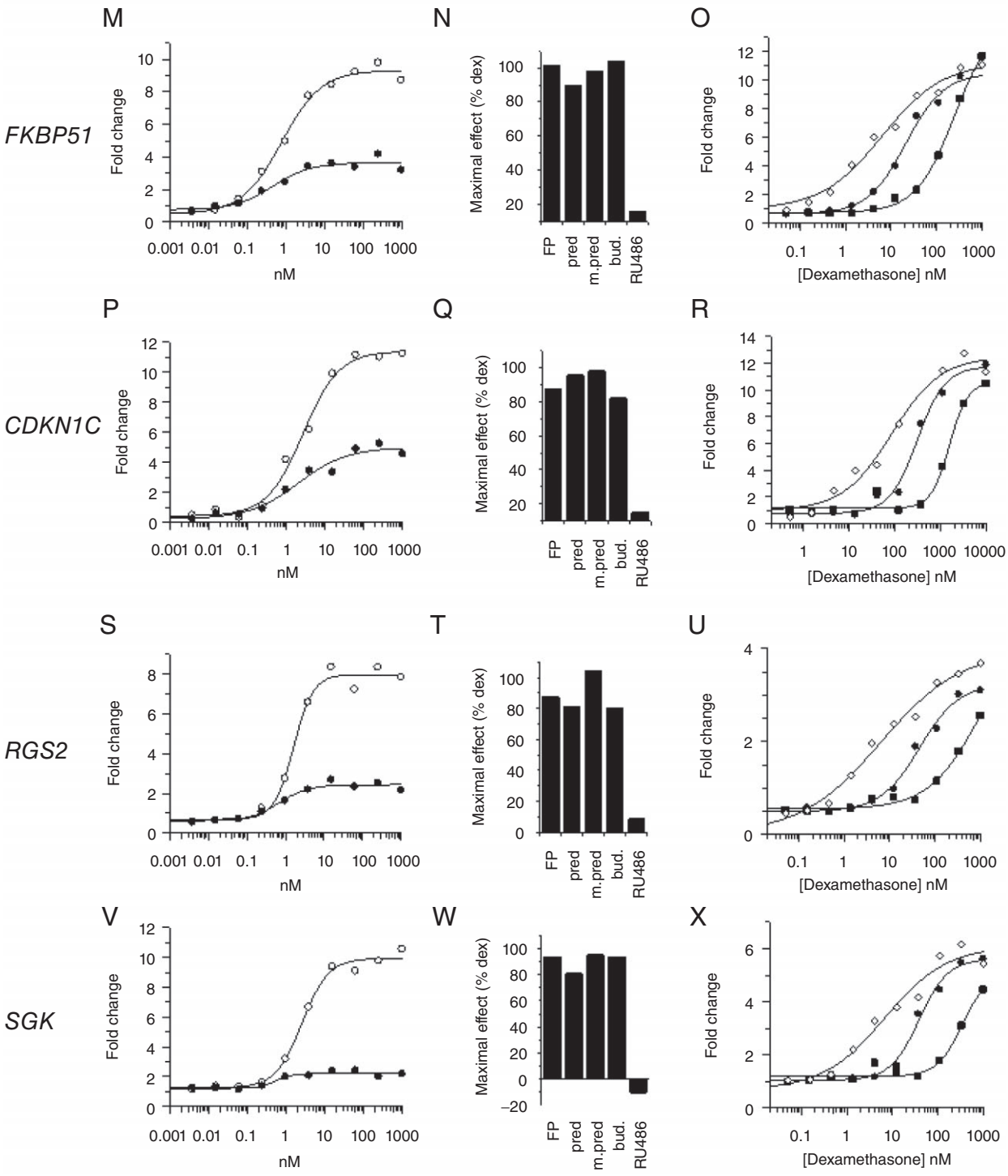

Figure 5

GW870086 shows a range of activity on glucocorticoid-up-regulated genes compared with classical glucocorticoids in A549 cells. Left panels: A549 cells were treated with indicated concentrations of dexamethasone (open circles) or GW870086 (closed circles) for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells. Middle panels: A549 cells were treated with 1nM FP ($n = 2$), 1 μM prednisolone (pred, $n = 2$), 1 μM methylprednisolone (m.pred., $n = 1$), 50 nM budesonide (bud., $n = 2$), 1 μM RU486 ($n = 3$) or 1 μM dexamethasone (dex) for 1 h prior to stimulation with 1·ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as a percentage of the effect of 1 μM dexamethasone in each experiment. Right panels: A549 cells were treated with dimethyl sulphoxide (open diamonds) or RU486 [100 nM (closed circles) or 1 μM (closed squares)] for 15 min before addition of the indicated concentrations of dexamethasone for 1 h prior to stimulation with 1 ng·mL^{-1} TNF-α for 5 h. RNA was extracted and gene expression determined by Q-PCR. Data are presented as fold change relative to untreated cells.

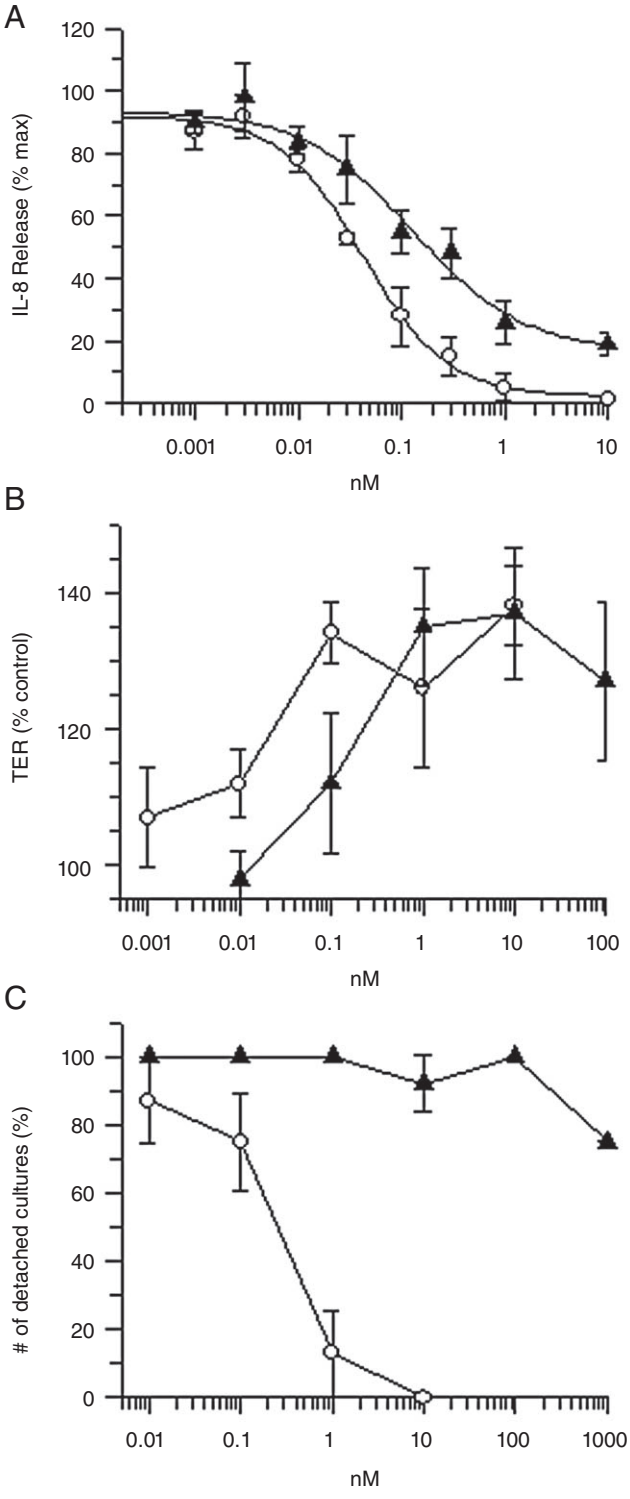

Figure 6

Effects of GW870086 on respiratory epithelial cell function. (A) 16HBE cells were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles) for 1 h prior to stimulation with TNF-α, and the amount of IL-8 released into the medium was assessed after 24 h incubation. (B) Monolayer cultures of 16HBE cells were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles) for 24 h before the transepithelial resistance was measured. (C) Monolayer cultures of 16HBE cells were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles) for 24 h before challenge with human sputum elastase. The number of cultures that remained attached as intact monolayers was assessed. All data are presented as mean and SEM for three independent experiments.

selective *in vitro* activity of these molecules did not translate into improved *in vivo* activity in animal models of inflammation (Belvisi *et al.*, 2001a,b) However, more recent non-steroidal agonists have been reported to retain novel pharmacology both *in vitro* and *in vivo* (Mohler *et al.*, 2007; Schacke *et al.*, 2007, 2008, 2009a,b) Given the subtleties of the pharmacological profile involved, we felt it was critical to demonstrate that GW870086 behaved in the same way in murine cells as in human cells in order to interpret the subsequent *in vivo* experiments. Reporter gene systems in murine NIH-3T3cells identical to those in human A549 cells were used, and very similar effects were observed, suggesting that the overall behaviour of the compound is similar. This means that the profound efficacy observed across the inflammatory models occurs despite the altered pharmacology, strongly suggesting that GW870086 has exciting potential as an anti-inflammatory therapy.

GW870086 shows minimal activity at the other steroid hormone receptors, and this makes it the most specific of the potent steroids available. As GW870086 is closely related to the topical steroid FP, it shares a similar pharmacokinetic profile, and further development of this molecule will thus focus on topical applications. Long-term studies are required to monitor corticosteroid side effects, but the low systemic exposure observed following topical dosing will make meaningful assessment of glucocorticoid side effects in the mice challenging.

Schacke *et al.* (2009b) have recently reported the optimization of a selective agonist for topical administration in conditions such as dermatitis. Their novel compound, ZK245186, is reported to be a potent anti-inflammatory agent *in vitro* and *in vivo*, with a superior therapeutic index as compared with the gold standard glucocorticoids used for treatment of dermatitis, and is reported to be in early clinical trials. It is now clear that it is possible to identify compounds that deliver a pharmacological profile consistent with an improved therapeutic index, and it will now be important to see how these novel and unique pharmacological profiles translate into efficacy and safety during clinical development.

Acknowledgements

We wish to thank Chris Clayton and the whole team for expert help with the transcriptomics studies.

Conflict of Interest

The authors are employees of GlaxoSmithKline.

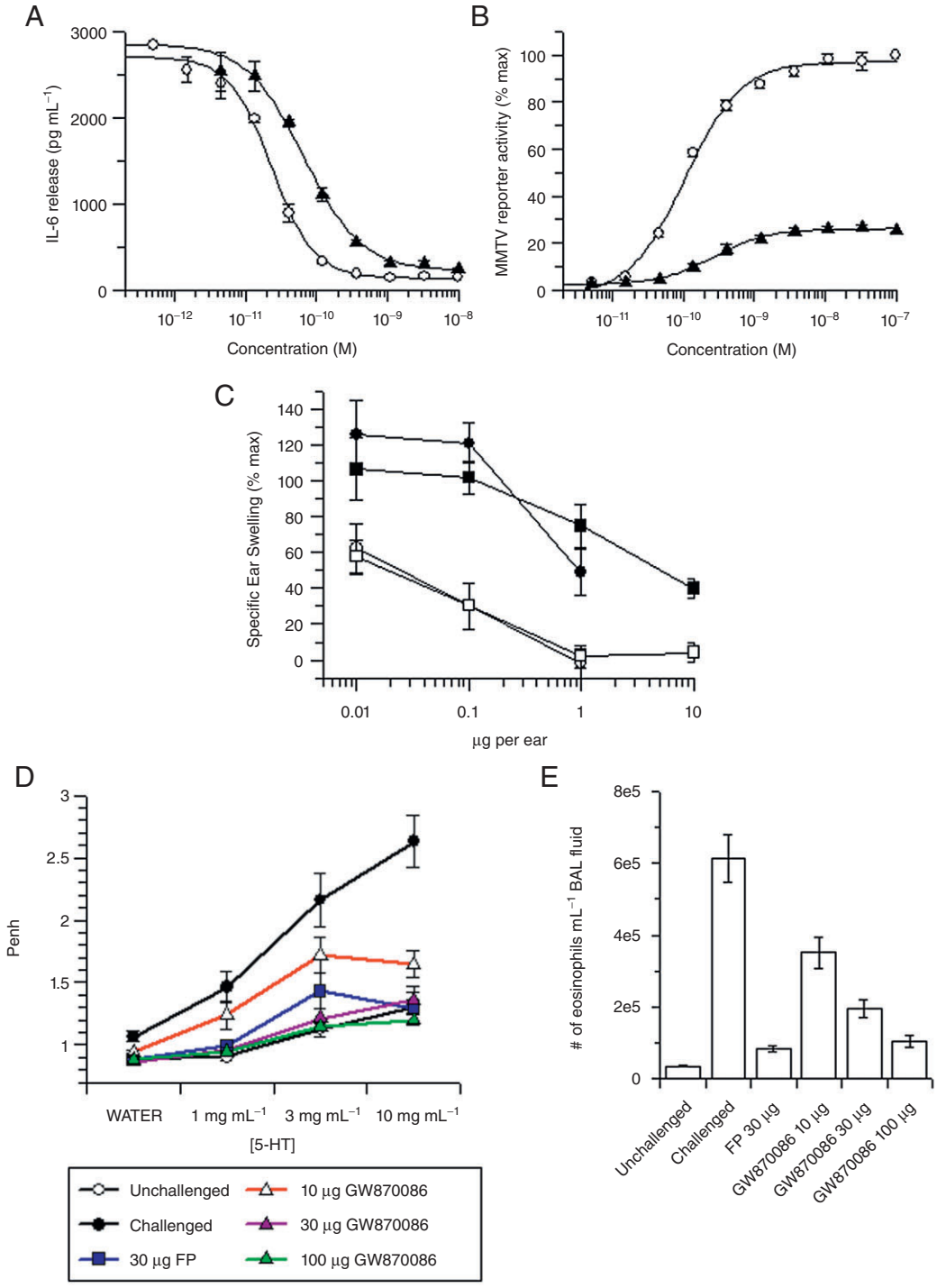

Figure 7

GW870086 retains efficacy in murine models of inflammation. (A) NIH3T3 cells were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles) for 1 h prior to stimulation with IL-1β, and the amount of IL-6 released into the medium was assessed after 24 h incubation. (B) NIH3T3 cells containing an MMTV-driven luciferase reporter construct were treated with indicated concentrations of FP (open circles) or GW870086 (closed triangles) for 24 h, and the amount of luciferase activity in the cells was measured. (C) Mice were sensitized to oxazolone, then treated topically with indicated amounts of FP (circles) or GW870086 (squares) onto one ear prior to challenge with oxazolone on both ears. Ear thickness was measured before and after challenge, and the specific ear swelling of both the treated (open symbols) and untreated (closed symbols) ears were calculated. There were six animals in each group assessed. Mice were sensitized to ovalbumin, then dosed intratracheally with FP (squares, 30 µg) or GW870086 (triangles; open 10 µg, grey 30 µg, filled 100 µg) for 3 days in parallel with intranasal ovalbumin challenge. There were five animals in each group assessed. (D) Airway function was assessed using whole-body plethysmography after challenge with indicated concentrations of 5-HT. Open circles indicate the response of animals that had not been challenged with ovalbumin, while closed circles indicate challenged animals dosed with vehicle. (E) Lung eosinophils were recovered by bronchioalveolar lavage and counted using flow cytometry.

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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:

Figure S1 Global gene expression patterns.
Figure S2 Example time profiles of TNF-regulated genes.
Figure S3 Example profiles of a range of genes.
Figure S4 GW870086 has no effect on non-GR target genes.
Table S1 TNF response genes.
Table S2 Glucocorticoid-up-regulated genes (>twofold).
Table S3 Glucocorticoid-down-regulated genes (>1.5-fold).
Table S4 Taqman Primer probesets.
Table S5 CEREP screening data.