Pharmacological properties of the enhanced-affinity glucocorticoid fluticasone furoate in vitro and in an in vivo model of respiratory inflammatory disease

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Fluticasone furoate (FF) is a novel enhanced-affinity glucocorticoid that has been developed as topical therapy for allergic rhinitis. The pharmacological properties of FF have been investigated using a number of in vitro experimental systems. FF demonstrated very potent glucocorticoid activity in several key pathways downstream of the glucocorticoid receptor (GR) as follows: the transrepression nuclear factor-κB (NF-κB) pathway, the transactivation glucocorticoid response element pathway, and inhibition of the proinflammatory cytokine tumor necrosis factor-α. Furthermore, FF showed the greatest potency compared with other glucocorticoids for preserving epithelial integrity and reducing epithelial permeability in response to protease- and mechanical-induced cell damage. FF showed a 30- to >330,000-fold selectivity for GR-mediated inhibition of NF-κB vs. the other steroid hormone receptors, substantially better than a number of other clinically used glucocorticoids. In studies examining the respiratory tissue binding properties of glucocorticoids, FF had the largest cellular accumulation and slowest rate of efflux compared with other clinically used glucocorticoids, consistent with greater tissue retention. The in vivo anti-inflammatory activity of FF was assessed in the Brown Norway rat ovalbumin-induced lung eosinophilic model of allergic lung inflammation. At a dose of only 30 μg, FF achieved almost total inhibition of eosinophil influx in the lung, an inhibition that was greater than that seen with the same dose of fluticasone propionate. In conclusion, the potent and selective pharmacological profile of FF described here could deliver an effective, safe, and sustained topical treatment of respiratory inflammatory diseases such as allergic rhinitis and asthma.

GLUCOCORTICOIDs are commonly used to treat a wide range of inflammatory conditions such as asthma, allergic rhinitis, chronic obstructive pulmonary disease, rheumatoid arthritis, inflammatory bowel disease, and autoimmune diseases (15). Despite advances in the compounds and formulations employed in clinical practice, there are limitations to currently available glucocorticoids. In particular, among the intranasal glucocorticoids used in the treatment of allergic rhinitis, incomplete symptom reduction, the need for twice-daily dosing, and unpleasant sensory attributes all contribute to suboptimal management of the disease. Therefore, there remains an unmet clinical need for intranasal glucocorticoids with enhanced symptom reduction, a longer duration of action, and improved sensory attributes.

Glucocorticoids mediate their effects on the body through the glucocorticoid receptor (GR). Activated GR affects a number of downstream pathways via DNA-binding-dependent (e.g., transactivation) and -independent (e.g., transrepression) mechanisms. DNA-binding-dependent effects are mediated via binding of the activated GR directly to DNA at specific recognition sites, known as glucocorticoid response elements (GREs), to effect either an increase or decrease in the transcription of certain gene products. Glucocorticoid-activated GR can also interact directly with certain transcription factors, such as the proinflammatory nuclear factor-κB (NF-κB; without binding directly to DNA), and consequently inhibit their ability to increase the transcription of a variety of proinflammatory gene products (2).

Fluticasone furoate (FF, GW685698X; see Ref. 20) is a novel enhanced-affinity glucocorticoid with a unique combination of pharmacodynamic and physicochemical properties. FF was identified following a detailed investigation of the effect of modification of the 17α-ester moiety of the glucocorticoid fluticasone propionate (FP). Although FF is structurally related to FP, they are distinct chemical and pharmacological entities (Fig. 1).

Some glucocorticoids, such as beclomethasone dipropionate and ciclesonide, require de-esterification [to beclometasone 17-monopropionate and des-isobutyryl-ciclesonide (active principle, Cic-AP), respectively] for pharmacological activity. Neither FF nor FP require de-esterification for activity; in both cases, the activity resides in the entire molecule. Both FF and FP have a fluoromethylthioester at the 17β-position that is cleaved during hepatic metabolism to inactivate any glucocorticoid that enters the systemic circulation. However, the 17α-ester group of FF and FP (furoate and propionate, respectively) is metabolically stable and is not cleaved from the rest of the molecule. FF and FP share no common metabolites, and neither compound is metabolized to fluticasone (Fig. 1). FF is therefore a distinct drug molecule and not a salt or a prodrug of fluticasone.

For optimal efficacy and minimal side effects, topical glucocorticoids should display high local efficacy with low systemic exposure and adverse effects. Previous studies have shown that FF binds with a greater affinity to the human GR than other clinically used glucocorticoids (22). The current...
paper presents a more detailed examination of the properties of FF using a number of in vitro and in vivo experimental systems to examine its efficacy, selectivity, and tissue binding. These properties demonstrate that FF has a preclinical profile, which should lead to a highly effective and well-tolerated topical treatment for rhinitis and other respiratory disorders.

**MATERIALS AND METHODS**

**Reagents**

Unless otherwise stated, chemicals and biochemicals were purchased from Sigma-Aldrich (Gillingham, Dorset, UK); tissue culture media, nutrients, serum, and antibiotics were obtained from Invitrogen (Carlsberg, CA); and cytokines were supplied by R&D Systems (Minneapolis, MN). Fugene-6 transfection reagent was purchased from Roche Diagnostics (Burgess Hill, UK).

All compounds were synthesized within GSK, dissolved in DMSO at a concentration of 10 mM, and appropriate dilutions were prepared in DMSO. The dilutions were transferred to the assay plates ensuring that the DMSO concentration was constant across the plate at 5% for binding and 1% for functional assays.

**Cell Culture**

Reporter cell lines derived from the human Caucasian lung carcinoma A549 cell line (ECACC No. 86012804) were maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with heat-inactivated FCS (FCS-HI; 10%), nonessential amino acids (1%), l-glutamine (2 mM), penicillin/streptomycin (1%), and geneticin (50 mg/ml).

African Green Monkey kidney fibroblast CV-1 cells (ECACC No. 87032605) were maintained in phenol red-free DMEM supplemented with FCS-HI (10%), L-glutamine (2 mM), and penicillin/streptomycin (1%).

Human breast epithelial ductal carcinoma T-47D cells (ATCC No. HTB-133) were maintained in DMEM supplemented with FCS-HI (10%) and glutamax (2 mM).

Passage 8 16HBE14o− cells were kindly supplied by Dieter Gruenert (University of California Los Angeles, San Francisco, CA) and maintained in Earles’ minimum essential medium (MEM) (Hyclone, Logan, UT) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (10 μg/ml), and l-glutamine (2 mM), and aliquots of passage 10 cells were frozen in liquid nitrogen for future use. Cultures used for the mechanical wound experiments were grown in the same medium except that the FCS was stripped of endogenous steroids using dextran-coated charcoal (9). The 6.5-mm Transwell clear cell culture inserts (Costar Europe, Badhoevedorp, The Netherlands) were coated with MEM containing 1% vitrogen (Cohesion, Palo Alto, CA), 0.001% fibronectin, and 0.1% BSA.

**Expression Plasmids**

The integrity of all expression plasmids used in this study was confirmed by direct DNA sequencing. The sequence of the expressed androgen receptor (AR) differs from the published sequence in that it has one additional glutamine residue (residue 79) and three additional glycine residues (position 475). In the BacMam construct expressing progesterone receptor b (PRb), the transcriptional start site of progesterone receptor a (PRA) has been modified from methionine to alanine to prevent PRA expression.

Where required, pBluescript II KS+ (Stratagene, La Jolla, CA) was added as bulk DNA to improve transfection efficiency.

**Assessment of Glucocorticoid Activity**

Repression of tumor necrosis factor-α-stimulated NF-κB activation. Human Caucasian lung carcinoma A549 cell line (ECACC No. 86012804) was stably transfected with a plasmid containing an NF-κB-responsive ELAM (E-selectin) promoter sequence, upstream of a secreted placent al alkaline phosphatase reporter gene, and a clonal cell line was derived and maintained. Cells were seeded at a density of 10,000 cells/well in 384-well plates containing glucocorticoids. After 1 h, cells were stimulated with tumor necrosis factor-α (TNF-α, 3.2 ng/ml) and incubated at 37°C for a further 15 h. Alkaline phosphatase activity was determined spectrophotometrically at 405 nm following addition of an equal volume of p-nitrophenylphosphate (2 mg/ml in 1 M diethanolamine, pH 9.8, 0.5 mM MgCl₂, and 0.28 M NaCl) as a substrate using a Fluoroskan Ascent reader (Thermo Electron, Waltham, MA).

**Glucocorticoid-mediated gene transcription.** A549 cells were stably transfected with a plasmid containing three copies of a consensus sequence for glucocorticoid response elements.
GRE linked to a herpes simplex virus thymidine kinase gene promoter upstream of the Renilla luciferase reporter. GRE cells were seeded at a density of 10,000 cells/well in 96-well plates and stimulated with glucocorticoids for 16 h. Renilla luciferase activity was determined by measuring the luminescence using a Viewlux reader (PerkinElmer, Wellesley, MA) 1 h after addition of coelenterazine (10 μM).

Repression of lipopolysaccharide-stimulated TNF-α production in human peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized human blood from normal volunteers by density gradient centrifugation using Accuspin System Histopaque-1077 (Sigma-Aldrich, Poole, UK). Cells were seeded at a density of 50,000 cells/well in 96-well plates containing glucocorticoid. Cells were stimulated with lipopolysaccharide (LPS; 1 ng/ml) and incubated at 37°C for 20 h. The supernatant was removed, and the concentrations of TNF-α were determined by electrochemiluminescence assay using the Meso Scale MSD technology (Meso Scale, Gaithersburg, MD).

Lung Epithelial Cell Function

Human lung epithelial 16HBE14o− cells (passage 10) were seeded on coated 6.5-mm-diameter Transwell clear membranes at a density of 10,000 cells/well. The cells were incubated at 37°C for 4 days to allow formation of an electrically tight monolayer. The growth medium was then removed and replaced with growth medium in which the FCS had been replaced with 0.1% BSA (defined medium). After 24 h, the medium was replaced with fresh serum-free medium containing different concentrations of glucocorticoid. After a further 24 h, the basolateral medium was replaced with medium containing 5 μg/ml human sputum elastase (Elastin Products, Owensville, MO). The morphology of the cell layer was examined under the microscope 24 h later. The transepithelial resistance was monitored throughout the experiment using a Millicell electrical resistance system (Millipore, Bedford, MA). An electrically tight monolayer was established as described above. The growth medium was then removed with replacement on the apical surface with 1 ml of serum-free medium containing glucocorticoid. The same quantity of dextran, and epithelial flux rates were expressed as dextran concentrations from a standard curve derived from known concentrations of dextran, and epithelial flux rates were expressed as picomole dextran per hour.

To measure epithelial permeability, electrically tight monolayers of 16HBE14o− cells (passage 10) were established as described above and treated with glucocorticoids for 7 days of steroid exposure, as previously described (25). Briefly, 50 μg of fluoresceinated dextran (neutral, mol wt = 4.4 kDa) were added to the apical compartment of each membrane. After 30 min, membranes were then transferred to new 24-well plates and incubated for a further hour, after which the membranes were carefully removed and the fluorescence of the basal medium was measured. Fluorescence measurements were interpreted as dextran concentrations from a standard curve derived from known concentrations of dextran, and epithelial flux rates were expressed as picomole dextran per hour.

To measure the extent of mechanically induced damage, 16HBE14o− cells (passage 10) were seeded in 96-well plates at a density of 10,000 cells/well and grown to confluence. Cells were treated with glucocorticoid for 24 h before wounding. Reproducible wounding was achieved by using a purpose-built wounding device, which created a circular wound halfway along the radius of each well. After wounding each well, the plate was removed, and cultures were fixed for 10 min using 50:50 methanol-acetone mixture. After removal of the fixative, cultures were air-dried, stained with 0.1% crystal violet, washed extensively in water, and finally air-dried. Images of the wound area were analyzed using a Leica Q5501W image analysis system (Leica Microsystems Imaging Solutions, Cambridge, UK).

To measure the retention of glucocorticoid in lung epithelial cells, electrically tight monolayers of 16HBE14o− cells (passage 10) were established as described above. The growth medium was then removed and replaced on the apical surface with 1 ml of serum-free MEM containing 500 nM radiolabeled FP or FF. The same quantity of serum-free MEM was added to the basolateral surface of the cells. After intervals of 20 and 40 min and 1, 2, 4, and 6 h, 500 μl of apical and basal medium were removed from three wells from each treatment group. The membranes were then washed eight times in ice-cold PBS and cut out of their holders. The radioactivity present in each sample was determined by scintillation counting and compared with values obtained for aliquots of the initial 500 nM solutions, which had been added to the cells.

Steroid Receptor Selectivity Assays

Androgen receptor assay. Monkey kidney CV-1 cells were transfected with plasmid androgen receptor (AR), encoding expression of the AR, and the reporter plasmid FBMMMTV using Fugene-6 reagent according to the manufacturer’s protocol. After transfection (24 h), cells were seeded at a density of 5,000 cells/well in white 384-well plates containing glucocorticoid. After a further 24 h at 37°C, luciferase activity was determined by addition of 10 μl of SteadyGLO reagent (Promega, Southampton, UK) to each well of the plates. Plates were incubated in the dark for 10 min before determination of the luminescence using a Viewlux reader (PerkinElmer).

Mineralocorticoid receptor assay. Monkey kidney CV-1 cells were transfected with plasmid mineralocorticoid receptor, encoding expression of the mineralocorticoid receptor; plasmid PGC1, encoding expression of the PGC1 coactivator; and the reporter plasmid MMTV luciferase using Fugene-6 reagent according to the manufacturer’s protocol. After transfection (24 h), cells were either left untreated (to measure agonism) or stimulated with 0.2 nM aldosterone (to measure antagonism) and seeded at a density of 7,000 cells/well in white 384-well plates containing glucocorticoid. After a further 24 h incubation at 37°C, luciferase activity was determined by addition of 10 μl of SteadyGLO reagent (Promega) to each well of the plates. Plates were incubated in the dark for 10 min before determination of the luminescence using a Viewlux reader (PerkinElmer).

PRb agonist assay. Monkey kidney CV-1 cells were transfected with 10% progesterone receptor (PRb)-BacMam and 10% MMTV-BacMam and seeded at a density of 10,000 cells/well in white 384-well plates containing glucocorticoid dissolved at the required concentration in DMEM. After a further 24 h incubation at 37°C, luciferase activity was determined by addition of 10 μl of SteadyGLO reagent (Promega) to each well of the plates. Plates were incubated in the dark for 10 min before determination of the luminescence using a Viewlux reader (PerkinElmer).

Estrogen receptor-α/β scintillation proximity binding assay. Biotinylated estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) proteins were captured on streptavidin-coated scintillation proximity assay beads (GE Healthcare, Little Chalfont, Bucks, UK). The beads were added to 384-well optical bottom plates containing glucocorticoids and 2 nM [3H]estradiol (170 Ci/mmol). The plates were sealed with 300 μl of scintillation cocktail on the bottom and incubated at room temperature for at least 2 h. The plates were read on a Wallac Microbeta Trilux 1450 reader (PerkinElmer) for 1 min/well.

Brown Norway rat ovalbumin model. All in vivo studies were ethically reviewed within GlaxoSmithKline and carried out in accordance with the UK Animal (Scientific Procedures) Act 1986. The Brown Norway rat ovalbumin-induced lung eosinophilia model is an established model of allergic inflammation of the respiratory system (17–19). Briefly, male Brown Norway rats (180–250 g) were obtained from Charles River (Ramsgate, UK) and assigned into groups of eight rats. Animals were sensitized by intraperitoneal injection of 1 mg ovalbumin and 10 mg aluminum hydroxide in 1 ml of PBS. After sensitization (14–21 days), animals were placed under gaseous general anesthesia using isoflurane and killed using intraperitoneal injection of pentobarbital sodium (euthatal; Merial Animal Health, Harlow, UK), and bronchoalveolar
Inhibition of NF-κB Pathway and Inhibition of LPS-induced TNF-α Production

Many of the inflammatory cytokine pathways are known to be activated by NF-κB, and glucocorticoids have been shown to potently inhibit NF-κB-mediated gene transcription (15). TNF is a key inflammatory cytokine, and its release and downstream activity are regulated by activation of NF-κB. Therefore, to evaluate the in vitro anti-inflammatory activity of FF, this study investigated the effects of FF and a range of clinically used glucocorticoids in NF-κB reporter assays using a human lung epithelial cell line and in an assay of LPS-induced TNF-α release from PBMCs. In both of these assays, FF showed the highest potency of all the glucocorticoids tested, with mometasone furoate (MF) demonstrating a similarly high potency in the NF-κB assay, summarized in Table 1.

Potent activation of GRE-mediated transcription and protection of respiratory epithelial cell integrity in vitro. Glucocorticoids are known to play a role in protecting epithelial cell barriers from inflammatory-mediated damage and in enhancing repair potential in damaged cells (24, 26). A number of gene products may be involved in this protective effect, such as the key anti-protease secretory leukocyte peptidase inhibitor (SLPI; 1, 21). The glucocorticoid-mediated transcription of this and other gene products is driven by the binding of the GR to GRE-binding sites on the respective promoter regions. The potency of FF and other clinically used glucocorticoids was therefore measured in an assay of GRE activation to determine whether there is a correlative potency between the activation of GRE and their protective effect in epithelial cells. Table 1 shows that FF is a very potent activator of the GRE pathway, with subnanomolar protranscriptomic activity.

The protection of human lung epithelial 16HBE14o− cells by FF and other glucocorticoids was assessed in a number of different ways. The maintenance of epithelial monolayer integrity can be monitored by measuring the detachment of the cell layer from the well membrane after treatment of the monolayer with the protease elastase. Figure 2A shows that FF had the greatest protective potency against elastase-induced monolayer damage as assessed by cell detachment. Pretreatment of an epithelial monolayer with glucocorticoid can also mitigate some of the secondary inflammatory damage caused as a consequence of an initial mechanical insult. Figure 2B shows that FF also had the most protective effect of all glucocorticoids tested in this system.

Table 1. Potency of glucocorticoids in human cellular assays of GR pathway activity (pEC50 ± SD)

<table>
<thead>
<tr>
<th>Assay system</th>
<th>FF</th>
<th>FP</th>
<th>MF</th>
<th>BUD</th>
<th>Cic-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>10.53 ± 0.27 (29 pM)</td>
<td>10.36 ± 0.34 (44 pM)*</td>
<td>10.59 ± 0.16 (26 pM)</td>
<td>9.64 ± 0.18 (229 pM)*</td>
<td>9.53 ± 0.35 (295 pM)*</td>
</tr>
<tr>
<td>LPS-TNF</td>
<td>9.93 ± 0.48 (117 pM)</td>
<td>9.63 ± 0.37 (234 pM)</td>
<td>ND</td>
<td>8.42 ± 0.32 (3,800 pM)*</td>
<td>8.50 ± 0.31 (3,160 pM)*</td>
</tr>
<tr>
<td>GRE</td>
<td>10.24 ± 0.07 (57 pM)</td>
<td>10.13 ± 0.26 (74 pM)</td>
<td>10.17 ± 0.11 (68 pM)</td>
<td>9.26 ± 0.38 (549 pM)*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SD. FF, fluticasone furoate; FP, fluticasone propionate; MF, mometasone furoate; BUD, budesonide; Cic-AP, ciclesonide active principle; GRE, glucocorticoid response element; LPS-TNF, lipopolysaccharide-induced tumor necrosis factor release; NF-κB, nuclear factor-κB; ND, not determined; pEC50, negative of the log of the mean molar concentration that produces 50% of the maximum possible response (calculated from the following no. of independent concentration curves: NF-κB FF n = 57, FP n = 312, MF n = 30, BUD n = 26, Cic-AP n = 12; TNF FF n = 16, FP n = 14, BUD n = 16, Cic-AP n = 15; GRE FF n = 11, FP n = 19, MF n = 8, BUD n = 4).*Difference vs. FF, P < 0.001 (ANOVA followed by Dunnet’s test).
concentration-dependent reduction in epithelial permeability to dextran, as shown in Fig. 3, with the rank order of potency being FF > FP > budesonide (BUD).

Selectivity for steroid hormone receptors. Ligands for GR often have affinity for other steroid hormone receptors [AR, estrogen receptor (ER), mineralocorticoid receptor (MR), and progesterone receptor (PR)] caused by the similarities between the ligand-binding domains of the receptors in this family (4, 5). These steroid hormone receptors play key roles in the regulation of many important aspects of physiology, including gestation, the menstrual cycle, expression of secondary sexual characteristics, and salt/water balance. Cross-reactivity of glucocorticoids for these receptors can therefore lead to potentially deleterious effects (8, 11). FF displays a highly selective profile for the GR vs. other steroid hormone receptors, with selectivity ranging from 30 (PRb) to >330,000 (AR, ER) for GR-mediated NF-κB inhibition vs. steroid hormone activity (Table 2). This level of selectivity is slightly better than that seen with FP but substantially better than that seen with MF, BUD, and the active component of ciclesonide (Cic-AP).

Retention of FF in Respiratory Epithelial Cells In Vitro

Prolonged residence time in the target tissue is a desirable property for topically applied glucocorticoids because it increases the opportunity for pharmacological action at the target site and reduces the risk of systemic exposure by slowing down its transport to the systemic compartment. The transport of FF across, and retention in, respiratory tissue was investigated using a monolayer of human lung epithelial (16HBE14o−) cells. FF (500 nM) was added to one side of the monolayer and was seen to associate with the cell monolayer at a level greater than that seen with other glucocorticoids (Fig. 4A). In separate experiments, it was shown that FF binds to human lung epithelial cells to a greater degree compared with Cic-AP (120%, data not shown). Additional studies examined the time dependence of cellular association of FF and FP. FF and FP reached a maximal cell association after ~30 min.

After association with the cell layer, the rate of flux of glucocorticoids out from the cell layer into the basal aqueous medium was measured (i.e., the opposite side of the monolayer to where the glucocorticoid was first added). The rate of transport of FF was lower than that seen with other glucocorticoids (Fig. 4B) even though the concentrations reached in the monolayer were greater than the other glucocorticoids. This is again consistent with a greater cellular retention of FF.

Anti-inflammatory Effects in a Respiratory Animal Model of Allergic Inflammation

The in vivo anti-inflammatory activity of FF was tested in the Brown Norway rat model of ovalbumin-induced respiratory allergic eosinophilia. Glucocorticoids that have been shown to be effective in rhinitis and inflammatory respiratory disease are also effective inhibitors of the eosinophilia elicited in this model (7, 23). FF, when given intratracheally at a dose of just 30 μg, essentially completely inhibited the lung eosinophilia in the Brown Norway rat (Fig. 5). This inhibition was significantly greater than that seen with the same dose of FP, FF also demonstrated a long anti-inflammatory duration of action in this model, still suppressing eosinophilia by 78 ± 9% 14 h postdosing.

Table 2. Potency of glucocorticoids for different human steroid hormone receptors (pEC50 ± SD) compared with potency for GR-mediated inhibition of NF-κB (pEC50 ± SD from Table 1)

<table>
<thead>
<tr>
<th>Steroid hormone receptor</th>
<th>FF</th>
<th>FP</th>
<th>MF</th>
<th>BUD</th>
<th>Cic-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR (NF-κB)</td>
<td>10.53±0.27</td>
<td>10.36±0.34</td>
<td>10.59±0.16</td>
<td>9.64±0.18</td>
<td>9.53±0.35</td>
</tr>
<tr>
<td>MR</td>
<td>7.63±0.44 (790)</td>
<td>7.64±0.4 (520)</td>
<td>9.33±0.41 (18)*</td>
<td>8.71±0.52 (8.6)*</td>
<td>8.74±0.48 (6.1)*</td>
</tr>
<tr>
<td>PRb</td>
<td>9.05±0.2 (30)</td>
<td>9.01±0.4 (22)</td>
<td>10.67±0.26 (0.82)*</td>
<td>9.58±0.15 (1.2)*</td>
<td>8.36±0.41 (15)</td>
</tr>
<tr>
<td>AR</td>
<td>&lt;5 (&gt;330,000)</td>
<td>&lt;6 (&gt;22,000)</td>
<td>6.65±0.61 (870)†</td>
<td>6.42±0.27 (1700)†</td>
<td>6.24±0.43 (1900)†</td>
</tr>
<tr>
<td>ER-α</td>
<td>&lt;5 (&gt;330,000)</td>
<td>&lt;6 (&gt;22,000)</td>
<td>&lt;5 (&gt;380,000)</td>
<td>&lt;5 (&gt;43,000)</td>
<td>&lt;5 (&gt;33,000)</td>
</tr>
<tr>
<td>ER-β</td>
<td>&lt;5 (&gt;330,000)</td>
<td>&lt;6 (&gt;22,000)</td>
<td>&lt;5 (&gt;380,000)</td>
<td>&lt;5 (&gt;43,000)</td>
<td>&lt;5 (&gt;33,000)</td>
</tr>
</tbody>
</table>

Values are means ± SD. Values in parentheses are the calculated selectivity of each glucocorticoid for GR receptor-mediated NF-κB vs. the respective steroid hormone receptor. GR, glucocorticoid receptor; AR, androgen receptor functional assay; ER-α, estrogen receptor-α binding assay; ER-β, estrogen receptor-β binding assay; MR, mineralocorticoid receptor functional assay; PRb, progesterone receptor b functional assay. pEC50 calculated from the following no. of independent concentration curves: MR FF n = 5, FP n = 48, MF n = 6, BUD n = 25, Cic-AP n = 18; PRb FF n = 12, FP n = 100, MF n = 4, BUD n = 7, Cic-AP n = 5; AR FF n = 15, FP n = 47, MF n = 7, BUD n = 6 Cic-AP n = 10; ERα FF n = 3, FP n = 29, MF n = 6, BUD n = 2, Cic-AP n = 7; ERβ FF n = 3, FP n = 34, MF n = 5, BUD n = 4, Cic-AP n = 4. Selectivity values are calculated for each glucocorticoid by dividing the molar EC50 for the respective steroid hormone receptor by the molar EC50 for GR-mediated NF-κB inhibition (shown to two significant figures). *Difference vs. FF, P < 0.0001 (fitted using a general linear model, e.g., ANOVA). Statistical comparisons cannot be made for ER because of the lack of absolute potency values and errors for all compounds, but comparisons can be made for the AR data (FF vs. MF, BUD, or Cic-AP) because of error residing with one of the means (±difference vs. FF statistically different at the 5% level).
DISCUSSION

FF is a new enhanced-affinity glucocorticoid with a unique combination of pharmacodynamic and physicochemical properties, which may contribute to a favorable efficacy profile in the topical treatment of various respiratory inflammatory disorders. X-ray crystal structure studies have previously shown that the 17α-furoate group of FF, in combination with the fluoromethylthio, keto, and hydroxyl groups on the fluticasone steroid backbone, enable better H-bond and Van de Waals interactions to be made with amino acids within the ligand-binding site of GR (3). These interactions are reflected in the enhanced affinity of FF for the GR. Previous studies on the human GR binding kinetics of FF have shown a very fast association and a slow dissociation resulting in a relative receptor affinity (RRA) of 2,988 (dexamethasone RRA = 100; see Ref. 22). By contrast, other topically active glucocorticoids displayed a significantly lower receptor affinity: MF, 2,244; FP, 1,775; Cic-AP, 1,212; and BUD, 855 (22).

Consistent with the above, results from the preclinical studies reported here show that FF has very potent activity in assays of glucocorticoid pathway activity and cell protection. GR agonist activity is known to suppress the activation of the downstream NF-κB pathway (15). Inhibition of the NF-κB pathway is thought to be intimately involved in the anti-inflammatory activity of glucocorticoids, since the NF-κB pathway is known to be a key factor in the synthesis of a wide number of inflammatory cytokines (10). In the experiments reported here, FF was the most potent inhibitor of NF-κB activation and TNF release of all the glucocorticoids tested, along with MF. These data demonstrate that the improved binding of the 17α-furoate group to GR contributes to the greater cellular potency seen in cellular GR-dependent pathways.

Binding of GR to GREs is a vital step in the induction of a number of key gene products such as the anti-protease SLPI and the phosphatase inhibitor MAPK phosphatase-1 (1, 21). As with the DNA-independent pathways described above, FF demonstrated very potent activation of this DNA-dependent glucocorticoid pathway. Protease enzymes, such as elastase, are believed to be important in the inflammatory cascade that damages respiratory tissue after continued exposure to allergen. Glucocorticoids are known to protect the airway epithelial barrier against a variety of cellular insults, including elastase, and to enhance the repair potential of damaged cells (24, 26). The induction of anti-inflammatory proteins, such as SLPI and MKP-1, via GRE activation, are likely to contribute to the cell protective properties of glucocorticoids. In assays of elastase-induced cell damage and mechanically induced cell damage, FF demonstrated a highly effective cellular protection with an affinity greater than that seen with other clinically used glucocorticoids, including MF and FP.

Fig. 5. Effect of FF and FP on allergen-induced lung eosinophilia in the Brown Norway rat (mean ± SD, n = 8 animals). Difference vs. FP (t-test): *P < 0.05. BAL, bronchoalveolar lavage; IT, intratracheal.
In addition, FF reduced epithelial permeability more potently than other glucocorticoids. Although the mechanism behind this increase in permeability is unknown for 16HBE14o− epithelial cells, studies on rat mammary tumor cells have shown that the glucocorticoid dexamethasone induces cell border localization of ZO-1 and β-catenin and that these changes correlate with increased electrical resistance (6). Moreover, in a further study, it was shown that dexamethasone induced stimulation of tight junction function and intercellular adhesion by downregulation of the actin-bundling protein fascin, which also binds to β-catenin (27). If similar effects occur in the airway epithelia of patients with lung disease, this could be a significant component in the efficacy of glucocorticoids, since epithelial damage incurred by shear forces within the airway should be reduced, as would the subsequent penetration of allergen and irritant in the submucosa. The importance of the latter is illustrated in studies where increased epithelial permeability resulted in greatly enhanced immune responses to intranasal antigens (13). Moreover, it has been shown that there is greatly increased susceptibility to adenoviral infection when epithelial integrity is disrupted (12). Thus, in addition to the anti-inflammatory effects of glucocorticoids, enhanced epithelial stability and reduced permeability could also contribute to their overall efficacy. However, more research is required to fully investigate the role of glucocorticoids in this complex process.

The GR is a member of the steroid hormone nuclear receptor family. Other family members include the MR, ER, AR, and PR. The ligand-binding domains of these receptors share significant homology with the GR (4, 5); however, many subtle differences exist in the topology of the ligand-binding sites. The GR, in particular, has a unique 17α-pocket, which may provide some selectivity for glucocorticoids because they often have larger substitutions at the 17α-position compared with estrogen, progesterone, and testosterone (4). However, some GR ligands have been shown to have affinity for other steroid hormone receptors (8, 11). Because of the important physiology that is regulated by the other steroid hormone receptors, it is important that a clinically used glucocorticoid should not interact significantly with these receptors. Table 2 shows that FF has substantially better selectivity than other clinically used glucocorticoids such as MF, BUD, and Cic-AP. Although at first glance it may appear that these glucocorticoids are structurally similar, the marked difference in their selectivity for steroid hormone receptors demonstrates that small structural changes can lead to substantial differences in their selectivity profiles. Although the 17α-ester groups of glucocorticoids may contribute to their GR selectivity, the results described here demonstrate that the steroid backbone plays a key role in defining GR selectivity, particularly against the PR. This is most clearly demonstrated by the difference in selectivity between FF and MF. Although they share the same 17α-furoate ester, they differ in the chemical structure of their fluticasone and mometasone steroid backbones. The fluticasone backbone of FF achieves a 30-fold selectivity for GR over PR, whereas the mometasone backbone of MF achieves no selectivity against PR at all. This total lack of selectivity for GR against PR is also seen with BUD; however, it is not clear whether this is because of its steroid backbone or its 17α-acetal moiety.

Although previous studies have shown that FF has a high affinity for and slow dissociation from GR (22), its duration of effect can also be extended by high affinity for the respiratory target tissue itself. Enhanced affinity for the target tissue may prolong residence time in the tissue, increase duration of the anti-inflammatory effect at the target site, and also reduce the risk of systemic exposure caused by delayed transit from the target site. In the experiments described in this paper, FF was rapidly transported in a human lung epithelial cell monolayer and reached maximal steady-state levels greater than that of any other glucocorticoid tested and approximately two times those seen with FP. This fast rate of transport of FF is consistent with studies showing that FF causes GR to translocate to its nuclear site of action faster than FP (16). Furthermore, the rate of transport of FF out of the cells in the basal aqueous layer was slower than the other glucocorticoids, again consistent with retention of FF in the respiratory tissue. Separate studies with freshly isolated human lung tissue also support the above data showing that FF was retained more avidly by lung tissue than FP (22). In addition, preliminary results suggest a similar trend in primary human nasal tissue. In a single experiment on human nasal tissue pooled from three donors, FF showed greater binding compared with FP (22). These properties will therefore encourage retention in respiratory tissue after topical dosing and, together with the previously demonstrated long duration of GR binding, may contribute to an extended duration of anti-inflammatory action.

After intranasal administration in humans, drugs are rapidly cleared from the lumen of the nose. A glucocorticoid will therefore need to enter the nasal tissue rapidly and effectively, since this portion of the drug will have to sustain anti-inflammatory activity until the next dose is given up to 24 h later. The very high association of FF with tissue will increase the likelihood that sufficient glucocorticoid will be contained in the tissue to maintain full anti-inflammatory activity over 24 h. This benefit will synergize with its very high affinity for GR and slow dissociation from the receptor. It is unlikely that weaker-potency glucocorticoids can overcome their weaker potency simply by being given at a higher dose because of the limited capacity for the nose to absorb drug after each dosing. The amount of drug available for binding to GR will therefore become limiting over the 24-h period; however, if the glucocorticoid has greater potency, then the drug that is left in the nasal tissue will have a proportionally greater anti-inflammatory effect.

A substantial proportion of intranasally administered glucocorticoids are swallowed, providing a potential route for systemic availability with consequent unwanted effects, such as cortisol suppression. FF has been shown to undergo very effective first-pass metabolic inactivation by the liver (3). In addition, FF also has a high affinity for plasma protein (>99%; see Ref. 16). These factors suggest that blood concentrations of free FF (i.e., those that are available for transport into tissues associated with glucocorticoid side effects such as the pituitary and hypothalamus) will be below the levels likely to elicit adverse effects. This hypothesis is consistent with the absence of cortisol suppression in humans after intranasal administration of supratherapeutic doses of FF (14).

It is known that allergy is a major factor in the pathogenesis of rhinitis and asthma, and, although no animal models can capture all aspects of human rhinitis or asthma, the Brown
Norway rat model shows a characteristic eosinophil inflammation of the lung, elicited by an allergic response to the administration of ovalbumin. Glucocorticoids that have been shown to be effective in rhinitis and inflammatory respiratory disease are also effective inhibitors of this eosinophilia (7, 23). In this model, FF demonstrated a more potent suppression of inflammation than that seen with FP, confirming the results of the in vitro studies.

In conclusion, this paper suggests that the combination of the fluticasone backbone and 17α-furoate ester in FF provides the optimal combination for potency, tissue binding, and steroid hormone selectivity. The enhanced affinity of GR binding of FF, coupled with its extended tissue association, could be expected to lead to greater and more prolonged anti-inflammatory effects compared with other clinically used glucocorticoids and should provide true once-daily efficacy. In addition, the high selectivity of FF for GR, coupled with its avid target tissue retention, rapid systemic clearance, and high plasma protein binding, should provide a safe clinical profile. FF is currently under investigation for the treatment of allergic rhinitis. The results of these studies will help establish the effectiveness of this novel enhanced-affinity glucocorticoid in a clinical setting.

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