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Discovery of a novel isoxazoline derivative of prednisolone endowed with a robust anti-inflammatory profile and suitable for topical pulmonary administration

E. Ghidini [a,*], A.M. Capelli [a], C. Carnini [c], V. Cenacchi [b], G. Marchini [c], A. Virdis [d], A. Italia [e], F. Facchinetti [c]

[a] Chemistry Research and Drug Design Department, Chiesi Farmaceutici S.p.A., Parma, Italy
[b] Pharmacokinetic Department, Chiesi Farmaceutici S.p.A., Parma, Italy
[c] Pharmacology and Toxicology Department, Chiesi Farmaceutici S.p.A., Parma, Italy
[d] Nikem Research Srl, Baranzate di Bollate (Mi), Italy
[e] Chiman Srl, via Reggio Calabria, 12 Rottofreno (PC), Italy

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ABSTRACT

A novel glucocorticoids series of (GCs), 6α,9α-di-Fluoro 3-substituted C-16,17-isoxazolines was designed, synthesised and their structure–activity relationship was evaluated with glucocorticoid receptor (GR) binding studies together with GR nuclear translocation cell-based assays. This strategy, coupled with *in silico* modelling analysis, allowed for the identification of Cpd **#15,** an isoxazoline showing a sub-nanomolar inhibitory potency (IC_{50} = 0.84 nM) against TNFα-evoked IL-8 release in primary human airways smooth muscle cells. In Raw264.7 mouse macrophages, Cpd **#15** inhibited LPS-induced NO release with a potency (IC_{50} = 6 nM) > 10-fold higher with respect to Dexamethasone. Upon intratracheal (i.t.) administration, Cpd **#15**, at 0.1 μmol/kg significantly inhibited and at 1 μmol/kg fully counteracted eosinophilic infiltration in a model of allergen-induced pulmonary inflammation in rats. Moreover, Cpd **#15** proved to be suitable for pulmonary topical administration given its sustained lung retention ($t_{1/2}$ = 6.5 h) and high pulmonary levels (>100-fold higher than plasma levels) upon intratracheal administration in rats. In summary, Cpd **#15** displays a pharmacokinetic and pharmacodynamic profile suitable for topical treatment of conditions associated with pulmonary inflammation such as asthma and COPD.

1. Introduction

An important limitation of GCs therapy is that the desired anti-inflammatory effects are accompanied by side effects such as loss of muscle mass, redistribution of body fat, osteoporosis, diabetes, glaucoma and depression [1]. In patients with asthma and chronic obstructive pulmonary disease (COPD), the adverse effects of GCs chronic use can be limited by topical pulmonary delivery via inhalation [2]. Nevertheless, a degree of systemic exposure inevitably occurs which may raise safety concerns in elderly patients as well as in patients requiring high dose regimen [3]. Hence, there is the need to enhance local anti-inflammatory potency of topical GCs while limiting their systemic exposure in order to minimize unwanted side effects.

A considerable amount of research is aimed at discovering novel steroidal GR agonists with high anti-inflammatory potency upon topical application and limited systemic exposure. Despite these efforts, only few novel steroidal molecules showing significant structural changes with respect to existing drugs have been developed [4,5]. The present study attempts to fill this gap by describing the design, synthesis and pharmacological profile of a novel series of 6α,9α-di-Fluoro 3-substituted isoxazolines. **Cpd #15,** in particular, proved to be a suitable compound for pulmonary topical administration given its robust anti-inflammatory potency, prolonged lung retention and low systemic exposure upon intratracheal administration.

2. Experimental section

2.1. Chemicals and reagents

All commercially available chemicals and solvents were purchased from Aldrich-Sigma (St. Louis, MO). Steroidal derivatives

Abbreviations: GCs, glucocorticoids; NO, nitric oxide; GILZ, glucocorticoid-induced leucine zipper; ASMCs, airway smooth muscle cells.

* Corresponding author. Tel.: +39 0521 279913; fax: +39 05212762 545567.
E-mail address: e.ghidini@chiesi.com (E. Ghidini).

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(compounds **#1–18)** were synthesized in our laboratory following the route described in Scheme 1. Starting from commercially available derivative **#21** and for **#22**, the reaction proceeded in ethyl acetate and $NaHCO_3$, together with a few drops of water, by stirring at room temperature for six days (Scheme 1; A). When derivatives Cpd **#21** were prepared by *in situ* chlorination of the corresponding aldoximes with $BTMAICl_4$ (benzyltrimethylammonium tetrachloroiodate) [6] or bleach, the reaction proceeded in dry dichloromethane (DCM) and triethylamine (TEA) at room temperature for 3 h (Scheme 1; B). All reactions details are reported in the Supporting Information. The structures of these compounds are shown in Table 1 and the steroidal drugs are:

(16S,17R)-3′-(4-chlorophenyl)-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#1**; (16S,17R)-3′-(4-methoxyphenyl)-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#4**; (16S,17R)-3′-methylacetate-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#5**; (16S,17R)-3′-propyl-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#6**; (16S,17R)-3′-methyl-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#7**; (16S,17R)-3′-(hydroxymethyl)-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#8**; (16S,17R)-3′-hydroxy-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#10**; (16S,17R)-3′-(thiophen-3-yl)-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#11**; (16S,17R)-3′-(furan-3-yl)-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#12**; (16S,17R)-3′-(thiophen-3-yl)-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#13**; (16S,17R)-3′-(furan-3-yl)-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#14**; (16S,17R)-3′-bromo-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#15**; (16S,17R)-3′-methyl-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#16**; (16S,17R)-3′-(4-methoxyphenyl)-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#17**; (16S,17R)-3′-phenyl-6,9-difluoro-11β,21-dihydroxy-4′H-pregna-1,4-dieno[16,17-d]isoxazole-3,20-dione, Cpd **#18**.

Preparation of Cpd **#2,** Cpd **#3** and Cpd **#9** was already described in literature. [7]

The purity of tested compounds determined by analytical UPLC was >98%. The standards Dexamethasone (Chart 1) is commercially available and was purchased from Acros Organics while Deflazacort active metabolite (Chart 1) was synthesized following a literature method [8].

Table 1
Compounds series.

Compound	R	X, Y
#1	p-Cl-Phenyl,	H, H
#2	COOEt	H, H
#3	COOH	H, H
#4	p-OMe-Phenyl	H, H
#5	CH_2OCOCH_3	H, H
#6	Propyl	H, H
#7	Methyl	H, H
#8	CH_2OH	H, H
#9	Br	H, H
#10	OH	H, H
#11	3-Thienyl	H, H
#12	3-Furyl	H, H
#13	3-Thienyl	F, F
#14	3-Furyl	F, F
#15	Br	F, F
#16	Methyl	F, F
#17	p-OMe-Phenyl	F, F
#18	Phenyl	F, F

2.2. Biological assay

2.2.1. Cell culture

Murine macrophagic cell line (RAW264.7) was purchased from ATTC (Manassas, USA) and cultured in RPMI 1640 medium (w/o Phenol Red) supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 μg/ml streptomycin (Invitrogen), in an atmosphere of 5% CO_2 at 37 °C.

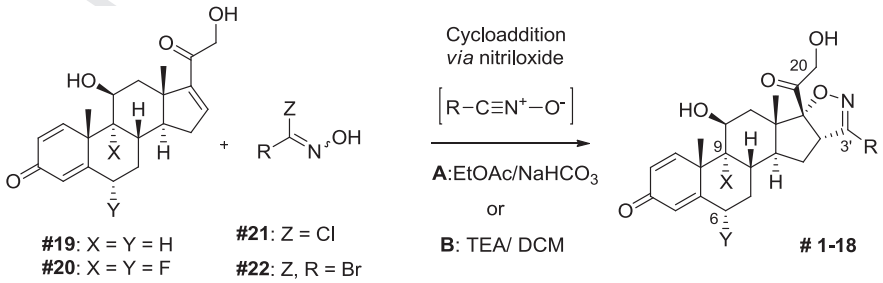
PathHunter™ CHO-K1 GR and MR Cell Line stably expressing EA-NLS-NRS and the ProLabel-tagged glucocorticoid and mineralcorticoid receptor respectively were purchased from DiscoverX (CA, United States). Cells were cultured in F-12 Nutrient Mixture (HAM) supplemented with 10% Fetal Bovine Serum (Invitrogen) plus 2 mM L-glutamine and antibiotics (100 U/ml Penicillin, 100 g/ml Streptomycin, 300 g/ml Hygromycin B, and 500 g/ml G418/Geneticin) in an atmosphere of 5% CO_2 at 37 °C.

Primary human airway smooth muscle cells (ASMCs) were purchased from LONZA (Basel, CH) and cultured in DMEM medium supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, 100 U penicillin and 100 μg/ml streptomycin (Invitrogen), in an atmosphere of 5% CO_2 at 37 °C.

2.2.2. Nitric measurement assay protocol

RAW264.7 cells were seeded in 0.3 ml RPMI (w/o Phenol Red) containing 10% FBS in 48-well tissue culture plates at the density of 7.5×10^4 cells/well and grown for 24 h at 37 °C with 5% CO_2. Then cells were treated with different concentration of corticosteroids (10^{-11}M-10^{-6} M, final DMSO concentration 0.1%) for 15 min. before stimulation with lipopolysaccharide from *Escherichia coli* (100 ng/ml as final concentration) and incubated for 18 h in RPMI (w/o Phenol Red) supplemented with 10% FBS.

Accumulation of nitrite in the medium was measured by a colorimetric assay method based on the Griess reaction. Briefly,



Scheme 1. Compounds **1–18** were synthesized starting from enone, #19 or #20 and hydroximoyl chlorides derivatives **#21** or hydroxycarbonimidic dibromide **#22** via 1,3-dipolar cycloaddition of nitrile oxides.

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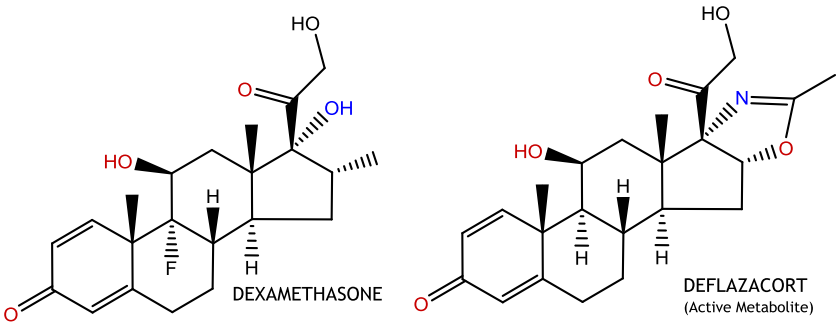

Q5 **Chart 1.**

samples were reacted with 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid at room temperature for 10 min, and nitrite concentration was determined by absorbance at 540 nm in comparison with sodium nitrite as a standard. Compound potencies were expresses as concentration able to inhibit the half maximal (50%) NO release [IC_{50}] in the dose–response curve obtained after stimulation with LPS.

2.2.3. Glucocorticoid receptor (GR) and mineralcorticoid receptor (MR) translocation assay protocol

The cell-based GR-translocation assay in Enzyme Fragment Complementation format developed by DiscoveRx (Fremont, CA) was employed to quantitatively measure GR nuclear translocation [9]. PathHunter CHO-K1 GR and PathHunter CHO-K1 MR cells were seeded in a 96-well plate at 15,000 cells/well in 100 μL medium without antibiotics and 24hours later the compounds were added (concentration ranging from 10^{-12}M to 10^{-6}M) for 3 h at 37 °C. Luminescence, estimated as relative light units (RLU), was detected by using a CENTRO LB 960 microplate reader (Berthold Technologies). Statistical analysis and determinations of EC_{50}s were performed by using Prism-version 3.0 Graphpad Software (San Diego, CA).

2.2.4. IL-8 release assay protocol

ASMCs were seeded in 0.5 ml DMEM containing 10% FBS in 48-well tissue culture plates at the density of 10^4 cells/well and grown for 24 h at 37 °C with 5% CO_2. Subsequently, cells were serum starved for 18 h and treated with different concentration of corticosteroids (final DMSO concentration 0.1%) for 60 min before stimulation with TNF (0.1 ng/ml). After 18 h incubation in DMEM serum free, the IL8 release in the supernatant was assayed using ELISA kit (Invitrogen). Compound potencies were expressed as concentration able to elicit half maximal inhibition of IL8 release [IC_{50}].

2.2.5. Real time PCR analysis of GILZ gene expression

ASMCs were seeded in 0.1 ml DMEM containing 10% FBS in 96-well tissue culture plates at the density of $4x10^3$ cells/well and grown for 24 h at 37 °C with 5% CO_2. Then cells were starved over-night in DMEM without FBS and then treated with different concentrations of compounds (10-^{12}M-10-^{7}M, final DMSO concentration 0.1%) for 4 h before mRNA extraction.

Total RNA was isolated using TaqMan Gene expression Cells-to-Ct kit (Applied Biosystems, Foster City, CA). Briefly, cells were lysed in Cell Lysis solution containing DNAse I for 5 min, followed by two-minute incubation with the stop solution. Reverse transcription reactions were performed using 10 μL of each cell lysate, according to the manufacturer's instructions. Two sets of primers-probes were designed for the human and rat cell lines using the Primer Express Software version 3.0 (Applied Biosystems). The chosen reporter fluorophores for TaqMan MGB probes were VIC for the endogenous reference β-actin gene (ACTB) and 6-carboxyfluorescein (FAM) for GILZ or TAT genes.

The two sets of primers-probes were the follows: set 1, ACTB-FW (forward) 5′-GGCGGCACCACCATGTAC-3′, ACTB-RE (reverse) 5′-CAGGGCAGTGATCTCCTTCTG-3′ ACTB probe5′-VIC-TGGCA TTG CCGACAGG-3′; set 2 GILZ-FW (forward) 5′-TGGCCATAGACAACAAG ATCGA-3′, GILZ-RE (reverse) 5′-TCACAGCATACATCAGATGATTC TTC-3′, GILZ probe 5′-FAM-AGGCCATGGATCTGG -3′.

The selected primers and probes were subjected to Basic Local Alignment Search Tool (BLAST) database searches to exclude any sequence similarities. Real-time quantitative PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems). All samples were run in triplicate in a final volume of 25 μL containing 12.5 μL of 2× TaqMan Gene Expression PCR Master Mix, 300 nM of each primer, 250 nM of each probe and 4 μL of RT reaction, according to the manufacturer's instructions (Applied Biosystems). Amplification conditions for GILZ/β-actin were: 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s and 60 °C for 1 min.

Relative expression of GILZ mRNA was calculated using the 2-(CT) comparative method, with normalization against the internal endogenous reference β-actin gene.

2.2.6. Competitive binding assay

Competitive binding assays to evaluate the affinity of the compounds to human glucocorticoid receptor and human mineralcorticoid receptor were performed in triplicate in a total volume of 160 μL, containing 5nM [^{3}H]Dexamethasone or 4.5 nM [^{3}H] D-Aldosterone (Amersharm Pharmacia Biotech) and various concentrations (0–1000 nM) of test compounds as previously described [10]. After 24 h incubation at 4 °C, unbound [^{3}H]Dexamethasone was removed by the treatment with DCC in TEDM buffer on ice. A 150 μL sample was pipetted into scintillation vial and 5 ml scintillation cocktail were added. By using, similar assay conditions were adopted for determining aldosterone binding. Non-specific binding was determined in the presence of 1000-fold excess of unlabelled Dexamethasone or Aldosterone respectively. The radioactivity was measured with scintillation counter (Beckman Instruments, Fullerton, CA). IC_{50} values (concentrations at which 50% of specific binding is displaced by the compounds) were determined from the best fit lines derived by least square regression lines of competitive displacement graph. The *Ki* values were calculated using the equation of Cheng and Prusoff.

2.2.7. Animals

Male Brown Norway rats (150–200 g) and male Sprague Dawley rats (250 g) were purchased from Charles River Laboratories Italy (Calco, Lecco). Prior to use animals were acclimated for at least 5 days to the local vivarium conditions (room temperature: 20–24 °C; relative humidity: 40–70%), having free access to standard rat chow and tap water. All the procedures were performed

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in animal operating rooms in conformity to the Italian legislation (D.L. vo 116/92) and the UE directive 2010/63/UE.

2.2.8. Intratracheal administration and pharmacokinetic

Compound **#15** was intratracheally administered to male Sprague Dawley rats.

Before intratracheal administration of 1 μmol/kg of **#15** as suspension at volume of 0.5 ml/kg (0.2% Tween 80 in 0.9% sodium chloride solution as vehicle) animals were anesthetized with isoflurane or sodium thiopental, in case of terminal sampling. Rats fasted from 1 h before the treatment. After i.t. treatment blood and lung were collected as follows: 0.083, 0.5, 1, 2, 4, 6, 24 and 48 h. Blood were put in heparinised plastic tubes, kept in an ice-water bath and then centrifuged within 0.5 h, for 3 min at 10,000×*g* at 4 °C for plasma separation. Lungs were collected, washed with 10 ml of 0.9% sodium chloride solution, then frozen over liquid nitrogen. Plasma and lungs were stored at −80 °C until the bioanalysis. 120 μL of plasma samples were purified adding 480 μL of acetonitrile and vortexed for 30 s., then the obtained samples were stored at −20 °C for 0.5 h and centrifuged at 3600×*g* for 0.25 h. The supernatant was later evaporated under N_2 stream. The samples were reconstituted with 200 μL of 1% acetic acid in acetonitrile and 1% acetic acid in water mixture (50:50, v:v), vortexed for 30 s and injected into the LC/MS-MS system. 70 μL of homogenated lung samples, obtained adding 3 ml of saline solution and acetonitrile mixture (50:50, v:v) to 1 g of rat lung, were purified with 280 μL of formic acid solution (0.2% in acetonitrile) and vortexed for 30 s. then the obtained samples were stored at -20 °C for 0.5 h and centrifuged at 3600×*g* for 0.25 h. 50 μL of an aqueous solution of formic acid solution (0.3%) were added to 150 μL of the supernatant and then vortexed for 30 s. The obtained samples (5 μL for lung and 20 μL for plasma) were injected into the LC/MS-MS system, Thermo Electron TSQ Quantum Access, as spectrometer and Accela HPLC system. The chromatographic analysis was performed in gradient mode with Formic acid in water (0.2%) (Solvent A) and Formic acid in acetonitrile (0.2%) (Solvent B), using Accucore (Thermo Fisher) C18 (50 × 2.1 mM) 2.6 μm column equipped with a Thermo Fisher in-line filter Javelin (88200) and Accucore C18 defender guard cartridge 10 × 2.1 mM, 2.6 μm, operating at 40 °C. The spectrometer was used in ESI positive ion mode, monitoring the following transitions: 502 → 462 (#15) and 501 → 293 (Fluticasone propionate, as IS).

The measured plasma concentrations were in the linear range: 0.48–533 ng/ml.

The measure lung concentrations properly adjusted to the corresponding weighs were in the linear range: 12.4–169680 ng/g.

2.2.9. Ovalbumin-induced pulmonary eosinophilia in sensitised Brown Norway rats

Male Brown-Norway rats were sensitized by intraperitoneal injection of 1 ml suspension containing ovalbumin (OVA, 1 mg/ml, Sigma Aldrich) and Al(OH)3 (100 mg/ml, Sigma Aldrich) for 3 consecutive days. Two weeks later the animals were nose-only exposed to an aerosol of OVA solution (1% in saline). The non challenged-vehicle-control treated animals were sensitised to OVA but exposed to aerosolized saline (sham). At 24 h after exposure either to OVA or saline aerosol, animals were anaesthetised with sevoflurane (4% in oxygen, Sevoflo, Abbott) and bronchoalveolar lavage fluid (BALF) was collected and subjected to total and differential cell counts. Cpd #15 and Dexamethasone were administered as suspension (vehicle: saline containing 0.2% Tween 80) by the intratracheal route 2 h before and 4 h after OVA aerosol.

Data were analysed using Graph Pad Prism™.The parametric tests performed to determine statistical significance were an analysis of variance (ANOVA) with Dunnett's post-test.

2.3. Modelling studies

The crystal structure of Dexamethasone in complex with human GR was retrieved from Brookhaven Protein Databank (PDB code: 1M2Z) [11] and utilized for binding site analysis using SiteMap (Schrodinger9.1) and for docking studies using GlideSP (Schrodinger9.1) [12].

Finally, log*P* and *pKa* values were calculated with the use of ACD/Labs (v12) [13].

3. Results

3.1. Chemistry

A series of isoxazolines, **Cpds #1–18** described in Table 1, was prepared following the synthetic pathway summarized in Scheme 1.

Intermediates 11β,21-dihydroxy-pregna-1,4,16-triene-3,20-dione (intermediate **#19**) and 6,9-difluoro-11β,21-dihydroxy-pregna-1,4,16-triene-3,20-dione (intermediate **#20**) were prepared respectively in four and two steps starting from commercially available Prednisone 21-O-acetate and Difluprednate by a method established in this laboratory [14]. For example, 1,3-dipolar cycloaddition of 4-chlorophenylformonitrile oxide (generated *in situ* by the treatment of 4-chloro-N-hydroxybenzimidoyl chloride with aqueous $NaHCO_3$ solution) [15] to an α,β-unsaturated enone **#19** gave a single adduct **#1** in a good yield. Only the 16,17-double bond reacted with the various nitrile oxides and ^1H NMR spectra are consistent with the proposed structures. The regiospecificity of 1,3-dipolar cycloaddition of nitrile oxides to an α, β-unsaturated enone and the stereospecificity of the cycloaddition to 16-ene steroid system are known [16,17].

3.2. In vitro and in vivo evaluation of novel isoxazolines derivatives

3.2.1. GR binding affinity

The affinity for the human GR receptor of this novel series of isoxazolines was measured in a binding assay and compared with Dexamethasone and Deflazacort as reference ligands. As reported in Table 2, the great majority of the compounds exhibited *Ki* values between 0.5 and 250 nM. In particular, the bromine derivative Cpd **#15** showed the lowest *Ki* value (0.5 nM).

3.2.2. GR nuclear translocation

The potency and efficacy of the isoxazoline derivatives in inducing nuclear translocation were evaluated in an enzyme fragment complementation format by using CHO-K1 PathHunters™ cells [18]. As reported in Table 2, EC_{50}s values lie between 9 and 884 nM. In particular, the bromine derivative Cpd **#15** stands out as the most potent derivative in this assay with an EC_{50} value of 9 nM, while Dexamethasone showed an EC_{50} value of 25 nM (Table 2 and Fig. 1).

3.2.3. Effect of varying concentrations of isoxazolines compounds on the LPS-evoked NO release in RAW 264.7

RAW 264.7 is a murine cell line of immortalized peritoneal macrophages which has been widely used for studying inflammatory responses in macrophages. In particular, LPS-evoked nitric oxide (NO) release is an index of inflammatory activation in these cells.

RAW264.7 were stimulated for 24 h with LPS (100 ng/ml) after 1 h pre-incubation with different concentrations of compounds tested (0.01–1000 nM).

Among the isoxazolines tested, **Cpd #6**, **Cpd #9, Cpd #14** and **Cpd #15** showed to be more potent or equipotent to

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Table 2
In vitro data.

Compound	*Ki* [nM][a]	GR traslocation EC_{50} [nM]	% Efficacy vs. dexamethasone	RAW NO[b] release IC_{50} (nM)
#1	107.3	161.7	16	[c]
#2	84.2	884.8	32	[c]
#3	1630.8	327	21	[c]
#4	249.5	[c]	–	[c]
#5	85.2	236.2	22	[c]
#6	66.6	212.4	41	135
#7	9.4	192.3	52	[c]
#8	55.3	272	25	[c]
#9	2.5	22.6	67	69.4
#10	>10000	[c]	–	[c]
#11	31.7	126.3	29	[c]
#12	59.7	243.9	28	[c]
#13	2.4	68.6	80	[c]
#14	3.2	56.4	66	53.7
#15	0.5	9.0	91	6.1
#16	1.5	129.4	76	–
#17	3.0	48.8	40	–
#18	2.5	22.9	42	–
Deflazacort [d]	10.8			
Dexamethasone	2.3	25	100	115

[a] Concentration displacing 50% of GR bound [^{3}H]Dexamethasone values represent the mean of at least two experiments. The standard deviation of the mean of GR *Ki* is ⩽18%.
[b] Order of magnitude: −9.
[c] No inhibition up to 1 μM.
[d] The des-acetyl-derivative.

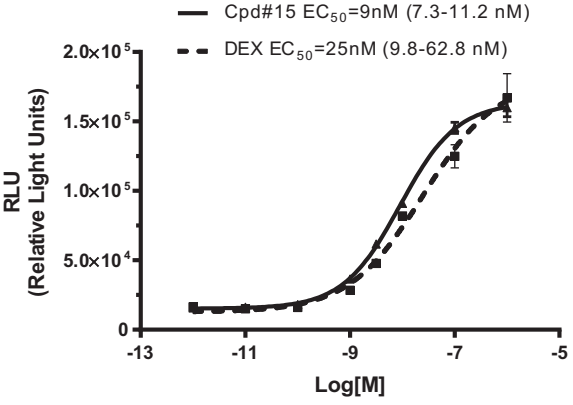

Fig. 1. Steroid-evoked stimulation of GR nuclear translocation. Concentration–response curves in CHO-K1 PathHunter™ cells incubated with Dexamethasone (DEX) or **Cpd #15** for 3 h. Data shown are mean ± SD of a representative experiment performed in triplicate.

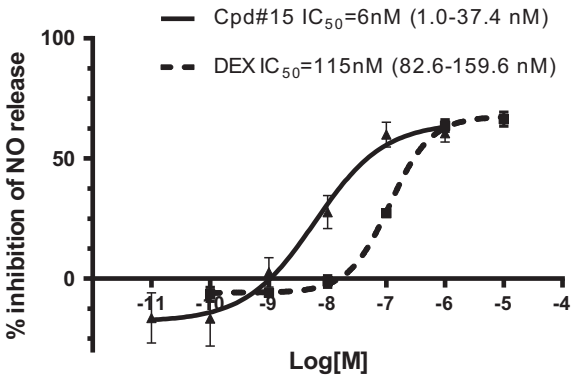

Fig. 2. Inhibitory effect of increasing concentrations of corticosteroids isoxazolines on the accumulation of nitrite induced by LPS. Concentration-dependent inhibition by Dexamethasone (DEX) and **Cpd #15** of LPS-evoked NO release in Raw 264.7 cells. Each data point is the mean ± SD of a representative experiment performed in triplicate.

Table 3
Mineralocorticoid receptor nuclear translocation.

Compound	EC_{50} [nM]	% Efficacy vs. aldosterone
#9	37.6	20
#13	76.5	30
#14	66.6	19
#15	6.1	50
Dexamethasone	9.2	50

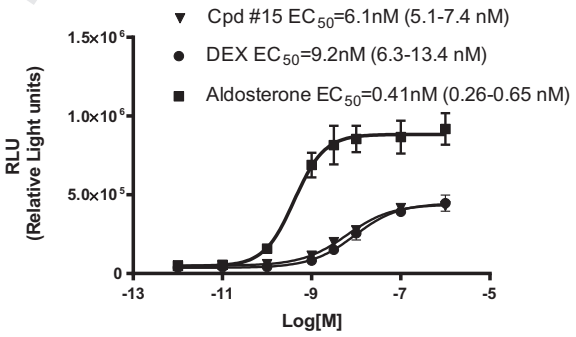

Fig. 3. Steroid-evoked stimulation of MR nuclear translocation. Concentration–response curves in CHO-K1 PathHunter™ cells incubated with Dexamethasone (DEX), **Cpd #15** and Aldosterone for 3hrs. Data are mean ± SD of a representative experiment performed in triplicate.

dexamethasone in inhibiting LPS-induced NO release from RAW264.7, whereas the other compounds tested exhibited poor inhibitory activity against NO release (IC_{50} > 1 μM) (Table 2, Fig.2).

3.2.4. MR nuclear translocation

The human mineralocorticoid receptor (MR) is highly homologous to GR [19,20]. In order to determine receptor selectivity, **Cpd #15** was tested in a competitive binding assay and in a MR nuclear translocation functional assay head to head with aldosterone and dexamethasone.

Cpd #15 and dexamethasone showed a *Ki* for the mineralcorticoid receptor of 45.0 nM (c.i. 28–74) and 12.5 nM (c.i. 5.3–30.6) respectively.

As reported in Table 3, **Cpd #15** showed potency and efficacy in eliciting MR translocation greatly inferior to aldosterone and similar to Dexamethasone (Fig. 3). The same experimental set up was run with cell culture medium containing charcoal stripped fetal bovine serum to delete cortisol, that could be present in cell culture medium. Indeed, results comparable to the non-stripped serum condition were obtained: EC_{50} = 7.2 nM (c.i. 6.8–7.6) for **Cpd #15** and EC_{50} = 7.5 nM (c.i. 7.2–7.7) for dexamethasone. **Cpd #9, #13, #14** tested in the MR translocation assay appeared to be 4–7-fold less potent than **Cpd #15**.

3.2.5. Effect of varying concentrations of isoxazoline compound Cpd #15 on the TNFα-evoked IL-8 release in ASMCs

In the airways smooth muscle cells (ASMCs) the secretion of IL-8 induced by inflammatory mediators is repressed by glucocorticoids [21] through a direct inhibitory interaction of the GR with activated transcription factors, such as NF-kB and AP-1 [22].

The anti-inflammatory effects of novel isoxazoline **Cpd #15** was assessed by measuring its potency in inhibiting TNFα-induced IL-8 release in ASMCs. As shown in Fig. 4, **Cpd #15** inhibited IL-8 release

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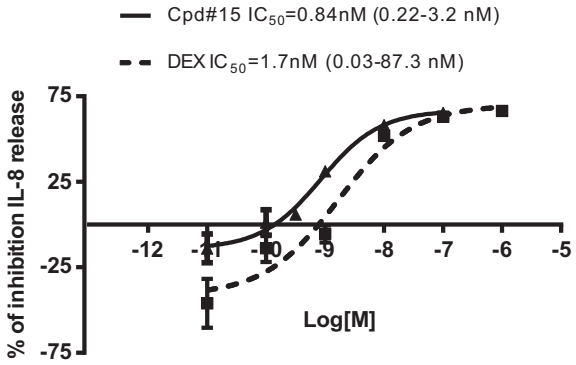

Fig. 4. Inhibitory effect of increasing concentrations of corticosteroids isoxazolines on IL-8 release induced by TNFα. Concentration-dependent inhibition by Dexamethasone (DEX) and **Cpd #15** of TNFα (0.1 ng/ml)–evoked IL-8 release in ASMCs. Data are mean ± SD of a representative experiment performed in triplicate.

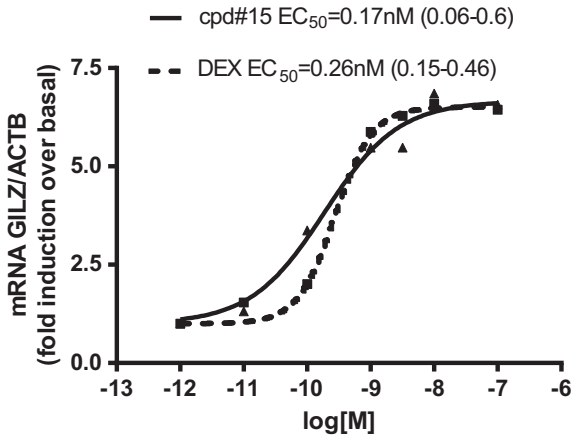

Fig. 5. Dose–response curves for reference compounds Dexamethasone (DEX) and isoxazoline **Cpd #15** on mRNA GILZ induction in airway smooth muscle cells. Relative GILZ mRNA levels were measured by Taqman real-time PCR and normalized to β actin. Data are mean ± SD of a representative experiment performed in triplicate.

with a sub-nanomolar potency (EC_{50} = 0.84 nM) comparable to that of Dexamethasone (EC_{50} = 1.7 nM).

3.2.6. Cpd #15 induces transactivation of the anti-inflammatory gene GILZ in human airway smooth muscle cells

Part of the anti-inflammatory properties of glucocorticoids depend also on their ability to trans-activate genes able to counteract inflammatory processes such as GC-induced leucine zipper (GILZ) [23].

Following incubation of ASMCs with increasing concentration of **Cpd #15** or Dexamethasone, relative GILZ mRNA amounts were measured by TaqMan real-time PCR and normalized with respect to β-actin. As shown in Fig. 5, **Cpd #15** displayed a potency (EC_{50} = 0.17 nM) and efficacy in inducing GILZ mRNA (about 6-fold induction over basal) comparable to those of Dexamethasone.

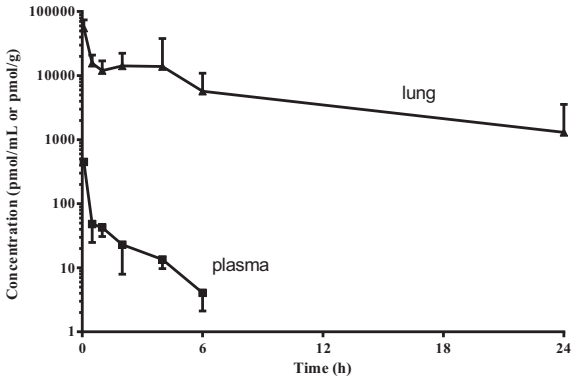

Fig. 6. Plasma (squares) and lung (triangles) levels after i.t. administration of **Cpd #15** (1 μmol/kg) to rats.

3.2.7. In vitro ADME and PK profile

Cpd #15 showed a medium rate of clearance in rat hepatocytes (25.8 μL/min 10^6 cells) and was stable in rat plasma (up to 5 h) as well as in S9 lung homogenates (45% after 1 h).

Following intra-tracheal (i.t.) administration in rat, **Cpd #15** (1 μmol/kg) showed a C_{max} value of 0.448 nmol/ml in plasma and the exposure achieved was 0.232 nmol/ml*h. In the lung, **Cpd #15** levels peaked at 0.083 h reaching a concentration of 55.37 nmol/g and its exposure (AUC_{last}) was 147.78 nmol/g*h, with a MRT_{last} of 5.35 h (Table 4). (See Fig. 6)

3.2.8. Effect of #15 on OVA-induced eosinophilia in sensitised Brown Norway rats

The effects of **Cpd #15** and the reference compound Dexamethasone in a rat model of asthma (OVA-induced pulmonary eosinophilia) were investigated. Animals were dosed i.t. with **Cpd #15** (0.1 – 1 μmol/kg) or Dexamethasone (1 μmol/kg) 2 h prior to and 4 h post OVA challenge.

Cpd #15 inhibited the OVA-induced increase in eosinophil number in the BAL in a dose-dependent fashion (Fig. 7). The inhibitory responses ranged from 43% (0.1 μmol/kg) to 95% (1 and 3 μmol/kg) and were statistically significant ($p < 0.05 - p < 0.01$) at all tested doses. The inhibitory effect of 1 μmol/kg Dexamethasone was superimposable to that of 1 μmol/kg of **Cpd #15**.

3.3. Modelling studies

Overlay of **Cpd #15** (GlideSP docked conformation) on Dexamethasone bound to the ligand binding domain of human GR is shown in Fig. 8. The contour map highlights that the steroid binding site is mainly hydrophobic (yellow grids). An extended polar region is located near R611 and N570 both involved in hydrogen bond interactions with the 3 – C = O of Dexamethasone. Besides, a small polar area (red and blue grids) is close to N564 which is involved in a hydrogen bond interaction with 11-OH of the ligand. Finally, the hydroxyl moiety of Dexamethasone at C17 is nicely accommodated in a polar pocket and makes a hydrogen bond interaction with Q642.

Table 4
PK data of Cpd #15.

	C_{max} (nmol/ml or nmol/g)	AUC_{last} (nmol/ml*h or nmol/g*h)	$T_{1/2}$ (h)	T_{last} (h)	MRT_{last} (h)
Plasma (i.t.)	0.448	0.232	1.59	6	1.09
Lung (i.t.)	55.37	147.78	6.47	24	5.35

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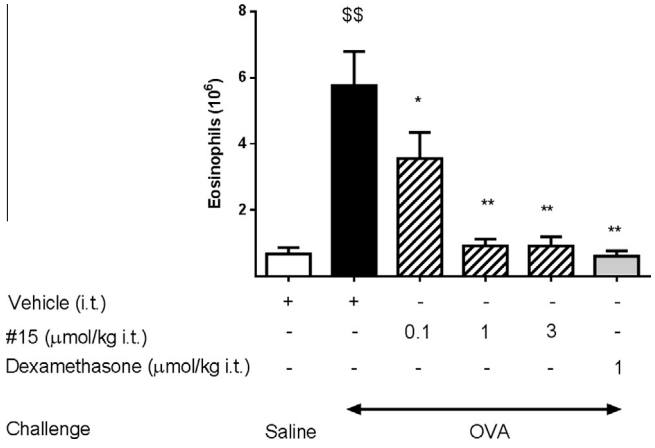

Fig. 7. Compound **#15** dose–response curve in the rat model of OVA-induced eosinophilia. Test compounds were administered i.t. to sensitized Brown Norway rats 2 h before and 4 h after OVA challenge. Each column represents the mean of total eosinophil number in BAL and each bar represents SEM. Changes were compared to the OVA challenged group (*) or to the sham challenged group ($) using ANOVA followed by Dunnett's test. $^{*}p < 0.05$ and $^{**}p < 0.01$, $^{\$\$}p < 0.01$, $n = 6–7$ animals/group.

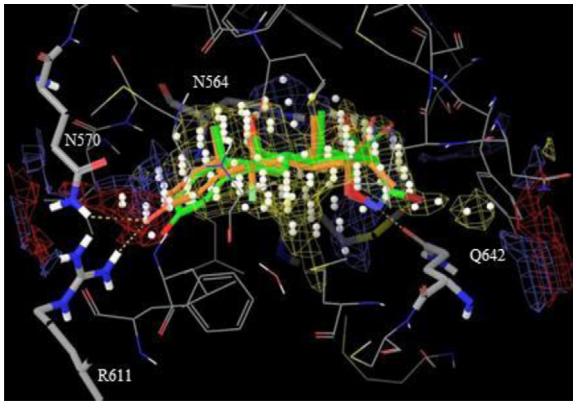

Fig. 8. Overlay of a docking solution of Cpd **#15** on the X-ray structure of Dexamethasone (orange) bound to the ligand binding domain of human GR (1M2Z PDB code) [11] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

4. Discussion

4.1. SAR interpretation

Cpd #7 (*Ki* = 22.33 nM)) shows a comparable binding affinity to the active metabolite of Deflazacort (see Chart 1, *Ki* = 10.84 nM). Small alkyl substituents are preferred at the C3′ position for the binding affinity. In particular, C3′-methyl-isoxazoline derivative shows better binding affinity than C3′-n-propyl-isoxazoline derivative **Cpd #6** (*Ki* = 66.59 nM). When the methyl group is replaced with a hydroxyl-methyl moiety, as in **Cpd #8**, the affinity is reduced (*Ki* = 55.29 nM), which suggests that polar groups are not tolerated. This is further confirmed by the lack of activity of **Cpd #10** (*Ki* = > 10,000). Improved affinity is obtained if the isoxazoline ring is decorated with a bromine at C3′ as in **Cpd #9** (*Ki* = 2.46 nM).

The inhibitory effect of compounds with R equal to hydrogen (see Scheme 1) on NO production in RAW 264.7 macrophage cells, described in Park et al. [5], is 10-fold lower than Cpd **#15**.

In order to expand the SAR of new chemical series, aromatic and heterocyclic moieties were introduced. The unsubstituted phenyl ring with X, Y equal to hydrogen showed very weak potency and efficacy in the Raw assay and was not further profiled (unpublished results). **Cpd #1**, bearing a p-chlorophenyl substituent at C3′ on the isoxazoline ring at the C16–17 position, displays only moderate binding affinity (*Ki* = 262.96 nM). Substitution of the p-chlorine moiety with p-methoxy group on the phenyl ring at C3′, as in **Cpd #4** (*Ki* = 249.54 nM) slightly improves the affinity in the binding assay. Replacement of the phenyl ring with either a furyl, **Cpd #12**, or a thienyl, **Cpd #11**, did not improve the affinity at GR (*Ki* = 59.66 and 31.73 respectively).

The relatively weak GR binding affinity of the compounds with X, Y = Hydrogen was improved with the incorporation of two fluorine atoms at C6, C9 on the corticosteroid core. These isoxazoline derivatives showed a 10-fold increase in their binding affinity while their potency did not change significantly.

The increase of the size and bulk of the para-substituent of phenyl derivative led to a reduction in both potency and efficacy in the GR nuclear translocation, e.g., **Cpd #18**.

These results were rationalized in light of the binding modes of this compound series by using docking methods. As shown in Fig. 8, the isoxazolidine ring of **Cpd #15** maps well the same polar region filled by the hydroxyl moiety at C20 of Dexamethasone, while the bromine substituent is nicely accommodated in a small hydrophobic pocket. The limited size of this cavity highlighted by the yellow grid map suggests that bulkier groups like phenyl (**Cpd #18**), thienyl (**Cpd #11**) and furyl (**Cpd #14**) are not tolerated due to their large steric hindrance which can be described with the use of calculated Molecular Refractivity (MR) parameter [24]. Mapping of this pocket appears to be suitable for small and hydrophobic substituents such as Br (**Cpd #9** and **Cpd #15**) and Me (**Cpd #7**). Both are characterized by low MR values (Br: MR = 8.88; Me: MR = 5.65) and medium lipophilicity (Br: cLog*P* = 1.73; Me: cLog*P* = 2.53) [14]. When either steric bulk or polarity increases, binding affinity significantly drops as observed for **Cpd #6** (R = propyl: MR = 14.96, cLog*P* = 3.55), **Cpd #8** (R = CH_2OH: MR = 7.19, cLog*P* = 1.38) and **Cpd #2** (R = COOEt: MR = 17.47, cLog*P* = 1.45) and is completely lost for **Cpd #3** which is likely charged at physiological pH (calculated *pKa* = 2.74).

4.2. Biological profile Cpd #15

The most interesting compound among the novel isoxazoline derivatives, described in this study, is the bromine derivative **Cpd #15**, which proved to be the most potent compound in the GR binding assay and in the cell-based GR nuclear translocation assay. **Cpd #15** showed *Ki* values pointing out a 50-fold higher affinity for GR vs. MR in the binding assay. The *Ki* ratio between MR and GR for dexamethasone is about 5, suggesting that **Cpd #15** is relatively more selective than dexamethasone. The observation that **Cpd #15** interaction with MR is also associated with nuclear translocation, suggests that such compound acts as an agonist at the MR. In order to evaluate its anti-inflammatory potency, we examined the effects on the release of relevant inflammatory mediators. **Cpd #15** dose-dependently inhibited the production of nitric oxide in LPS-stimulated Raw 264.7 macrophages, with a potency superior to the reference compound Dexamethasone. Inhibition of nitric oxide release in Raw 264.7 macrophages occur with relatively high EC_{50} with respect to *Ki* values due likely to the mechanism of suppression that requires transcriptional and post-transcriptional down regulation of the enzyme nitric oxide synthase [25]. It should also be kept in mind that Raw 264.7 are immortalized cell lines which are known to express relatively high levels of P-glycoproteins [26]. Upregulation of P-glycoprotein may cause drug resistance as well as reduced glucocorticosteroid responsiveness [27]. On the other hand, the repression of inflammatory responses by glucocorticoids also involves the up-regulation of anti-inflammatory genes,

including GILZ, whose expression is up-regulated by steroids in several cellular type including smooth muscle cells [28]. In human ASMCs, **Cpd #15** evoked a significant concentration-dependent induction of GILZ expression and suppression of TNFα-stimulated production of IL-8 with a potency and efficacy comparable to dexamethasone. In both assays, **Cpd #15** and dexamethasone show sub/low nanomolar EC_{50} values close to respective Ki values.

Having demonstrated that **Cpd #15** is a potent anti-inflammatory agent in vitro, and that its pharmacokinetic profile is suitable for topical administration (pulmonary levels > 100-fold higher than plasma levels upon i.t. delivery), we tested it in an *in vivo* model of allergen-induced pulmonary inflammation. Ovalbumin (OVA) inhalation to sensitized rats is a well characterized and recognized model for asthma [29]. This model is commonly used to investigate the anti-inflammatory activity of compounds developed for the treatment of allergic inflammatory airway diseases, such as corticosteroids and phosphodiesterase 4 (PDE4) inhibitors [30–32]. **Cpd #15,** when administered via the intratracheal route, proved to be as efficacious as Dexamethasone in counteracting eosinophilic infiltration in the broncho-alveolar lavage following OVA challenge (ED_{50} < 0.3 μmol/kg).

In conclusion, through a rational drug design approach we identified **Cpd #15,** a novel isoxazoline with a potent and robust anti-inflammatory profile which exhibits a pharmacokinetic and pharmacodynamic profile suitable for topical pulmonary delivery.

Therefore, Cpd **#15** could potentially be developed as an inhalable agent for the treating of pulmonary inflammation such as asthma and COPD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.12.016.

References

[1] Stanbury RM, Graham EM. Systemic corticosteroid therapy-side effects and their management. Br J Ophthalmol 1998;82:704–8.
[2] Barnes NC. The properties of inhaled corticosteroids: similarities and differences. Prim Care Respir J 2007;16:149–54.
[3] Ernst P, Suissa S. Systemic effects of inhaled corticosteroids. Curr Opin Pulm Med 2012;18:85–9.
[4] Kwon T, Heiman AS, Oriaku ET, Yoon K, Lee HJ. New steroidal anti-inflammatory antedrugs: steroidal [16,17-d]-3-carbethoxyisoxazolines. J Med Chem 1995;38:1048–51.
[5] Kwan-k Park, Dong-H Ko, You Z, Omar M, Khan F, Lee HJ. In vitro anti-inflammatory activities of new steroidal antedrugs: [16α,17α-d] Isoxazoline and [16α,17α-d]-3-hydroxy-iminoformyl isoxazoline derivatives of prednisolone and 9α-fluoroprednisolone. Steroids 2006;71:183–8.
[6] Kanemasa S, Matsuda H, Kamimura A, Kakinami T. Tetrahedron 2000;56:1057–64.
[7] Kwon T, Heiman AS, Oriaku ET, Yoon K, Lee HJ. J Med Chem 1995;38:1048–51; Bacher E, Demnitz FWJ, Hurni T. Tetrahedron 1997;53(42):14317–26.
[8] Yuan L; Yanchang L; Jinlu L; Chaohui S CN 101177443 A 20080514.
[9] Fung P, Peng K, Kobel P, Dotimas H, Kauffman L. A homogeneous cell-based assay to measure nuclear translocation using beta-galactosidase enzyme fragment complementation. Assay Drug Dev Technol 2006;4:263–72.
[10] Wambach G, Casals-Stenzel J. Structure-activity relationship of new steroidal aldosterone antagonists. Comparison of the affinity for mineralocorticoid receptors in vitro and the antialdosterone activity in vivo. Biochem Pharmacol 1983;32:1479–85;
Mulatero P, Panarelli M, Schiavone D, Rossi A, Mengozzi G, Kenyon CJ. Impaired cortisol binding to glucocorticoid receptors in hypertensive patients. Hypertension 1997 Nov;30(5):1274–8.
[11] Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 2002 Jul 12;110(1):93–105.
[12] [Schrödinger, Maestro version 9.1 Schrödinger, LLC, New York 2009 http://www.schrodinger.com/.
[13] ACD/LogP v12, www.acdlabs.com.
[14] Armani E, Ghidini E, Peretto I, Virdis A. Isoxazolidine derivatives WO 2011/029547 A8.
[15] Mukaiyama T, Hoshino T. The reactions of primary nitroparaffins with isocyanates. J Am Chem SOC 1960;2:5339–42.
[16] Moersch GW, Wittle EL, Neuklis WA. The decarboxylation of 3-carboxy-2-isoxazolines. 3β,17α-dihydroxypregna5-en-20-one-16α-carbonitrile. J Org Chem 1967;32:1387–91.
[17] Moersch GW, Wittle EL, Neuklis WA. The decarboxylation of 3-carboxy-2-isoxazolines. J Org Chem 1966;30:1272–3.
[18] Plumb J, Robinson L, Lea S, Banyard A, Blaikley J, Ray D. Evaluation of glucocorticoid receptor function in COPD lung macrophages using beclomethasone-17-monopropionate. PLoS One 2013;8:e64257.
[19] Pippal JB, Fuller PJ. Structure-function relationships in the mineralocorticoid receptor. J Mol Endocrinol 2008;41:405–13.
[20] Fuller PJ, Yao Y, Yang J, Young MJ. Mechanisms of ligand specificity of the mineralocorticoid receptor. J Endocrinol 2012;213:15–24.
[21] John M, Au BT, Jose PJ, Lim S, Saunders M, Barnes PJ. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. Am J Resp Cell Mol 1998;18:84–90.
[22] King EM, Holden NS, Gong W, Rider CF, Newton R. Inhibition of NF-kB-dependent transcription by MKP-1. J. Biol. Chem. 2009;284:26803–15.
[23] Ayroldi E, Riccardi C. Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. FASEB J 2009;23(11):3649–58.
[24] Tabulated MR parameters for aromatic ring substituents were retrieved from the following papers Hansch et al. J Med Chem 1973;16(11):1208;
Skagerberg B et al. Quant Struct Act Relat 1989;8:32–8.
[25] Söderberg M, Raffalli-Mathieu F, Lang MA. Regulation of the murine inducible nitric oxide synthase gene by dexamethasone involves a heterogeneous nuclear ribonucleoprotein I (hnRNPI) dependent pathway. Mol Immunol 2007;44(12):3204–10.
[26] Roy KR, Arunasree KM, Dhoot A, Aparna R, Reddy GV, Vali S. C-Phycocyanin inhibits 2-acetylaminofluorene-induced expression of MDR1 in mouse macrophage cells: ROS mediated pathway determined via combination of experimental and in silico analysis. Arch Biochem Biophys 2007;459(2):169–77.
[27] Montano E, Schmitz M, Blaser K, Simon HU. P-glycoprotein expression in circulating blood leukocytes of patients with steroid-resistant asthma. J Investig Allergol Clin Immunol 1996;6(1):14–21.
[28] Kelly MM, King EM, Rider CF, Gwozd C, Holden NS, Eddleston J. Corticosteroid-induced gene expression in allergen-challenged asthmatic subjects taking inhaled budesonide. Br. J. Pharmacol. 2012;165:1737–47.
[29] Leung SY, Williams AS, Nath P, Dinh QT, Oates T, Blanc FX. Dose-dependent inhibition of allergic inflammation and bronchial hyperresponsiveness by budesonide in ovalbumin-sensitised Brown-Norway rats. Pulm Pharmacol Ther 2008;21:98–104.
[30] Huang TJ, Eynott P, Salmon M, Nicklin PL, Chung KF. Effect of topical immunomodulators on acute allergen inflammation and bronchial hyperresponsiveness in sensitised rats. Eur J Pharmacol 2002;437:187–94.
[31] Wollin L, Bundschuh DS, Wohlsen A, Marx D, Beume R. Inhibition of airway hyperresponsiveness and pulmonary inflammation by roflumilast and other PDE4 inhibitors. Pulm Pharmacol Ther 2006;19:343–52.
[32] Chapman RW, House A, Richard J, Prelusky D, Lamca J, Wang P. Pharmacology of a potent and selective inhibitor of PDE4 for inhaled administration. Eur J Pharmacol 2010;643:274–81.