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Macrolactonolides: A novel class of anti-inflammatory compounds

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ABSTRACT

A new concept in design of safe glucocorticoid therapy was introduced by conjugating potent glucocorticoid steroids with macrolides (macrolactonolides). These compounds were synthesized from various steroid 17β-carboxylic acids and 9a-*N*-(3-aminoalkyl) derivatives of 9-deokso-9a-aza-9a-homoeritromicin A and 3-descladinosyl-9-deokso-9a-aza-9a-homoeritromicin A using stable alkyl chain. Combining property of macrolides to preferentially accumulate in immune cells, especially in phagocyte cells, with anti-inflammatory activity of classic steroids, we designed molecules which showed good anti-inflammatory activity in ovalbumin (OVA) induced asthma in rats. The synthesis, in vitro and in vivo anti-inflammatory activity of this novel class of compounds are described.

1. Introduction

Inflammation is one of the most complex pathophysiological processes involved in the host response to injury, whatever its intensity and origin.[1] Despite its initial beneficial aspect (host defense) it can become excessive, either by reacting to a non-injurious challenge (allergies or autoimmunity) or by generating excessive acute (shock or systemic inflammatory response syndrome) or long-lasting (chronic inflammatory diseases) deleterious responses. This beneficial/pathological event involves a host of cellular effectors, redundant humoral mediators and enzyme cascades whose importance may vary depending on the nature of the trigger.[2]

Corticosteroids (CS) are among the most widely used drugs in the world and are effective in many inflammatory and immune diseases. The most common use of CS is in the treatment of asthma, where inhaled CS have become first-line therapy and by far the most effective anti-inflammatory treatment.[3]

This utility resides in their gene transcriptional regulation effects on multiple biological mediators involved in the inflammatory process.[4] However, long-term systemic exposure to CS results in well-documented side effects such as growth retardation, osteoporosis, and suppression of the hypothalamic-pituitary-adrenal function and of the immune system.[5]

Several strategies have been employed in development of 'safer' anti-inflammatory steroids.

An approach well elaborated recently was the development of 'soft' or 'antedrug' steroids (e.g., loteprednol etabonate, GR250495X[6]).[6–9] Such steroids are designed to act topically at the site of application but that are transformed into inactive metabolites upon entry into the systemic circulation. The development of ciclesonide, however, followed a different concept. Ciclesonide is a pro-drug-soft steroid, which is cleaved by esterases at the site of action to produce an active entity (desisobutyryl-ciclesonide; des-CIC) and then is rapidly metabolised.[10]

Lee tested the hypothesis that the topical potencies of moderately potent steroidal antedrugs could be enhanced by conjugation to nonsteroidal anti-inflammatory drug, ibuprofen or indomethacin.[11] However, the topical potencies were not enhanced which might be attributed to the decreased affinity to glucocorticoid receptor (GR) of the conjugates, but showed remarkable reduction in systemic side effects with significantly improved local/systemic activity ratio.

Our attempt to make conjugate of potent steroidal agents with selected macrocyclic moieties resulted in development of a new generation of conjugated molecules: macrolactonolides. This is a novel concept in which a known active corticosteroid is covalently linked, through a suitable linker, to a macrolide scaffold that has the ability to accumulate in inflammatory cells.[12]

Macrolide antibiotics accumulate in inflammatory cells at concentrations up to several hundred-fold higher than those in

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extracellular fluid enabling phagocytic cells to deliver concentrated active drug to sites of infection[13,14] The mechanism of intracellular accumulation is not clear, but exhibits characteristics of an active (protein mediated) process.[13] Concentration occurs in the cytoplasm and azurophilic granules of neutrophils, thus favouring antibiotic delivery to bacteria phagocytosed by leukocytes. Cytokines stimulate in vitro accumulation of macrolides into macrophages, suggesting that at the site of inflammation (infection), cells may accumulate even more macrolide antibiotics than under physiological conditions.[15] Efflux or release of macrolides from leukocytes varies among macrolides, being very fast with erythromycin and clarithromycin, but very slow with azithromycin,[16,17] so that the latter agent is retained much longer in the cells.

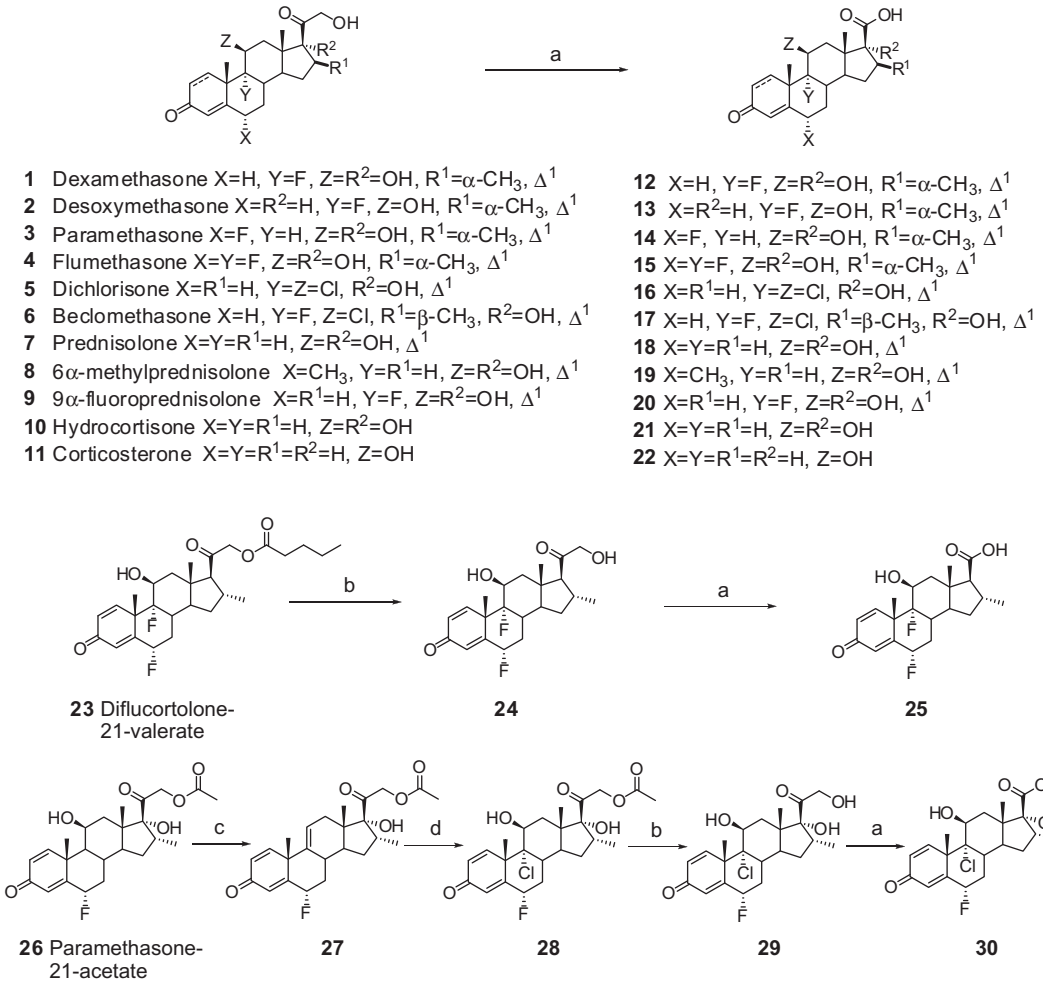
Macrolactonolides were conceived to retain the initial pharmacological activity of the selected pharmacophore, and have improved pharmacokinetic behavior (primarily cellular and tissue accumulation) associated with the macrolide scaffold. As some pharmacodynamic effects of the macrolide moiety are expected as well, the final biological effects of these novel compounds would be in part related to the individual components and in part to the inherent activity of the whole molecule.

In this paper we disclose the synthesis and in vitro biological profiles of this novel class of anti-inflammatory compounds, together with the in vivo profile of selected example.

2. Chemistry

Starting steroid 17β-carboxylic acids **12**–**22**, **25** and **30** were prepared as shown in Scheme 1. Oxidative cleavage of commercially available steroids **1**–**11** with periodic acid in aqueous dioxane, according to literature procedure,[18] gave high yields of 17β-carboxylic acids **12**–**22**. Compound **24**, required for the synthesis of acid **25**, was obtained from diflucortolone-21-valerate **23** by cleavage with $KHCO_3$ in aqueous methanol/dioxan solution.[19a] Two additional steps on the route to acid **30** from paramethasone-21-acetate **26** comprise elimination of the 11β-hydroxy group from the steroidal nucleus with methanesulfonyl chloride in pyridine-dimethylformamide solution at 80–85 °C,[19] followed by reaction of 9,11 anhydro derivative **27** with 1,3-dichloro-5,5-dimethylhydantoin (DDH)/$HClO_4$ in aqueous tetrahydrofuran to afford 9α-chloro intermediate **28**.[20] Two final steps to carboxylic acid **30** are completed as for compound **25**.

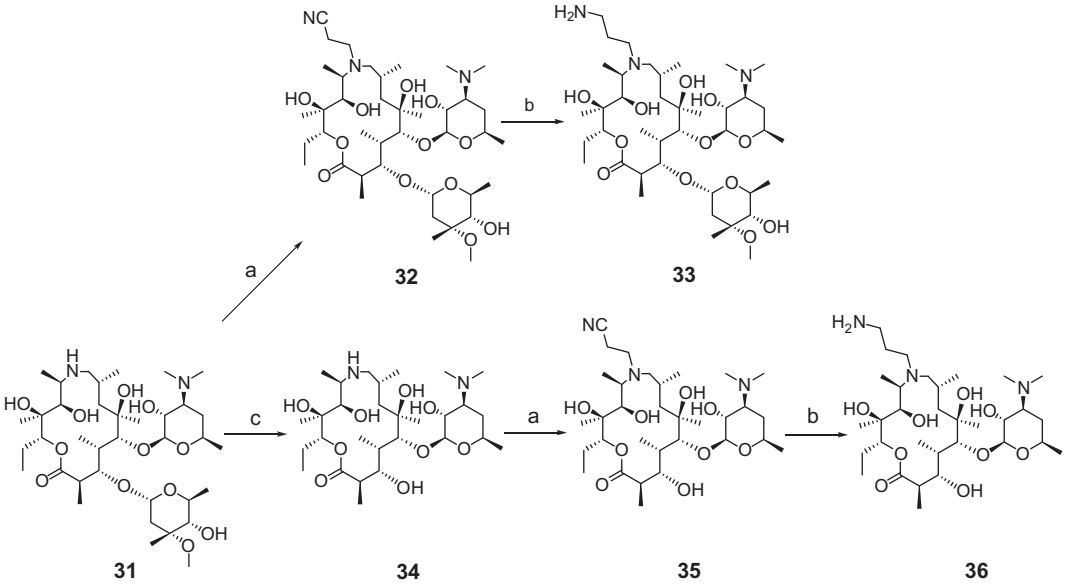
Macrolide precursors containing 9a-aminoalkyl chain were prepared using 9-deokso-9a-aza-9a-homoeritromicin A **31** as a starting material (Scheme 2). 9-Deokso-9a-aza-9a-(2-cyanoethyl) derivative **32** was prepared as reported by Bright and co-workers,[21] by Michael addition of compound **31** with acrylonitrile. Subsequent catalytic hydrogenation of **32** to 9a-(3-aminopropyl) derivative **33** was conducted by modified procedure using PtO_2 catalyst at hydrogen pressure of 40–50 atm.[22]



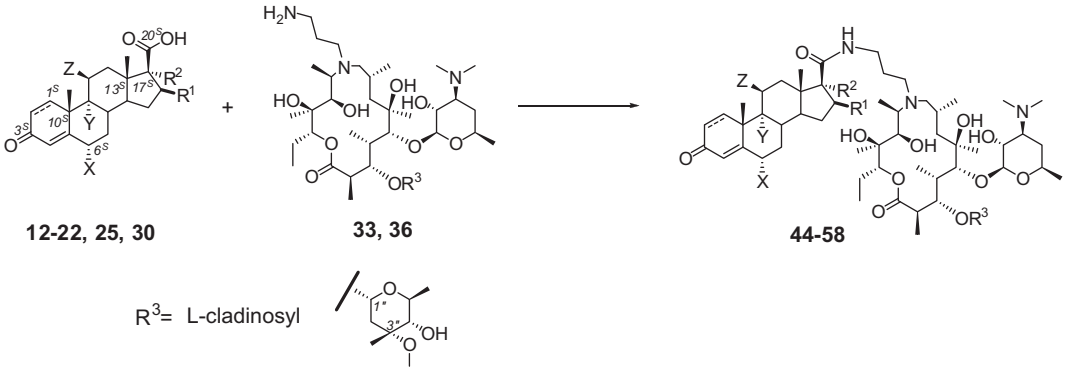
1 Dexamethasone X=H, Y=F, Z=R^2=OH, R^1=α-CH_3, Δ^1
2 Desoxymethasone X=R^2=H, Y=F, Z=OH, R^1=α-CH_3, Δ^1
3 Paramethasone X=F, Y=H, Z=R^2=OH, R^1=α-CH_3, Δ^1
4 Flumethasone X=Y=F, Z=R^2=OH, R^1=α-CH_3, Δ^1
5 Dichlorisone X=R^1=H, Y=Z=Cl, R^2=OH, Δ^1
6 Beclomethasone X=H, Y=F, Z=Cl, R^1=β-CH_3, R^2=OH, Δ^1
7 Prednisolone X=Y=R^1=H, Z=R^2=OH, Δ^1
8 6α-methylprednisolone X=CH_3, Y=R^1=H, Z=R^2=OH, Δ^1
9 9α-fluoroprednisolone X=R^1=H, Y=F, Z=R^2=OH, Δ^1
10 Hydrocortisone X=Y=R^1=H, Z=R^2=OH
11 Corticosterone X=Y=R^1=R^2=H, Z=OH

12 X=H, Y=F, Z=R^2=OH, R^1=α-CH_3, Δ^1
13 X=R^2=H, Y=F, Z=OH, R^1=α-CH_3, Δ^1
14 X=F, Y=H, Z=R^2=OH, R^1=α-CH_3, Δ^1
15 X=Y=F, Z=R^2=OH, R^1=α-CH_3, Δ^1
16 X=R^1=H, Y=Z=Cl, R^2=OH, Δ^1
17 X=H, Y=F, Z=Cl, R^1=β-CH_3, R^2=OH, Δ^1
18 X=Y=R^1=H, Z=R^2=OH, Δ^1
19 X=CH_3, Y=R^1=H, Z=R^2=OH, Δ^1
20 X=R^1=H, Y=F, Z=R^2=OH, Δ^1
21 X=Y=R^1=H, Z=R^2=OH
22 X=Y=R^1=R^2=H, Z=OH

Scheme 1. Reagents and conditions: (a) H_5IO_6, dioxane/H_2O, rt, 2 h (b) $KHCO_3$/H_2O, dioxane/MeOH, rt, 72 h; (c) MsCl, DMF/Py, reflux, 2.5 h; (d) 1,3-dichloro -5,5-dimethylhydantoin (DDH), $HClO_4$,/ H_2O, THF, 0 °C-rt.



Scheme 2. Reagents and conditions: (a) $H_2CCH{=}CN$, reflux, 16-19 h (b) PtO_2, EtOH, rt, 40–50 atm, 48–72 h; (c) HCl/H_20, rt, 48 h.



Scheme 3. Reagents and conditions: EDCxHCl, HOBT, Et_3N, CH_2Cl_2, rt, 16-18 h.

Synthetic routes for preparation 3-descladinosyl-9-deokso-9a-aza-9a-(3-aminopropyl)-9a-homoeritromicin A **36** is illustrated in Scheme 2. On this route additional steps comprise hydrolysis of the cladinose in 2 M HCl/H_2O solution to afford descladinosyl intermediate **34**.[23]

As shown in Scheme 3, steroid-macrolide conjugates were prepared from various steroid 17β-carboxylic acids and macrolide intermediates with ω-alkylamino group at N(9) by standard amide coupling procedures with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBT).[24]

All prepared compounds and their in vitro activities are listed in the Table 1.

3. Biological evaluation

Each compound was first evaluated for its ability to bind for glucocorticoid receptor (GR) in cell-free medium. Most of the macrolactonolides show much lower affinity in cell-free binding assay when compared to dexamethasone **1** (EC_{50} = 0.0037 μM). The most active compound is **49** with EC_{50} value of 0.09 μM, followed by compounds **57**, **55**, **56** and **48** with EC_{50} values in the range of 0.35–0.91 μM. These are mainly halogen substituted compounds on positions 6 and 9 of steroid moiety. Also, the majority of the compounds with affinity for the GR more than 10 μM are non-halogenated compounds (**51**, **52** and **53**) with the exception of compound **47**. This compound possess chlorine atom on position 9, but in comparison with the other molecules is the only one with β-CH_3 group at position 16 of steroid moiety. Furthermore, the glucocorticoid activity is dependent on the presence of L-cladinose in macrolide scaffold.

In most pairs without cladinose (compounds **54**, **55**, **56**, **57** and **58**) versus parent compound with cladinose (compounds **45**, **46**, **48**, **50** and **53**, respectively), later one has slight lower affinity for GR. It seems that macrolide moiety has a modest impact on the interaction of the novel molecules with the ligand binding domain of GR.

Since macrolides exhibit different cellular kinetics, we have also tested the novel compounds in the cellular binding assay which is influenced by both cellular kinetics and GR affinity. We observed differences in potency which depend on the biological complexity of the in vitro assay. Compound **49** retains its affinity in cellular assay also (see Table 1). This activity might not be dependent only on cell-free receptor affinity, since compounds with similar affinity like **55**, **56** and **57** do not exert such potency in cellular assays. This might be attributed to different cellular accumulation of these molecules. From the obtained results (Table 1), it has been also noticed that among novel macrolactonolides compound **45** showes

Table 1
Biological evaluation of macrolactonolides in cell free and cellular GR binding assays

Compd.	X	Y	Z	Δ^1	R^1	R^2	R^3	EC_{50}[a] (μM) GR	EC_{50}[a] (μM) GR
1	H	F	OH	+	α–CH_3	OH	–	0.0037	0.0125
44	H	F	OH	+	α–CH_3	OH	L-clad.	2.09	23
45	H	F	OH	+	α–CH_3	H	L-clad.	4.16	0.43
46	F	H	OH	+	α–CH_3	OH	L-clad.	1.44	>25
47	H	Cl	OH	+	β–CH_3	OH	L-clad.	>10	>25
48	F	F	OH	+	α–CH_3	OH	L-clad.	0.91	>25
49	F	F	OH	+	α–CH_3	H	L-clad.	0.09	0.53
50	F	Cl	OH	+	α–CH_3	OH	L-clad.	1.50	3.81
51	H	H	OH	+	H	OH	L-clad.	>10	>25
52	H	H	OH	–	H	H	L-clad.	>10	>25
53	H	H	OH	–	H	OH	L-clad.	>10	>25
54	H	F	OH	+	α–CH_3	H	H	2.81	>25
55	F	H	OH	+	α–CH_3	OH	H	0.55	5.91
56	F	F	OH	+	α-CH_3	OH	H	0.69	3.36
57	F	Cl	OH	+	α–CH_3	OH	H	0.35	1.68
58	H	H	OH	–	H	OH	H	6.20	>25

Values are mean of at least two assays.
[a] Cell free GR binding assay.

the highest affinity for GR in the cellular assay (EC_{50} = 0.43 μM), despite its week affinity in cell-free GR binding. If one compares potency of compound **45** with potency of dexamethasone, compound **45** shows one thousand time lower potency in cell free assay, while in the cell assay the difference significantly decreases to the ratio of 35.

For that reason, compound **45** was evaluated further in glucocorticoid induced T-cell hybridoma (H13) apoptosis assay. Glucocorticoid induced T-cell hybridoma apoptosis is a functional assay, which is dependent on receptor affinity, cellular kinetics and ligand dependant GR–DNA and GR-protein interactions. Dexamethasone was used as a reference standard (EC_{50} = 0.0097 μM). Consistently with previous results compound **45** was less active than dexamethasone in this assay, but still retained significant activity with EC_{50} value 0.05 μM, indicating that it not only binds to GR, but also induces GR related activity.

Next step was to test the effects of selected compound **45** and dexamethasone on the production of different cytokines in stimulated peripheral blood mononuclear cells (PBMCs), Table 2.

Unsurprisingly, dexamethasone, a potent anti-inflammatory drug, inhibited the majority of measured cytokines across all used stimuli (lipopolysaccharide (LPS), anti-CD3 and phytohemagglutinine (PHA) panels) with EC_{50} values ranging from low nanomolar to subnanomolar concentrations. On the contrary, compound **45** inhibited cytokine production induced after stimulation with anti-CD3 and PHA, but inhibition was much weaker after stimulation with LPS. The highest inhibition effects of compound **45** were observed on TNFalpha, IL-1beta, IL12p70 and GM-CSF levels after anti-CD3 stimulation with EC50 values ranging from 0.017-0.11 μM. In addition, IFNgamma, IL-4, IL-5 and IL-13 levels were significant reduced with compound **45** upon PHA stimulation.

Macrolides accumulate and have prolonged residence time in many tissues and host defense cells, predominantly macrophages and polymorphonuclear leukocytes. Conceptually, it was envisioned that conjugate molecules would display improved accumulation in comparison to the parent steroid subunit by retaining macrolide-like accumulation behavior. The uptake and retention of compound **45** were tested in J774A.1 (murine macrophage cell line) (Figs 1 and 2) and compared with desoxymethasone and dexamethasone.

Standard steroids, like dexamethasone and desoxymethasone, do not accumulate in cells. Moreover, they are easily washed out (Fig 2). Accumulation of compound **45** in cells is significantly higher than that of parent steroid, desoxymethasone. In addition, it

Table 2
Effect of compound **45** and dexamethasone on cytokine production in PBMCs

LPS	EC_{50} (μM)	
	Compound **45**	Dexamethasone
TNFalpha	3	0.01
IL-1beta	>20	0.014
IL-6	>20	0.055
IL-8	9	0.072
MIP1alpha	7.9	0.066
Anti-CD3		
TNFalpha	0.037	0.0019
IL-1beta	0.03	0.0021
IL-12p70	0.017	0.0041
GM-CSF	0.11	0.00047
PHA		
TNFalpha	1.33	0.0061
IL-1beta	13.75	0.0094
IL-6	4.03	0.082
IL-8	3.05	0.088
MIP1alpha	2.63	0.13
IFNgamma	0.067	0.0076
IL-4	0.21	0.00092
IL-13	0.42	0.0012
IP-10	>20	0.0024
MCP-1	19	>0.2
IL-2	>20	0.0036
IL-3	1.267	0.0035
IL-5	0.606	0.001
IL-10	5.124	0.0055

shows moderate release from cells and in that aspect they are more similar to macrolide compounds. This characteristic may enable compound **45** to stay in cells and tissues for a longer period of time ensuring prolonged action even when the compound is no longer detected extracellularly.

Therefore, in order to observe its behaviour in vivo we decided to topically administer compound **45** to the lungs of mice (Fig. 3).

Following in. dosing compound **45** reaches maximum levels between 5 and 15 min in lung tissue and blood/plasma, respectively. The exposure in the lung is 53-fold higher compared to blood and plasma, with an elimination half life of 20 in comparison to 0.9 h. (Table 3). In comparisons to other steroids, for example, desoxymethasone, the exposure in the lung is ca. 8-fold higher (GSK unpublished data).

These findings seem to support earlier observed in vitro cellular acummulation and retention.

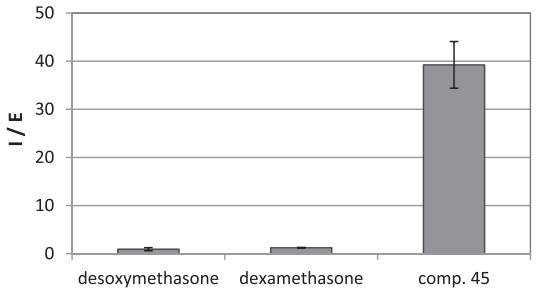


Figure 1. Accumulation of compound **45** and standard steroids in J774A.1 murine macrophage cells. Cells were incubated with 10 μM (E) compounds for 3 h Intracellular concentration (I) was measured by LC–MS/MS. Results are expressed as I/E ratio. Mean values of triplicate samples ± SD from one representative experiment are shown.

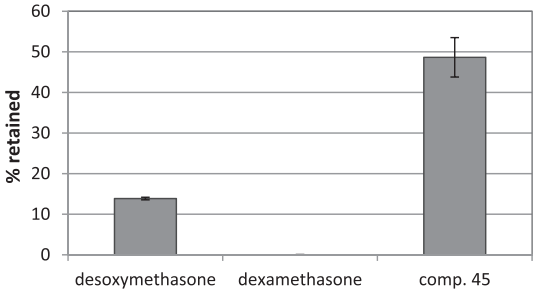


Figure 2. Retention of compound **45** and standard steroids in J774A.1 murine macrophage cells. Cells were incubated with 10 μM compounds for 3 h, washed and incubated for next 3 h in pure medium. Intracellular concentration was measured by LC–MS/MS. Results are expressed relative to initially accumulated amount of each compound. Mean values of triplicate samples ± SD from one representative experiment are shown.

We have postulated that compound **45** with lower GR affinity than standard steroids, but modified cellular accumulation and possibly different pattern of ligand dependant GR cellular functions, might yield good topical effects. In order to prove this, we have chosen a compound **45** to be profiled in vivo in the ovalbumin (OVA) induced eosinophilia in Brown-Norway (BN) rats following dry powder application. The compound **45** (32 mg/kg) was compared with the beclomethasone dipropionate (BDP) (11 mg/kg).

The results are presented in Figures 4–9. Following inhalation of OVA in actively-sensitized BN rats a marked, statistically significant increase in the number of inflammatory cells in the bronchoalveolar lavage fluid (BALF), was measured. The mean absolute number of cells increased from 4.06×10^6 in actively-sensitized, non-challenged animals (negative control) to 26.73×10^6 in challenged animals (positive control) representing a 6.6-fold increase in number (p <0.01, Fig. 4).

In the OVA challenged animals absolute values of all major inflammatory cell types including neutrophils, eosinophils, lymphocytes and macrophages were all significantly elevated in the BALF (p <0.01). When expressed as a percentage of the total cells, eosinophils, key players in asthma, were markedly increased from 7.9% to 53.1% of total cells (Fig. 6).

Beclomethasone dipropionate (BDP, 11 mg/kg) induced a significant reduction in the percent of eosinophils in the bronchoalveolar lavage fluid (BALF) from 53.1% (positive control) to 43% (p <0.01, Fig. 6). In addition, in the presence of BDP absolute values of eosinophils (p <0.05) were significantly reduced when compared with air treated but challenged controls (Fig. 5).

Compound **45** (32 mg/kg) induced a significant reduction in total cells (p <0.01) and eosinophils (p <0.01) in the lungs of rats challenged with ovalbumin. When expressed as a percentage of total cells, eosinophils were notably decreased from 53.1% to 43.6% (p <0.05, Fig. 6). In this model BDP showed no effect on the number of neutrophils neither on macrophages in BALF (Figs. 7 and 9.). In contrast to the BDP, compound **45** significantly reduced absolute number of neutrophils (p <0.01) as well as number of macrophages (p <0.01), demonstrating interesting anti-inflammatory profile, possibly reflecting the presence of macrolide component in its structure.

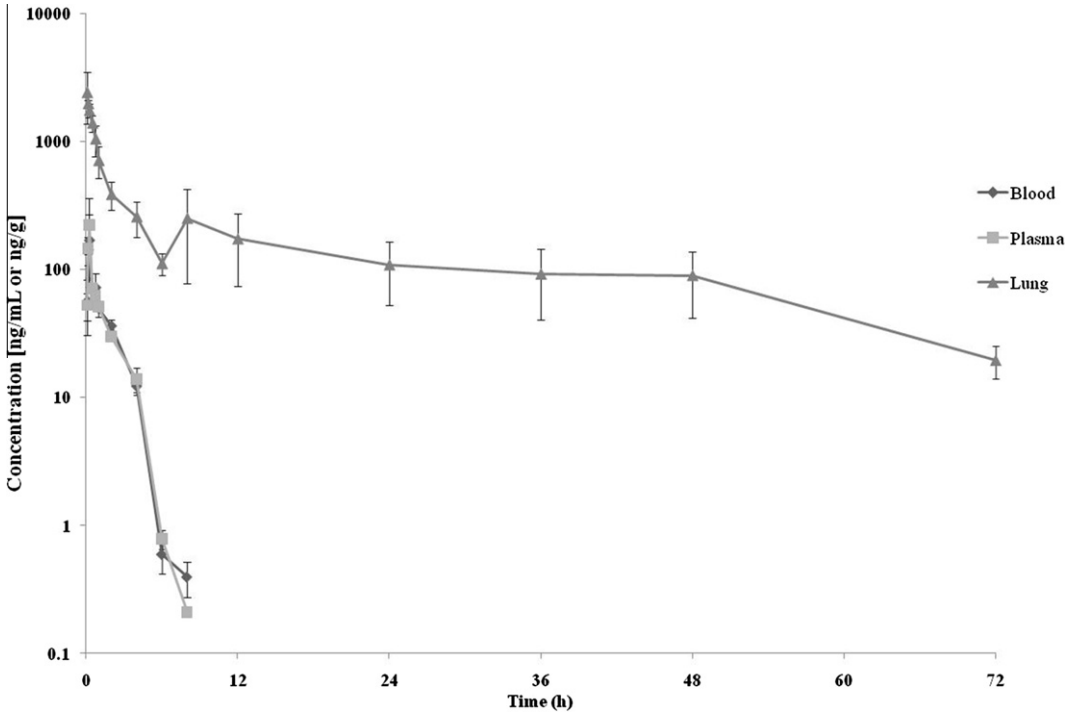


Figure 3. Pharmacokinetic profile of compound **45** in the blood, plasma and lung tissue after in. dosing in mice.

Table 3
Pharmacokinetic parameters following i.n. dosing in mice (2 mg/kg)

Matrix	Cmax ng/mL or ng/g	Tmax h	AUC(0-inf) ng*h/mL	T1/2 h	Tissue to blood/plasma AUC ratio
Blood	169	0.25	181	0.9	.
Plasma	223	0.25	181	0.9	
Lung	2414	0.08	9683	20	53

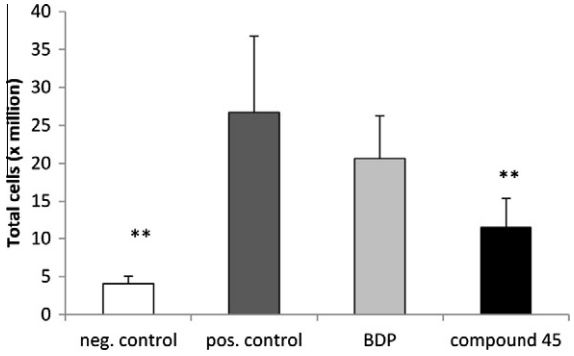


Figure 4. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on total cells (x million) recovered from the BALF of actively-sensitised BN rats. Values are mean + SD; $n = 10$. ∗/∗∗: Dunnett-test based on pooled variance sig. at 5% or 1% level.

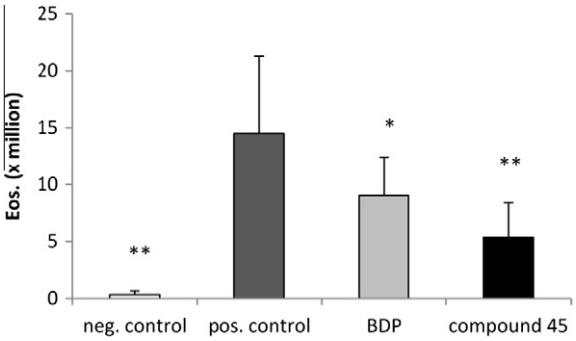


Figure 5. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on absolute number of eosinophils (x million) recovered from the BALF of actively-sensitised BN rats. Values are mean +SD; $n = 10$. ∗/∗∗: Dunnett-test based on pooled variance sig. at 5% or 1% level.

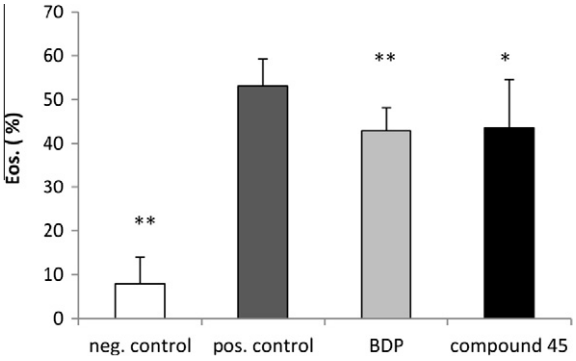


Figure 6. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on the number of eosinophils expressed as a percentage of total cell numbers recovered from the BALF of actively-sensitised BN rats. Values are mean +SD; $n = 10$. ∗/∗∗: Dunnett-test based on pooled variance sig. at 5% or 1% level.

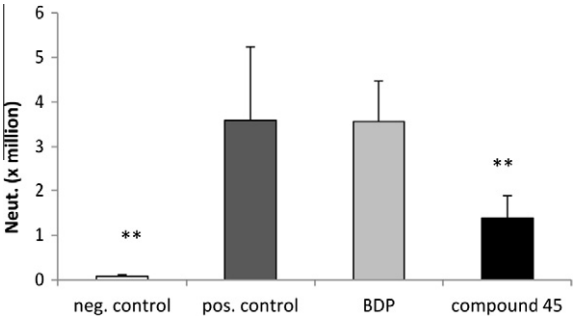


Figure 7. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on absolute number of neutrophils (x million) recovered from the BALF of actively-sensitised BN rats. Values are mean +SD; $n = 10$.

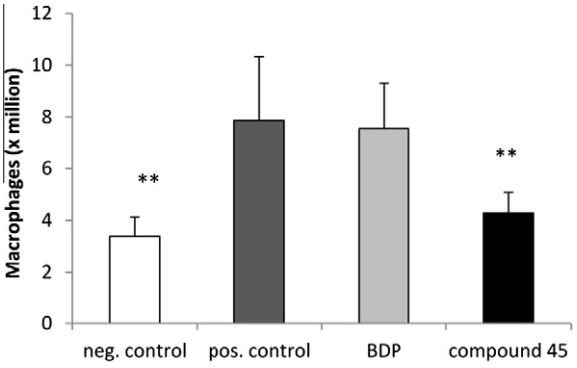


Figure 8. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on absolute number of macrophages (x million) recovered from the BALF of actively-sensitised BN rats. Values are mean +SD; $n = 10$. ∗/∗∗: Dunnett-test based on pooled variance sig. at 5% or 1% level.

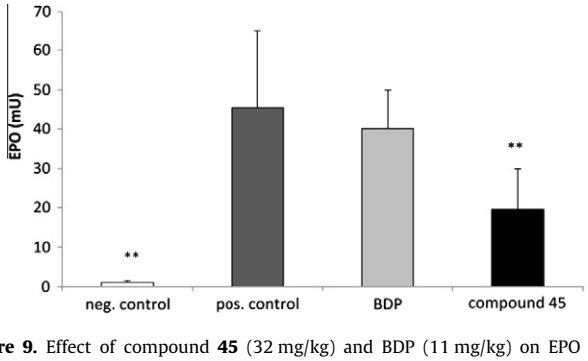


Figure 9. Effect of compound **45** (32 mg/kg) and BDP (11 mg/kg) on EPO (mU) recovered from the BALF of actively-sensitised BN rats. Values are mean +SD; $n = 10$. ∗/∗∗: Dunnett-test based on pooled variance sig. at 5% or 1% level.

Furthermore, eosinophil peroxidase (EPO) activity, a clear sign of eosinophil activation, was increased in eosinophils from actively sensitized challenged animals when compared with non-challenged control animals from 45.4 mU versus 1.1 mU, respectively ($p < 0.01$, Fig. 9). In BALF from animals treated with compound 45 (32 mg/kg) eosinophil peroxidase activity was significantly

Table 4
Antibacterial activity of compound **45**, given as minimum inhibitory concentration (MIC) in units of mg/L.

Strain	Phenotype	AZM	Compd **45**	Strain	Phenotype	AZM	Compd **45**
S. aureus ATCC13709		0.5	8	*S. pyogenes* 3565	eryS	⩽0.125	0.25
S. aureus 90265/97	iMLS	>64	>64	*S. pyogenes* Finland 11	iMLS	16	32
S. aureus PK2	cMLS	>64	>64	*S. pyogenes*166 GR-Micro	cMLS	>64	>64
S. aureus PK1	M	>64	>64	*S. pyogenes* Finland 2	M	8	32
S. pneumoniae SP 030	ery S	⩽0.125	⩽0.125	*M. catarrhalis* ATCC 23246		⩽0.125	8
S. pneumonia 58 Spain	cMLS	>64	64	*H. influenzae* ATTC 49247		1	64
S. pneumoniae 134 GR-M	iMcLS	>64	>64	*E. faecalis* ATCC 29213		4	64
S. pneumoniae Ci137	M	8	32	*E. coli*/ATCC 25922		2	>64

AZM = azithromycin. All ATCCstrains are erithromycin susceptible. iMLS = inducible resistance to macrolide, licosamide and streptogramin (MLS) antibiotics; iMcL = inducible resistance to macrolide and constitutive resistance to lincosamide antibiotics; cMLS = constitutive MLS resistance; M = efflux mediated macrolide resistance.

reduced to 19.6 mU (p <0.01). reaching more than 50% of reduction. This effect was apparent but to a much lesser extent in BALF cells from BDP treated animals (approximately a 12% reduction, not significant).

As macrolides are well established and clinically widely used antimicrobial agents, and antibacterial activity is undesirable for an anti-inflammatory drug, it was important to assess antibacterial potency of conjugate molecules. Minimal inhibitory concentrations (MICs) of azithromycin and compound **45** were determined by a standard broth microdilution method as described by CLSI guidelines (Table 4).[29]

Compared to azithromycin, compound **45** showed decreased anti-bacterial activity on most of the tested bacteria, Gram negative strains (*Moraxella catarrhalis*, *Haemophilus influenzae* and *Escherichia coli*) especially. However, relatively good potency was retained against *Streptococcus.pneumoniae* and *Streptococcus pyogenes*. Therefore, full abolishment of antibacterial activity will be the subject of further investigations.

4. Conclusion

Macrolactonolides were conceived to retain the initial pharmacological activity of the selected pharmacophore, and have improved pharmacokinetic behavior (primarily cellular and tissue accumulation) associated with the macrolide scaffold. Compound **45** shows improved accumulation/release behavior in murine macrophage cell line in comparison to classical steroids, as well as prolonged residence in murine lungs upon topical administration. Despite weak GR binding and modest activity in vitro, tested compound showes good in vivo efficacy in the OVA induced eosinophilia with interesting efficacy profile which might even indicate additional, macrolide-related effects. Compound **45** retains some antimicrobial activity, whereas activity against certain strains is abolished.

5. Experimental

5.1. Chemistry

Commercial reagents were used as received without additional purification. All used chemicals and solvents were p.a. purity. IR spectra were recorded as potassium bromide pastilles on Nicolet Magna IR 760 FT IR-spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity 600, Bruker Avance DPX 300 spectrometer at 300 MHz, or Bruker Avance DRX 500 spectrometer at 500 MHz, respectively, in the solvent indicated. Chemical shifts are reported in ppm using tetramethylsilan (TMS) as an internal standard. Purity of the compounds was determined by on HPLC-MS system Waters 2690 + Micromass Quattro Micro and on HPLC-UV system Waters 2690 + Waters 996 Photodiode Array Detector. HRMS data were acquired using Q-TOF 2 Waters system. Thin layer chromatography (TLC) was performed on aluminum plates Merck Silica gel 60 F_{254} and visualized with UV light at 254 nm and/or by spraying with 5% H_2SO_4/EtOH solution followed by heating at 140 °C. Column chromatography was performed on silica gel 60 (Merck, 0063–0200 nm). Steroid intermediates **12–22**, **24**, **25**, **27–30** and macrolide intermediates **32** and **34** were synthesized as described before.[18–21,23,25]

5.1.1. Preparation of compound 33

A solution of compound **32** (3.00 g, 3.79 mmol) in abs. EtOH (100 ml) was hydrogenated over 500 mg of PtO_2 for 2 days at 40 atm. The catalyst was filtered off and the filtrate evaporated. Crude product was purified by silica gel column chromatography ($CHCl_3$/CH_3OH/NH_4OH, 6:1:0.1) to afford 700 mg (23%) of pure compound **33**. MS (ESI) 792.4 $(MH)^+$, 397.0 $[(MH_2)^{++}]$. IR KBr 3451 (N–H, O–H), 2972, 2937, 1724 (C=O lactone), 1659, 1638, 1546, 1462, 1378, 1281, 1168, 1111, 1078, 1054, 1000, 958, 899, 835, 729, 639 (cm^{-1}). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.06 (H-1″), 4.92 (H-13), 4.46 (H-1′), 4.15 (H-3), 3.58 (H-5), 3.32 (3″–OCH_3), 2.28 [3′–$N(CH_3)_2$], 1.29 (6–CH_3), 0.84 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.03, 102.50, 95.11, 83.52, 78.66, 77.27, 49.08, 47.91, 39.99, 39.33, 28.46, 25.94, 10.62.

5.1.2. Preparation of compound 35

A solution of compound **34** (4.00 g; 6.93 mmol) in acrylonitrile (30 ml) was refluxed for 24 h, then concentrated under reduced pressure. Compound **35** (3.90 g) was used in the next step without further purification. MS (ESI) 630.2 $(MH)^+$, 315.8 $[(MH_2)^{++}]$. IR KBr 3451 (O–H), 2975, 2939, 2879, 2248 (C≡N), 1707 (C=O lactone), 1638, 1459, 1382, 1348, 1261, 1172, 1112, 1074, 1050, 978, 957, 934, 902, 865, 835, 757 (cm^{-1}). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.65 (H-13), 4.50 (H-1′), 3.89 (H-3), 3.71 (H-5), 2.44 [3′–$N(CH_3)_2$], 1.30 (6–CH_3), 0.91 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.21, 118.70, 105.48, 95.23, 77.87, 76.24, 39.97, 26.32, 17.74, 10.78.

5.1.3. Preparation of compound 36

Compound **36** (985 mg, 28%) was prepared from compound **35** (3.50 g, 5.55 mmol) by the procedure described for the preparation of compound **33**. MS (ESI) 634.38 $(MH)^+$, 317.89 $[(MH_2)^{++}]$. IR KBr 3478, 3445, 3358 and 3294 (N–H, O–H), 2985, 2941, 2870, 2803, 2722, 1722 (C=O lactone), 1655, 1608, 1463, 1381, 1348, 1307, 1267, 1175, 1112, 1078, 1049, 1036, 1021, 1003, 976, 933, 896, 870, 831, 798, 758, 670 (cm^{-1}). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.00 (H-13), 4.52 (H-1′), 3.75 (H-3), 3.57 (H-5), 2.28 [3′–$N(CH_3)_2$], 1.34 (6–CH_3), 0.86 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.02, 106.24, 95.38, 77.92, 77.45, 40.23, 38.82, 28.21, 26.03, 10.65.

5.1.4. General procedure for preparation of 44–58

To the suspension of acid **12** (110 mg; 0.29 mmol) in dry CH_2Cl_2 (5 mL) cooled to 0 °C under argon, Et_3N (0.380 mL; 2.73 mmol) was added resulting in a clear solution. Subsequently, HOBT (80 mg;

0.59 mmol), amine **33** (230 mg; 0.29 mmol) and EDC×HCl (235 mg; 1.23 mmol) were added. The reaction mixture was stirred at room temperature overnight (18 h). The solvent was evaporated under reduced pressure and the residue purified on a silica gel column (eluant $CHCl_3$/MeOH/NH_4OH, 6:1:0.1). 224 mg (67%) of compound **44** were obtained. MS (ESI) 1152.49 (MH^+), 577.07 [$(MH_2)^{++}$]. HRMS (ESI) calcd for $C_{61}H_{104}FN_3O_{16}$ [$(MH_2)^{++}$]: 576.8700; found 576.8698. IR (KBr) 3517 and 3433, 3600–3100 (O–H, N–H), 2975, 2943 and 2871 (C–H), 1722 (C=O lactone), 1665 (C=O ketone), 1647 (C=O amide), 1624 and 1606 (C=C), 1560, 1535, 1459, 1439, 1420, 1377, 1321, 1268, 1223, 1176, 1166, 1127, 1108, 1094, 1054, 1004, 956, 895, 796, 752, 701, 670 (cm^{-1}). ^1H NMR (500 MHz, DMSO-d_6) δ 7.34 (NH), 7.31 (H-1^S), 6.21 (H-2^S), 5.99 (H-4^S), 4.83 (H-13), 4.79 (H-1″), 4.42 (H-1′), 4.11 (H-11^S), 4.07 (H-5″), 4.01 (H-3), 3.66 (H-5′), 3.51 (H-5, H-11), 3.22 (3″–OCH_3), 3.07 (H_a–CONH$CH_2CH_2CH_2$), 3.04 (H-2′, H-16^S), 3.00 (H_b-CONH$CH_2CH_2CH_2$), 2.91 (H-4″), 2.82 (H_a–CONHCH$_2$CH$_2$$CH_2$), 2.71 (H-2, H-10), 2.62 (H_a-6^S), 2.55 (H_a-9), 2.47 (H-3′, H_b–CONHCH$_2$CH$_2$$CH_2$), 2.27 ($H_a$-2″), 2.34 (H-$8^S$), 2.31 ($H_b$-$6^S$), 2.22 [3′–N$(CH_3)_2$], 2.07 ($H_b$-9), 2.03 (H-$14^S$), 2.03 ($H_a$-$12^S$), 1.94 (H-8), 1.91 (H-4), 1.77 (H_a-13–CH_2, H_a-7^S), 1.67 (H_a–CONHCH$_2$$CH_2CH_2$), 1.61 ($H_a$-7), 1.59 ($H_a$-4′, H_a-15^S), 1.57 (H_b–CONHCH$_2$$CH_2CH_2$), 1.50 ($H_b$-2″), 1.48 ($10^S$–$CH_3$), 1.42 ($H_b$-$12^S$), 1.35 ($H_b$-$15^S$), 1.34 ($H_b$-13–$CH_2$, H_b-7^S), 1.20 (6–CH_3), 1.15 (5″–CH_3), 1.13 (3″–CH_3), 1.09 (10–CH_3), 1.07 (5′–CH_3), 1.04 (H_b-7, H_b-4′), 1.02 (12–CH_3), 1.01 (2–CH_3), 0.98 (4–CH_3), 0.95 (13^S–CH_3), 0.84 (8–CH_3), 0.80 (16^S–CH_3), 0.79 (13–CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 186.19, 177.32, 173.13, 168.11, 153.85, 129.79, 124.98, 102.96, 102.41, 95.83, 87.24, 83.49, 78.79, 78.35, 77.39, 75.99, 75.24, 74.42, 73.66, 71.93, 71.54, 68.03, 65.80, 65.61, 63.71, 60.74, 49.71, 48.96, 48.40, 45.14, 43.94, 41.48, 41.23, 38.45, 36.40, 35.74, 35.74, 35.73, 34.78, 32.90, 31.25, 30.96, 28.21, 28.19, 28.05, 28.05, 23.79, 23.30, 22.28, 22.35, 21.91, 19.37, 18.19, 17.78, 16.00, 15.76, 11.91, 10.44, 10.29.

5.1.5. Preparation of compound 45

Compound **45** (224 mg, 89%) was prepared from acid **13** (80 mg; 0.22 mmol) and amine **33** (174 mg; 0.22 mmol). MS (ESI) 1136.53 (MH^+), 569.08 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{61}H_{104}FN_3O_{15}$ [$(MH_2)^{++}$]: 568.8726; found 568.8716. IR (KBr) 3513 and 3423, 3600–3100 (O–H, N–H), 2972, 2942 and 2873 (C–H), 1720 (C=O lactone), 1666 (C=O ketone), 1647 (C=O amide), 1630 and 1610 (C=C), 1561, 1534, 1459, 1377, 1294, 1271, 1248, 1166, 1109, 1094, 1054, 1005, 958, 890, 829, 752 (cm^{-1}). ^1H NMR (500 MHz, DMSO-d_6) δ 7.44 (NH), 7.30 (H-1^S), 6.21 (H-2^S), 6.00 (H-4^S), 4.89 (H-13), 4.79 (H-1″), 4.42 (H-1′), 4.08 (H-11^S), 4.08 (H-5″), 4.01 (H-3), 3.66 (H-5′), 3.51 (H-5, H-11), 3.22 (3″–OCH_3), 3.02 (H–CONH$CH_2CH_2CH_2$, H-2′), 2.91 (H-4″), 2.82 (H_a–CONHCH$_2$CH$_2$$CH_2$), 2.71 (H-2, H-10), 2.63 (H_a-6^S), 2.53 (H_a-9), 2.50 (H-16^S), 2.44 (H-3′, H_b–CONHCH$_2$CH$_2$$CH_2$), 2.32 ($H_b$-$6^S$), 2.29 (H-$8^S$), 2.27 ($H_a$-2″), 2.23 [3′–N$(CH_3)_2$], 2.09 ($H_b$-9), 1.95 (H-4), 1.90 (H-8), 1.77 (H_a-13–CH_2, H_a-7^S), 1.77 (H_a-12^S), 1.64 (H_a–CONHCH$_2$$CH_2CH_2$, H_a-7), 1.63 (H_b-12^S), 1.60 (H_a-15^S), 1.59 (H_a-4′), 1.51 (H_b–CONHCH$_2$$CH_2CH_2$, H_b-2″), 1.49 (10^S–CH_3), 1.37 (H_b-13–CH_2), 1.35 (H_b-7^S, H_b-15^S), 1.20 (6–CH_3), 1.15 (H_b-7, 5″–CH_3), 1.14 (3″–CH_3), 1.10 (2–CH_3), 1.07 (5′–CH_3), 1.04 (H_b-4′), 1.01 (12–CH_3), 1.00 (10–CH_3), 0.98 (4–CH_3), 0.90 (13^S–CH_3), 0.88 (8–CH_3, 16^S–CH_3), 0.80 (13–CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 185.15, 176.32, 170.75, 166.96, 152.80, 128.83, 124.04, 101.89, 101.44, 94.80, 82.45, 77.77, 77.27, 76.24, 74.93, 74.42, 74.24, 72.65, 70.54, 70.14, 66.97, 64.78, 64.76, 64.55, 62.84, 59.88, 48.71, 48.02, 48.37, 44.17, 43.86, 42.57, 40.40, 40.26, 37.20, 34.69, 33.32, 32.60, 30.90, 30.13, 29.96, 27.91, 27.07, 27.07, 27.07, 27.07, 22.34, 22.33, 21.33, 21.20, 20.88, 18.36, 18.35, 18.11, 15.72, 14.83, 10.92, 9.31, 9.26.

5.1.6. Preparation of compound 46

Compound **46** (107 mg, 32%) was prepared from acid **14** (110 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1153.5 (MH^+), 577.3 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{61}H_{104}FN_3O_{16}$ [$(MH_2)^{++}$]: 576.8700; found 576.8690. IR (KBr) 3434, 3600–3100 (O–H, N–H), 2973, 2937 and 2875 (C–H), 1727 (C=O lactone), 1666 (C=O ketone), 1629 and 1609 (C=C), 1520, 1456, 1378, 1318, 1281, 1271, 1167, 1110, 1094, 1054, 1014, 958, 899, 823, 716 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.28 (H-1^S), 6.29 (H-4^S), 6.27 (H-2^S), 5.31 (H-6^S), 4.97 (H-1″), 4.77 (H-13), 4.49 (H-1′), 4.46 (H-11^S), 4.10 (H-3), 3.58 (H-5), 3.32 (3″–OCH_3), 2.48 [3′–N$(CH_3)_2$], 1.43 (10^S–CH_3), 1.36 (6–CH_3), 1.10 (13^S–CH_3), 0.92 (16^S–CH_3), 0.89 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.74, 178.40, 155.66, 127.86, 118.02, 102.83, 96.45, 87.71, 83.74, 79.31, 77.74, 69.50, 49.43, 40.52, 26.87, 21.16, 17.49, 14.68, 11.11.

5.1.7. Preparation of compound 47

Compound **47** (170 mg, 61%) was prepared from acid **17** (96 mg; 0.24 mmol) and amine **33** (197 mg; 0.24 mmol). MS (ESI) 1169.2 (MH^+), 585.3 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{61}H_{104}ClN_3O_{16}$ [$(MH_2)^{++}$]: 584.8553; found 584.8555. IR (KBr) 3422, 3600-3100 (O–H, N–H), 2971, 2938 and 2874 (C–H), 1732 (C=O lactone), 1662 (C=O ketone), 1619 and 1609 (C=C), 1512, 1454, 1378, 1317, 1247, 1168, 1111, 1052, 1013, 959, 886, 836, 755 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.29 (H-1^S), 6.29 (H-2^S), 6.07 (H-4^S), 4.98 (H-1″), 4.72 (H-13), 4.54 (H-11^S), 4.46 (H-1′), 4.10 (H-3), 3.58 (H-5), 3.32 (3″–OCH_3), 2.38 [3′–N$(CH_3)_2$], 1.67 (10^S–CH_3), 1.36 (6–CH_3), 1.10 (13^S–CH_3), 1.08 (16^S–CH_3), 0.88 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 186.76, 177.83, 172.89, 152.89, 129.25, 124.93, 102.97, 95.97, 82.61, 79.26, 76.38, 75.51, 49.48, 40.51, 26.70, 24.41, 20.58, 18.38, 11.12.

5.1.8. Preparation of compound 48

Compound **48** (258 mg, 76%) was prepared from acid **15** (115 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1171.0 (MH^+), 586.1 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{61}H_{103}F_2N_3O_{16}$ [$(MH_2)^{++}$]: 585.8653; found 585.8641. IR (KBr) 3433, 3600–3100 (O–H, N–H), 2969, 2938 and 2876 (C–H), 1728 (C=O lactone), 1671 (C=O ketone), 1634, 1524, 1456, 1378, 1316, 1283, 1167, 1110, 1055, 1032, 999, 960, 933, 899, 821, 728, 711 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.20 (H-1^S), 6.40 (H-4^S), 6.33 (H-2^S), 5.38 (H-6^S), 5.03 (H-1″), 4.67 (H-13), 4.45(H-1′), 4.33 (H-11^S), 4.09 (H-3), 3.60 (H-5), 3.33 (3″–OCH_3), 2.30 [3′–N$(CH_3)_2$], 1.53 (10^S–CH_3), 1.35 (6–CH_3), 1.10 (13^S–CH_3), 0.94 (16^S–CH_3), 0.88 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.86, 178.70, 172.77, 151.50, 129.85, 120.97, 102.87, 95.72, 86.82, 83.01, 79.39, 77.84, 71.76, 49.50, 40.34, 27.15, 23.04, 16.52, 14.65, 11.08.

5.1.9. Preparation of compound 49

Compound **49** (105 mg, 69%) was prepared from acid **25** (50 mg; 0.13 mmol) and amine **33** (115 mg; 0.14 mmol). MS (ESI) 1155.2 (MH^+), 578.3 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{61}H_{103}F_2N_3O_{15}$ [$(MH_2)^{++}$]: 577.8679; found 577.8678. IR (KBr) 3444, 3600–3100 (O–H, N–H), 2969, 2940, 2876 and 2782 (C–H), 1731 (C=O lactone), 1671 (C=O ketone), 1634, 1525, 1456, 1379, 1317, 1293, 1263, 1176, 1167, 1110, 1057, 1015, 959, 938, 899, 835, 816, 756, 709 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.29 (H-1^S), 6.39 (H-4^S), 6.34 (H-2^S), 5.37 (H-6^S), 4.98 (H-1″), 4.79 (H-13), 4.48 (H-1′), 4.30 (H-11^S), 4.10 (H-3), 3.61 (H-5), 3.32 (3″–OCH_3), 2.42 [3′–N$(CH_3)_2$], 1.53 (10^S–CH_3), 1.39 (6–CH_3), 1.02 (13^S–CH_3), 0.98 (16^S–CH_3), 0.90 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.31, 178.00, 171.63, 151.13, 129.25, 120.31, 102.44, 95.30,

86.20, 83.05, 78.53, 77.23, 70.62, 48.89, 39.91, 26.00, 22.47, 20.77, 15.23, 10.60.

5.1.10. Preparation of compound 50

Compound **50** (224 mg, 65%) was prepared from acid **30** (120 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1187.0 (MH^+), 594.2 [$(MH_2)^{++}$]. HRMS (ESI) calcd for $C_{61}H_{103}ClFN_3O_{16}$ [$(MH_2)^{++}$]: 593.8505; found 593.8509. IR (KBr) 3430, 3600–3100 (O–H, N–H), 2972, 2938 and 2876 (C–H), 1729 (C=O lactone), 1668 (C=O ketone), 1633, 1520, 1455, 1379, 1318, 1167, 1123, 1055, 958, 900, 819, 756 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.31 (H-1^S), 6.37 (H-4^S), 6.32 (H-2^S), 5.35 (H-6^S), 4.91 (H-1″), 4.75 (H-13), 4.57 (H-11^S), 4.48 (H-1′), 4.08 (H-3), 3.55 (H-5), 3.30 (3″–OCH_3), 2.21 [3′–$N(CH_3)_2$], 1.65 (10^S–CH_3), 1.36 (6–CH_3), 1.12 (13^S–CH_3), 0.95 (16^S–CH_3), 0.89 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.96, 151.77, 129.52, 120.94, 103.05, 95.92, 86.80, 82.80, 79.25, 77.83, 75.30, 49.49, 40 .58, 26.54, 24.51, 17.63, 14.74, 11.19.

5.1.11. Preparation of compound 51

Compound **51** (285 mg, 88%) was prepared from acid **18** (100 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1120.7 (MH^+), 561.6 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{60}H_{103}N_3O_{16}$ [$(MH_2)^{++}$]: 560.8669; found 560.8651. IR (KBr) 3432, 3600–3100 (O–H, N–H), 2972, 2937 and 2875 (C–H), 1731 (C=O lactone), 1660 (C=O ketone), 1621, 1523, 1456, 1378, 1262, 1167, 1111, 1053, 1013, 958, 889, 804, 755 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.33 (H-1^S), 6.24 (H-2^S), 6.00 (H-4^S), 5.02 (H-1″), 4.72 (H-13), 4.46 (H-1′, H-11^S), 4.10 (H-3), 3.60 (H-5), 3.32 (3″–OCH_3), 2.37 [3′–$N(CH_3)_2$], 1.45 (10^S–CH_3), 1.36 (6–CH_3), 1.01 (13^S–CH_3), 0.88 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 186.76, 178.32, 173.94, 156.90, 127.60, 122.23, 102.86, 95.75, 83.28, 79.23, 77.86, 70.14, 49.48, 40.41, 26.89, 20.99, 18.36, 11.09.

5.1.12. Preparation of compound 52

Compound **52** (238 mg, 74%) was prepared from acid **22** (96 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1106.5 (MH^+), 553.8 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{60}H_{105}N_3O_{15}$ [$(MH_2)^{++}$]: 553.8773; found 553.8765. IR (KBr) 3444, 3600–3100 (O–H, N–H), 2971, 2936, 2877 and 2788 (C–H), 1731 (C=O lactone), 1667 (C=O ketone), 1531, 1456, 1379, 1268, 1245, 1167, 1109, 1093, 1053, 1013, 1000, 957, 902, 867, 754, 665 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 5.67 (H-4^S), 4.99 (H-1″), 4.64 (H-13), 4.47 (H-1′), 4.35 (H-11^S), 4.17 (H-3), 3.65 (H-5), 3.33 (3″–OCH_3), 2.35 [3′–$N(CH_3)_2$], 1.44 (10^S–CH_3), 1.36 (6–CH_3), 1.09 (13^S–CH_3), 0.87 (13–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.86, 177.73, 172.78, 122.63, 103.33, 96.07, 84.05, 79.73, 78.40, 68.37, 49.82, 40.80, 27.25, 21.22, 15.81, 11.52.

5.1.13. Preparation of compound 53

Compound **53** (75 mg, 23%) was prepared from acid **21** (100 mg; 0.29 mmol) and amine **33** (230 mg; 0.29 mmol). MS (ESI) 1123.0 (MH^+), 562.1 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{60}H_{105}N_3O_{16}$ [$(MH_2)^{++}$]: 561.8747; found 561.8743. IR (KBr) 3430, 3600–3100 (O–H, N–H), 2972 and 2937 (C–H), 1729 (C=O lactone), 1660 (C=O ketone), 1520, 1456, 1378, 1268, 1245, 1167, 1121, 1078, 1054, 1014, 1000, 959, 897, 868, 835, 804, 755 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 5.67 (H-4^S), 4.98 (H-1″), 4.69 (H-13), 4.48 (H-1′), 4.47 (H-11^S), 4.12 (H-3), 3.68 (H-5), 3.32 (3″–OCH_3), 2.43 [3′–$N(CH_3)_2$], 1.44 (10^S–CH_3), 1.36 (6–CH_3), 1.00 (13^S–CH_3), 0.87 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.70, 178.33, 172.69, 122.20, 102.85, 95.82, 83.83, 79.25, 77.85, 68.35, 49.45, 40.50, 27.00, 20.91, 17.59, 11.12.

5.1.14. Preparation of compound 54

Compound **54** (121 mg, 78%) was prepared from acid **13** (57 mg; 0.16 mmol) and amine **36** (100 mg; 0.16 mmol). MS (ESI) 978.3 (MH^+), 489.3 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{53}H_{100}FN_3O_{12}$ [$(MH_2)^{++}$]: 489.8254; found 489.8227. IR (KBr) 3426 and 3343, 3600–3100 (O–H, N–H), 2972, 2942, 2876 and 2874 (C–H), 1696 (C=O lactone), 1661 (C=O ketone), 1625, 1550, 1457, 1379, 1352, 1253, 1170, 1109, 1054, 979, 958, 897, 832, 696 (cm^{-1}). ^1H NMR (500 MHz, DMSO-d_6) δ 7.42 (NH), 7.31 (H-1^S), 6.21 (H-2^S), 6.01 (H-4^S), 5.02 (H-13), 4.51 (H-1′), 4.07 (H-11^S), 3.45 (H-3), 3.41 (H-5), 2.22 [3′–$N(CH_3)_2$], 1.49 (10^S–CH_3), 1.14 (6–CH_3), 0.89 (13^S–CH_3), 0.88 (16^S–CH_3), 0.77 (13–CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 185.15, 175.24, 170.65, 152.71, 128.85, 124.03, 103.11, 89.07, 76.34, 75.84, 70.34, 40.34, 26.57, 22.75, 21.47, 15.73, 10.59.

5.1.15. Preparation of compound 55

Compound **55** (138 mg, 58%) was prepared from acid **14** (90 mg; 0.24 mmol) and amine **36** (150 mg; 0.24 mmol). MS (ESI) 995.0 (MH^+), 498.2 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{53}H_{100}FN_3O_{13}$ [$(MH_2)^{++}$]: 497.8229; found 497.8216. IR (KBr) 3435, 3600–3100 (O–H, N–H), 2974, 2937 and 2876 (C–H), 1712 (C=O lactone), 1666 (C=O ketone), 1628, 1520, 1456, 1380, 1317, 1260, 1174, 1112, 1073, 1050, 988, 956, 930, 899, 863, 823, 755, 718 (cm^{-1}). ^1H NMR (500 MHz, $CDCl_3$) δ 7.32 (H-1^S), 6.30 (H-4^S), 6.29 (H-2^S), 5.31 (H-6^S), 4.70 (H-13), 4.54 (H-1′), 4.42 (H-11^S), 3.74 (H-3), 3.63 (H-5), 2.34 [3′–$N(CH_3)_2$], 1.43 (10^S–CH_3), 1.32 (6–CH_3), 1.10 (13^S–CH_3), 0.94 (16^S–CH_3), 0.85 (13–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.74, 180.84, 173.98, 155.26, 128.12, 118.20, 106.43, 95.05, 87.74, 78.83, 77.92, 69.63, 40.34, 27.89, 21.20, 17.43, 14.64, 10.86.

5.1.16. Preparation of compound 56

Compound **56** (163 mg, 67%) was prepared from acid **15** (94 mg; 0.24 mmol) and amine **36** (150 mg; 0.24 mmol). MS (ESI) 1012.9 (MH^+), 507.3 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{53}H_{89}F_2N_3O_{13}$ [$(MH_2)^{++}$]: 506.8182; found 506.8170. IR (KBr) 3429, 3600–3100 (O–H, N–H), 2974, 2940 and 2876 (C–H), 1711 (C=O lactone), 1669 (C=O ketone), 1633, 1520, 1456, 1379, 1316, 1262, 1174, 1111, 1073, 995, 957, 899, 863, 820, 756, 709 (cm^{-1}). ^1H NMR (300 MHz, $CDCl_3$) δ 7.20 (H-1^S), 6.41 (H-4^S), 6.33 (H-2^S), 5.37 (C-6^S), 4.68 (H-13), 4.45 (H-1′), 4.35 (H-11^S), 3.75 (H-3), 3.61 (H–5), 2.26 [3′–$N(CH_3)_2$], 1.54 (10^S–CH_3), 1.32 (6–CH_3), 1.11 (13^S–CH_3), 0.95 (16^S–CH_3), 0.87 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.50, 178.04, 172.13, 150.83, 129.92, 120.84, 106.40, 94.67, 87.45, 78.07, 77.09, 71.47, 40.10, 26.36, 22.99, 16.85, 14.32, 10.77.

5.1.17. Preparation of compound 57

Compound **57** (235 mg, 85%) was prepared from acid **30** (110 mg; 0.27 mmol) and amine **36** (169 mg; 0.27 mmol). MS (ESI) 1029.0 (MH^+), 515.2 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{53}H_{89}ClFN_3O_{13}$ [$(MH_2)^{++}$]: 514.8034; found 514.8025. IR (KBr) 3433, 3600–3100 (O–H, N–H), 2972, 2935 and 2875 (C–H), 1711 (C=O lactone), 1668 (C=O ketone), 1633, 1520, 1456, 1381, 1320, 1262, 1174, 1114, 1071, 1051, 993, 957, 935, 899, 818, 754 (cm^{-1}). ^1H NMR (300 MHz, $CDCl_3$) δ 7.20 (H-1^S), 6.41 (H-4^S), 6.34 (H-2^S), 5.36 (C-6^S), 4.66 (H-13), 4.55 (H-11^S), 4.47 (H-1′), 3.75 (H-3), 3.62 (H-5), 2.27 [3′–$N(CH_3)_2$], 1.65 (10^S–CH_3), 1.33 (6–CH_3), 1.10 (13^S–CH_3), 0.96 (16^S–CH_3), 0.85 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.54, 177.99, 172.04, 150.94, 129.62, 120.90, 106.36, 94.29, 86.53, 78.06, 77.04, 74.87, 40.10, 26.42, 24.36, 17.34, 14.33, 10.76.

5.1.18. Preparation of compound 58

Compound **58** (112 mg, 48%) was prepared from acid **21** (84 mg; 0.24 mmol) and amine **36** (150 mg; 0.24 mmol). MS (ESI) 965.0.0 (MH^+), 483.2 [$(MH_2)^{++}$]. HRMS (ESI) Calcd for $C_{52}H_{91}N_3O_{13}$ [$(MH_2)^{++}$]: 482.8276; found 482.8270. IR (KBr) 3430, 3600-3100 (O–H, N–H), 2973, 2937 and 2877 (C–H), 1709 (C=O lactone), 1660 (C=O ketone), 1520, 1456, 1380, 1348, 1267, 1231, 1173, 1112, 1075, 1050, 999, 957, 898, 866, 754 (cm^{-1}). ^1H NMR (300 MHz, $CDCl_3$) δ 5.67 (H-4^S), 4.70 (H-13), 4.47 (H-1′, H-11^S), 3.70 (H-3), 3.59 (H-5), 2.29 [3′–$N(CH_3)_2$], 1.44 (10^S–CH_3), 1.33 (6–CH_3), 0.99 (13^S–CH_3), 0.85 (13–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.66, 177.75, 172.60, 122.21, 106.40, 94.87, 77.91, 77.37, 68.21, 40.25, 26.32, 20.84, 17.40, 10.78.

5.2. Biology

5.2.1. Cell free GR binding assay

Compounds are evaluated for binding to the steroid receptor by a fluorescence polarization competitive binding assay (FP). FP assay is performed using Invitrogen assay based on partial purified glucocorticoid receptor, according to manufacturer's instructions. FP was measured using Tecan spectrofluorimeter, with fluorescein set of the filters. EC50 values were calculated using GraphPad Prism software.

5.2.2. Cellular assay of binding to GR

The gene for the alpha isoform of human glucocorticoid receptor was cloned by reverse polymerase chain reaction. The total RNA was isolated from human peripheral blood lymphocytes according to the instructions of the manufacturer (Qiagen), transcripted into cDNA with AMV reverse transcriptase (Roche) and the gene was multiplied by specific primers 1) 5′ATATGGATCCCTGATGGACTCCAAAGAATCATTAACTCC3′ and 2) 5′ATATCTCGAGGGCAGTCACTTTTGATGAAACAGAAG3′. The reaction product obtained was cloned into the XhoI/BamHI site of Bluescript KS plasmid (Stratagene), subjected to sequencing by the dideoxy fluorescent method with M13 and M13rev primers (Microsynth) and then it was cloned into the XhoI/BamHI site of pcDNA3.1 hygro(+)plasmid (Invitrogen Life Technologies). 293 MSR cell line (Invitrogen) was transfected with expression plasmid. 48 h post transfection, cells were plated with hygromicin B to obtain single cell clones. Two weeks individual clones were picked and expanded, and clones with the highest expression of human GRα were chosen by measuring specific dexamethasone binding. All compounds were tested on one clone showing highest specific binding.

One day prior to the binding assay, 2×10^5 GR expressing 293 MSR cells were plated in 48 well plate. On the day of experiment, serial dilutions were prepared from 10 mM DMSO stock solution in DMEM medium. 24 nM [^{3}H]dexamethasone (Pharmacia) in DMEM medium was added. The cells were incubated for 60 min at 37 °C in an atmosphere with 5% CO_2, washed three times with PBS buffer (Sigma) and then lysed in 0,5% SDS solution (Sigma). After the addition of UltimaGold XR scintillation liquid (Packard), the radioactivity was read in a Tricarb (Packard) scintillation counter. Results were analyzed using GraphPad Prism software.

5.2.3. H13 cell line (T-cell hybridoma) proliferation

In a 96-well plate, triplicates of test steroid dilution in RPMI medium (Institute of Immunology, Zagreb) with 10% FBS are performed. To the solutions of compounds, 30,000 cells per well are added and are incubated overnight at 37 °C in an atmosphere with 5% CO_2, then 1 μCi of [^{3}H]thymidine (Pharmacia) is added and the mixture is incubated for additional 3 hours. The cells are harvested by applying a vacuum over GF/C filter (Packard). Onto each well, 30 μl of Microscynt O scintillation liquid (Packard) is added and the incorporated radioactivity is measured on the scintillation counter (Packard). The specificity of apoptosis induction by glucocorticoids is proven by antagonising the proliferation inhibition with mifepristone (Sigma) (results not shown).

5.2.4. Cytokine production in stimulated PBMCs

MDS Pharma Services, study number 0626381.

Compounds were tested in 10pt EC-50 titrations, starting at 20 μM. All samples were run in duplicate. Cytotoxicity was assessed in parallel Alamar Blue assay, using PHA stimulation for 48 h. Compounds were diluted in culture media (CM), DMSO held constant across all concentrations. Thawed cryopreservec PBMCs were seeded in 30 × 96 well plates in CM, incubated for 1 h at 37 °C at 5% CO_2. After adding compounds, cells were incubated for 1 h at 37 °C at 5% CO_2, inducers (LPS, anti-CD3 and PHA) diluted in CM were added, plates were incubated at 37 °C, 5% CO_2, for 6, 24 or 48 h, depending on panel, plates spinned at 1200 rpm for 10 min, supernatants collected and store at −80 °C. Cell culture supernatants were quantified for cytokine multiplex detected by Luminex and Upstate Bead kit. Peak cytokine production of TNFalpha, IL-1beta, IL-6, IL-8 and MIP1alpha in LPS stimulation was at 6 h. Peak cytokine production of TNFalpha, IL-1beta, GM–CSF and IL-12p70 in anti-CD3 stimulation was 48 h. Peak cytokine production of TNFalpha, IL-1beta, IL-6 IL-8 and MIP1alpha in PHA stimulation was 6 h, IFNgamma, IL-4, IL-13, IP-10 and MCP-1 was 24 h and IL-2, IL-3, IL-5 and IL-10 was 48 h. Activity of compound **45** therefore are estimated at dose timepoints.

5.2.5. Cellular accumulation and retention in J774A.1 cell line

Murine macrophage cell line J774A.1 (ATCC, TIB-67) was grown in DMEM medium (Gibco, Invitrogen) with 1% Glutamax (Gibco, Invitrogen), 10% fetal bovine serum (FBS, Biowest). Two days before experiment, cells were seeded in 12 well plates at density of 2×10^5 cells in 1.2 mL per well in the culture medium.

Cellular accumulation and retention experiments were performed as described previously.[26] Briefly, compounds were dissolved in dimethyl sulphoxide (Sigma) at concentration of 20 mM. Cells were incubated with 10 μM compounds for 3 h in DMEM medium with 25 mM Hepes without other supplements. To determine cellular accumulation, cells were washed with ice cold PBS, and lysed in 0.5% TritonX-100 (Sigma) in deionized water. To measure cellular retention drug-loaded cells were incubated in fresh medium for another 3 h, washed, and lysed. Concentrations in samples were determined by LC–MS/MS method as described previously.[27] Intracellular concentration was calculated using cellular volume of 1.68 μL per million cells.[28] Accumulation is expressed as intracellular (I) to extracellular (E) concentration ratio where E, due to large volume of incubation medium (3 mL), was taken as constant (10 μM). Retention in cells is expressed as a percentage of initially accumulated amount of tested substance that remained in the cells after 3 h washout period.

5.2.6. Mouse pharmacokinetic studies

The pharmacokinetics of compound **45**, was evaluated following intranasal (in.) administration to male Balb/c mice (21–25 g), purchased from Charles River Laboratories (Sultzfled, Germany). The formulation for compound **45** was prepared freshly in 1% dimethyl sulfoxide (DMSO) and 0.125% carboxymethyl cellulose (CMC). Blood and lung samples were obtained by terminal bleeding at 5, 10, 15, 30, 45 min and 1, 2, 6, 6, 8, 12, 24, 36, 48 and 72 h following i.n. administration. Blood samples were collected into tubes containing K_2EDTA as anticoagulant. An aliquot of blood (50 μL) was removed and hemolyzed in an equivalent volume of water in a 1:1 ratio. The remainder was and centrifuged at 3000 rpm for 10 min. to obtain plasma and plasma was aliquoted as well. Lunt tissue samples were collected at each time point. All samples were frozen at −20 °C until analysis.

Animal experiments were approved by the GSK Research Center Zagreb Institutional Committee for Animal Research and conducted in accordance with GSK's policy and International and Croatian rules and regulations on Animal Welfare.

5.2.6.1. Sample preparation and bioanalysis. All samples were extracted by protein precipitation and analysed by LC–MS/MS. Standard solutions were prepared on the day of analysis from an intial stock solution prepared in DMSO (1 mg/mL). Calibarations curves were prepared by spiking blank matrix with standard stolutions in the approprate matrix (blood, plasma or lung homogenate). Samples were and calibration curve standards were extracted by addition of six volumes of mixture of acetonitrile/methanol (2:1) containing internal standard (roxithromycin, 0.5 ng/mL). Samples and standards were mixed and centrifuged at 4000 rpm for 15 minutes and superantants were transferred to a 96-well plate for LC–MS/MS analysis. Lung homogenates were obtained from by homogenizing tissue in analytical grade water, in a 1:5 ratio and aliquots of homogenates (100 μL) are processed in the same manner as blood.

Compound **45** was analysed using Turbo IonSpray ionization on an Applied Biosystems API4000 triple quadrupole mass spectrometer coupled to an Agilent 1200 HPLC system and CTC autosampler. Muliptle reaction monitoring was use in positive ion mode with a transition of 1136.8→979 for compound X and 837.6→158 for the internal standard. Chromatographic separation was achieved on a Supelco Ascentis Express C18, 30 × 2.1 mm (2.7 μm) column with a flow of 0.7–0.8 mL/min and a total run time of 1.5 minutes. Gradient conditions with a mobile phase composed of acetonitrile with 1% formic acid and 0.1% formic aci din water were used. Standard curve rages of 0.5–2500 ng/mL were used, with lower limits of quantifications of 0.5 ng/mL for blood and plasma and 2.5 ng/mL for lung tissue.

5.2.7. Ovalbumin (OVA) induced eosinophilia in Brown-Norway (BN) rats

This study was performed in RCC, Switzerland, study number 848029.

Animals: experiment was performed on rats, Brown-Norway Orllco, SPF quality.

Accomodation*:* groups of five in Makrolon-type 4 cages with wire mesh tops and softwood bedding (Lignocel, Schill AG, 4132 Muttenz/ Switzerland).

Diet*:* Pelleted standard Provimi Kliba 3433 rat and mouse maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst/ Switzerland), Batch No. 24/03 *ad libitum.*

Conditions*:* Standard Laboratory Conditions. Air-conditioned with 10–15 air changes per hour, and continuously monitored environment with ranges for temperature 22 ± 3 °C and relative humidity 30–70%. There was a 12 h fluorescent light/12 hours dark cycle with music during the light period.

Experimental procedures*:* Forty eight hours after the inhalation of an allergen (OVA), a significant increase in the number of inflammatory cells was induced in the airways of sensitized BN rats. The sensitization procedure includes one initial injection of OVA on test day 1, followed by two similar injections (boosts) performed on test days 14 and 20, respectively. On day 27 animals were exposed to compound **45** (32 mg/kg), control item (air) or the reference item BDP (11 mg/kg). One hour after the end of the treatment on day 27 animals were challenged with OVA by inhalation for 60 minutes. The accumulation of eosinophils in the airways was measured approximately 48 h after the OVA challenge (day 29), by differentiation of cells collected by bronchoalveolar lavage. Results from the treated groups were compared to those of a 'positive control' group which was sensitised and challenged but not treated. A 'negative control' group, which was sensitised but neither treated nor challenged, was also included to determine the amplitude of the inflammatory cell response in the positive control animals.

5.2.8. In vitro antibacterial activity assays

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to guidelines of the Clinical Laboratory Standards Institute,[29] except that for *Streptococcus* medium, lysed blood was substituted with 5% horse serum. Double dilutions of tested compounds in 96-well microtitre plates were prepared using TECAN Genesis 150. Bacteria were grown on appropriate agar plates (by Becton Dickinson, USA)—Columbia agar with 5% sheep blood for Streptococci, Mueller–Hinton chocholate agar for *H. influenzae* and Mueller–Hinton agar for Staphylococci.

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