EXPERIMENTAL STUDY

Transactivation via the human glucocorticoid and mineralocorticoid receptor by therapeutically used steroids in CV-1 cells: a comparison of their glucocorticoid and mineralocorticoid properties

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Abstract

Background: Glucocorticoids (GCs) are commonly used for long-term medication in immunosuppressive and anti-inflammatory therapy. However, the data describing gluco- and mineralo-corticoid (MC) properties of widely applied synthetic GCs are often based on diverse clinical observations and on a variety of *in vitro* tests under various conditions, which makes a quantitative comparison questionable.

Method: We compared MC and GC properties of different steroids, often used in clinical practice, in the same *in vitro* test system (luciferase transactivation assay in CV-1 cells transfected with either hMR or hGRα expression vectors) complemented by a system to test the steroid binding affinities at the hMR (protein expression in T7-coupled rabbit reticulocyte lysate).

Results and Conclusions: While the potency of a GC is increased by an 11-hydroxy group, both its potency and its selectivity are increased by the Δ1-dehydro-configuration and a hydrophobic residue in position 16 (16-methylene, 16α-methyl or 16β-methyl group). Almost ideal GCs in terms of missing MC effects, as defined by our *in vitro* assay, are therefore prednylidene, budesonide, beclomethasone and betamethasone.

The MC potency of a steroid is increased by a 9α- or a 6α-fluoro substituent. A hydrophilic substituent in position 16 (like 16-hydroxylation in triamcinolone) decreases both MC and GC properties. As no substituent that leads to an isolated reduction of GC activity could be characterized in our experiments, 9α-fluorocortisol, the most frequently used steroid for MC substitution, seems to be the best choice of available steroids for this purpose.

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Introduction

Glucocorticoids (GCs) are potent suppressors of the immune response, which makes them frequently used agents in long-term anti-inflammatory therapy (1–3). Besides their GC properties, all of these steroids possess mineralocorticoid (MC) properties causing unwanted side effects such as fluid-electrolyte imbalance and hypertension (4). This is most likely due to the close structural relationship of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (5) and their overlapping signalling pathways. Both receptors belong to the steroid receptor superfamily, a subgroup of the nuclear receptors. The first one also includes the androgen, progesterone and estrogen receptor, the latter the receptors for thyroid hormone, vitamin D and retinoic acids, and a large group of orphan receptors (5–7). The physiological human ligands of the MR and the GR are aldosterone and cortisol respectively. In the absence of ligands, the MR and the GR are associated with a heteromeric complex containing hsp70, hsp90 and other chaperone molecules (8–10). After ligand binding, a conformational change and partial dissociation of the complex occur, followed by nuclear translocation (11, 12). Within the nucleus, the ligand-activated receptors regulate transcription via three major pathways. The one described best, transactivation, requires receptor dimerization and binding to *cis*-activating palindromic glucocorticoid response elements (GREs) located in the promoter region of target genes (13, 14). So far no response elements specific for the mineralocorticoid receptor have been described. Another way of gene regulation is the binding to negative glucocorticoid response elements (nGREs),

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leading to transrepression (15). The third one is transrepression by inactivation of transcription factors such as AP-1, NF-κB and NF-AT, which seems to be the main mechanism in the anti-inflammatory action of natural and synthetic glucocorticoids (1, 16, 17). Additional extragenomic effects have been described both for mineralo- and gluco-corticoids (18–20).

The scientific origin of many data describing the agonistic properties of widespread synthetic GCs is elusive. As they are based on several *in vivo* and *in vitro* tests under varying conditions and with different parameters measured (4, 21–28), it is almost impossible to compare them in a reliable way. Our aim was to evaluate the glucocorticoid potencies of different therapeutically used steroids systematically by employing a human GR (hGR)-dependent transactivation assay in CV-1 cells. In a second step, we intended to compare their mineralocorticoid potencies in an equivalent assay differing from the first one merely by the presence of the human MR (hMR) instead of the hGR. To complement the latter transactivation data, we investigated their prerequisite, the steroid binding to the hMR. The intention of this approach was to facilitate the optimizing of long-term steroid therapies and the prediction of GC and MC properties of future synthetic steroids.

Materials and methods

Steroids

The steroids were puchased from Bristol Myers Squibb GmbH (Regensburg, Germany), Glaxo Research and Development (Stevenage, UK), Merck, MMDRI-Lepetit Research Center (Gerenzano, Italy), Paesel and Lorei (Hannover, Germany), Schering (Berlin, Germany), Sigma Chemical (St Louis, MO, USA) (Table 1). For a better understanding of the formulas, see Fig. 1.

Plasmids

pRShMR and pRShGR were gifts from Prof. R Evans (The Salk Institute, La Jolla, CA, USA). The plasmids contain the coding sequence for the hMR and the hGRα respectively, under the control of the Rous sarcoma virus long terminal repeat and the SV40 origin of replication. The structure of the pRShGR is identical to that of the pRShMR with the exception of the part containing the hMR coding nucleotides which has been replaced by the coding sequence of the hGRα (5, 29).

pMMTV-Luc was kindly provided by Dr B Gellersen (Hamburg, Germany). It contains the cDNA for firefly luciferase, which catalyses a light emitting reaction on addition of the proper substrate. Transcription is controlled by glucocorticoid responsive elements present in the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (30) immediately upstream from the luciferase sequence (31).

The pRL-SV40 vector (Promega, Madison, WI, USA) causes constitutive expression of Renilla luciferase and does not require post-translational modification for activity (32). It served as an internal standard to normalize firefly luciferase light emission measurements with regard to transfection efficiency and the number of cells in each well.

Cell culture, transfection and transactivation assay

The transfections and transactivation assays were carried out in CV-1 cells (african green monkey kidney

Table 1 Steroids.

Substance	Formula	Source
Dehydroepiandrosterone	5-androstene-3β-ol-17-one	Sigma
Progesterone	4-pregnene-3,20-dione	Sigma
Aldosterone	4-pregnene-18-al-11β,21-diol-3,20-dione	Sigma
Cortisone	4-pregnene-17β,21-diol-3,11,20-trione	Sigma
Cortisol	4-pregnene-11β,17α,21-triol-3,20-dione	Sigma
6α-fluorocortisol	4-pregnene-6α-fluoro-11β,17α,21-triol-3,20-dione	Schering
9α-fluorocortisol	4-pregnene-9α-fluoro-11β,17α,21-triol-3,20-dione	Merck
Prednisone	1,4-pregnadiene-17α,21-diol-3,11,20-trione	Sigma
Prednisolone	1,4-pregnadiene-11β,17α,21-triol-3,20-dione	Sigma
6α-methylprednisolone	1,4-pregnadiene-6α-methyl-11β,17α,21-triol-3,20-dione	Schering
Prednylidene	1,4-pregnadiene-16-methylen-11β,17,21-triol-3,20-dione	Merck
Budesonide	1,4-pregnadiene-16,17-butylidenbis(oxy)-11β,21-diol-3,20-dione	Sigma
Deacetyldeflacacort	1,4-pregnadiene-16,17-methyloxazoline-11β,21-diol-3,20-dione	MMDRI
Deflazacort	1,4-pregnadiene-16,17-methyloxazoline-11β,21-diol-3,20-dione 21-acetate	MMDRI
Isoflupredone	1,4-pregnadiene-9α-fluoro-11β,17α,21-triol-3,20-dione	Paesel
Dexamethasone	1,4-pregnadiene-9α-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione	Sigma
Betamethasone	1,4-pregnadiene-9α-fluoro-16β-methyl-11β,17α,21-triol-3,20-dione	Glaxo
Triamcinolone	1,4-pregnadiene-9α-fluoro-11β,16α,17α,21-tetrahydroxy-3,20-dione	Squibb
Beclomethasone	1,4-pregnadiene-9α-chloro-16β-methyl-11β,17α,21-triol-3,20-dione	Sigma
Flumethasone	1,4-pregnadiene-6α,9α-difluoro-16α-methyl-11β,17α,21-triol-3,20-dione	Sigma

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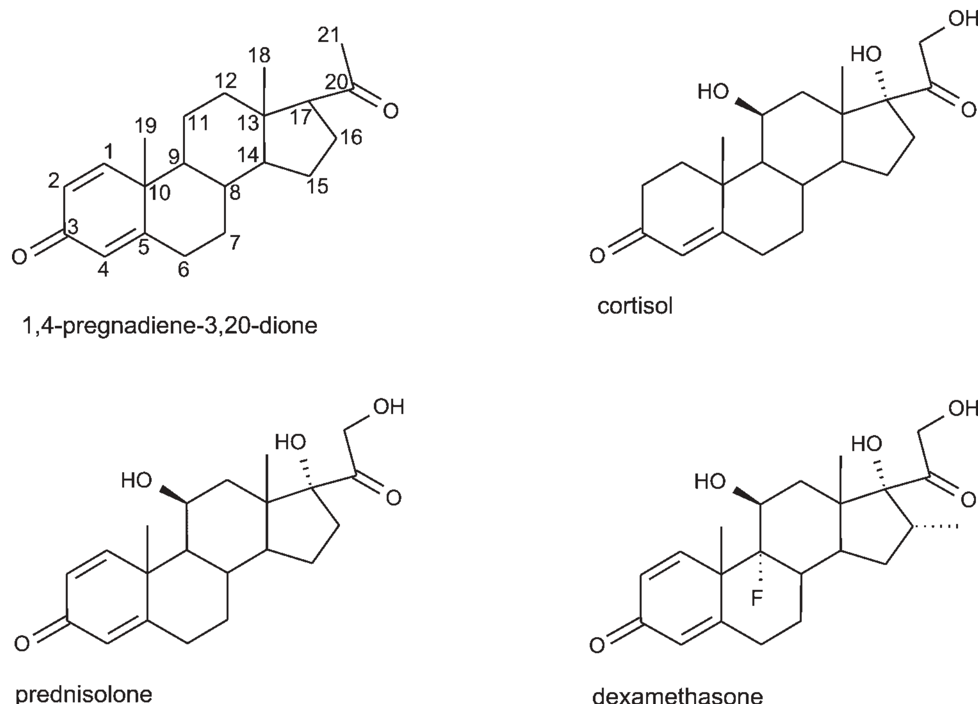

Figure 1 Structural formulas for 1,4-pregnadiene-3,20-dione, cortisol (4-pregnene-11β,17α,21-triol-3,20-dione), prednisolone (1,4-pregnadiene-11β,17α,21-triol-3,20-dione) and dexamethasone (1,4-pregnadiene-9α-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione).

cells; American Type Culture Collection) in 24-well plates as described previously (33). The 80–90% confluent cells (approximately 50 000 per well) were transiently transfected with a cationic lipid reagent (Lipofectamine Plus Method, Life Technologies, Karlsruhe, Germany). In each well, 0.02 μg of pRL-SV40 and 0.15 μg of pMMTV-Luc were co-transfected together with either 0.3 μg of pRShMR or pRShGR. After 24 h of incubation, different steroids were added in charcoal stripped fetal calf serum in concentrations ranging from 10^{-10} to 10^{-5} M for the GR and from 10^{-11} to 10^{-6} M for the MR. The CV-1 cells were lysed 24 h later in passive lysis buffer from the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The activity of constitutively expressed Renilla luciferase and hormone-dependently expressed firefly luciferase were measured in a Berthold LB 9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). The transactivational response was determined as the ratio of firefly to Renilla luciferase activity normalized to the maximum activity of reference substances: dexamethasone for GR- and aldosterone for MR-mediated transactivation. The resulting graphs start at a baseline effect and asymptotically approach individual maximal effects (Fig. 2). To characterize these curves, we calculated the concentration (EC_{50}) at which 50% of the asymptotical maximum effect (E_{max}) was achieved by iterative nonlinear regression using the Prism software (Version 3.02) from GraphPad (San Diego, CA, USA). Differences in EC_{50} values were tested for significance with the same software by means of a modified two-tailed *t*-test.

Coupled cell-free transcription and translation of hMR

Expression of hMR from the plasmid pchMR (kindly provided by Prof. M Rafestin-Oblin, Institut National de la Sante et de la Recherche Medicale, Paris, France) containing the hMR cDNA (34) was performed in a coupled rabbit reticulocyte lysate system for transcription and translation (TNT[R] from Promega, Madison, WI, USA). T7 RNA polymerase was used for synthesis of hMR mRNA which was translated to hMR during incubation of 90 min at 30 °C according to the manufacturer's instruction.

Steroid-binding competition at the hMR

H-aldosterone (5 nM) was incubated in the absence or presence of unlabelled aldosterone (5 μM) for determination of specific and nonspecific ligand binding. Different unlabelled steroids were tested for competition at the hMR in increasing concentrations (10^{-11} to 10^{-6} M) as duplicates. The incubation volume was 25 μl, and steroids were dissolved in 12.5 μl of TEGDW buffer (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate and 10% glycerol) mixed with 0.2% ethanol. 12.5 μl aliquots of reticulocyte lysate containing synthesized hMR were added to

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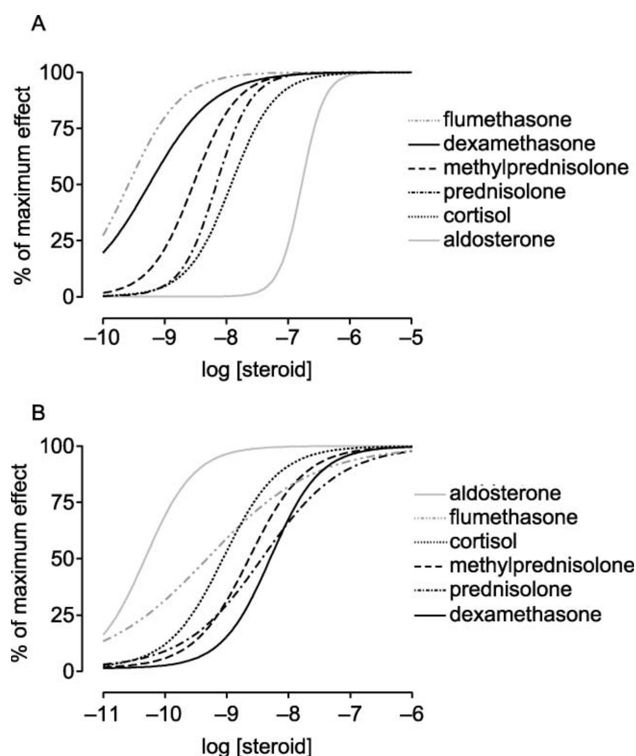

Figure 2 Nonlinear regression curves for the concentration-response-relationship of transactivation by six representative steroids in CV-1 cells. To facilitate the comparison of EC_{50} values, the response is shown as percentage of the maximum effect which differs for each steroid-receptor combination and is given in Tables 2 and 3. (A) Transactivation via hGRα at steroid concentrations from 10^{-10} to 10^{-5} M. (B) Transactivation via hMR at steroid concentrations from 10^{-11} to 10^{-6} M.

ice-cold steroid solution in TEGDW and incubated for 4 h at 4 °C for steroid binding. Unbound steroids were separated using dextrane-coated charcoal (DCC): 1 ml of 0.5% DCC in TEGDW was added to each 25 μl binding assay mixture, shaken for 7 min on ice and centrifuged at 14 000 ***g*** for 10 min at 4 °C. Aliquots of 780 μl from the supernatant were added to 10 ml of Ultima Gold solution (Packard Bioscience, Groningen, Netherlands) and analyzed in a β-counter (Winspectral, 1414 PerkinElmer, Turku, Finland). D.p.m. values were calculated online using an external standard. The affinities of the steroids to the hMR were calculated as IC_{50} values by nonlinear regression with Prism (see above) assuming one site competition.

Results

To ensure that our CV-1 cell system does not contain relevant amounts of endogenous GR or MR, the transactivation activities of the two reference substances, dexamethasone and aldosterone, were measured in CV-1-cells that had not been transfected with steroid receptor expression vectors. Likewise, the system was tested for endogenous steroids by measuring the transactivation activity without prior addition of steroids but after transfection with hMR or hGR expression vectors. Neither approach led to relevant transactivation. In assays with receptor transfection, aldosterone induced half-maximal transactivation via the MR at concentrations about one order of magnitude below the EC_{50} observed with dexamethasone and the GR (Fig. 2).

All GC potencies calculated by our *in vitro* transactivation assay are listed in Table 2. Based on these results, the main structural features determining GC potency are the size and the polarity of the substituent in position 6 or 16 (Table 4). A hydrophobic residue increases GC activity of a steroid (statistically significant enhancement with 6α-methyl and 16-methylene substitution, tendency not reaching significance with 16α- and 16β-methyl and 16,17-methyloxazoline groups). The more polar 16-hydroxy substitution decreases GC potency.

The other important position for GC activity is the 11-hydroxy group, present in the glucocorticoids tested but not in aldosterone (hemiacetal form), which consequently possesses a low GC potency. The 6α- and 9α-fluorination leads to increased GC transactivation, which also applies to the Δ1-dehydro-configuration (in prednisolone).

6α- or 9α- fluorination and the keto configuration of the 11-hydroxy group affect the MC potencies (Table 3) qualitatively in the same way as the GC potencies (Table 4). However, opposite to the effect observed with the GR, the Δ1-dehydro-configuration and the 16-methylene, 16α-methyl and 16β-methyl groups attenuate MC potency.

In binding competition experiments with the hMR (Fig. 3), we found displacement of the ligand 3H-aldosterone decreasing in strength from 9α-fluorocortisol, 6α-fluorocortisol and cortisol to aldosterone, 6α-methylprednisolone and prednisolone (nearly the same displacement by the last three steroids), followed by dexamethasone, prednylidene, oxo-dexamethasone and cortisone. Thus, the MR affinity parallels the strength of MC agonism in our transactivation assay, with the exception of a discrepancy between the relatively weak binding of aldosterone (e.g. compared with cortisol) and its high transactivational potency.

Discussion

The traditional ways to measure GC and MC potencies of steroids refer to anti-inflammatory (rat ear edema test, McKenzie vasoconstriction test) or metabolic (liver glycogen assay) effects and sodium retention. Depending on the testing conditions, absolute potency values vary considerably. It is therefore extremely difficult, if not impossible, to arrange the individual results from different research groups to compile a comprehensive list (4, 21–28). Considering these very complex

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Table 2 Transactivation via the human glucocorticoid receptor (hGR). EC_{50} values and E_{max} of steroids tested in CV-1 cells cotransfected with pRShGR, pMMTV-Luc and pRL-SV40. The substances are arranged from top to bottom from highest to lowest glucocorticoid (GC) potency. The EC_{50} values of cortisone, dehydroepiandrosterone, prednisone and progesterone could not be calculated because no effect saturation was reached under the given experimental conditions (n.c. = not calculable). For the relative GC potencies, cortisol was chosen as the standard substance. GC potencies in literature are taken from references (4, 21–28) n.d.: no data found.

Steroid	E_{max}±SE	EC_{50} [M]	GC potency	GC potency in literature
Budesonide	0.95±0.03	4.57×10^{-11}	263	250
Prednylidene	1.37±0.03	6.61×10^{-11}	182	4
Beclomethasone	0.98±0.04	1.91×10^{-10}	63	20
Flumethasone	1.57±0.07	2.60×10^{-10}	46	n.d.
Betamethasone	0.95±0.02	2.64×10^{-10}	45	25
Dexamethasone	1.00±0.02	5.61×10^{-10}	21	25
Isoflupredone	1.24±0.03	8.77×10^{-10}	14	20
9α-fluorocortisol	0.90±0.02	1.90×10^{-9}	6.3	10
6α-fluorocortisol	0.90±0.02	2.63×10^{-9}	4.6	n.d.
6α-methylprednisolone	1.13±0.03	2.92×10^{-9}	4.1	5
Desacetyldeflazacort	1.28±0.03	4.37×10^{-9}	2.7	4
Prednisolone	0.78±0.02	6.90×10^{-9}	1.7	4
Cortisol	1.13±0.03	1.20×10^{-8}	1	1
Deflazacort	1.02±0.04	1.25×10^{-8}	0.96	4
Triamcinolone	0.78±0.02	3.42×10^{-8}	0.35	4
Aldosterone	1.11±0.03	1.66×10^{-7}	0.07	0–0.1
Cortisone	n.c.	n.c.	n.c.	0.8
Dehydroepiandrosterone	n.c.	n.c.	n.c.	0
Prednisone	n.c.	n.c.	n.c.	4
Progesterone	n.c.	n.c.	n.c.	0–0.3

in vivo actions, we tried to focus on a level that allows the comparison of several steroids *in vitro*. However, even though the results from our transactivation assays with the GR and MR were surprisingly congruent with GC and MC potency lists contained in most standard textbooks of medicine and pharmacology (see Tables 2 and 3 for comparison of relative GC and MC potencies), it is obvious that conclusions with respect to the use of GCs in humans must be drawn with caution.

Table 3 Transactivation via the human mineralocorticoid receptor (hMR). EC_{50} values and E_{max} of steroids tested in CV-1 cells cotransfected with pRShMR, pMMTV-Luc and pRL-SV40. The substances are arranged from top to bottom from highest to lowest mineralocorticoid (MC) potency. The EC_{50} values of prednisone, cortisone, deflazacort and dehydroepiandrosteorne could not be calculated because no effect saturation was reached under the given experimental conditions (n.c. = not calculable). For the relative MC potencies, aldosterone was chosen as the standard substance. MC potencies in literature are taken from references (4, 21–28) (n.d.: no data found).

Steroid	E_{max}±SE	EC_{50} [M]	MC potency	MC potency in literature
9α-fluorocortisol	1.814±0.22	4.78×10^{-12}	10	1
Isoflupredone	1.513±0.47	7.00×10^{-12}	7	n.d.
6α-fluorocortisol	1.794±0.12	4.14×10^{-11}	1.2	n.d.
Aldosterone	1.00±0.02	4.80×10^{-11}	1.0	1
Flumethasone	1.669±0.07	4.94×10^{-10}	0.097	n.d.
Cortisol	1.020±0.04	8.95×10^{-10}	0.054	0.0025
6α-methylprednisolone	1.845±0.05	2.31×10^{-9}	0.021	0.0013
Prednisolone	1.456±0.05	3.78×10^{-9}	0.013	0.002
Dexamethasone	1.502±0.06	5.09×10^{-9}	0.0094	0
Budesonide	0.822±0.04	7.62×10^{-9}	0.0063	n.d.
Progesterone	0.239±0.01	9.02×10^{-9}	0.0053	0
Desacetyldeflazacort	0.497±0.02	1.02×10^{-8}	0.0047	n.d.
Betamethasone	0.902±0.02	1.26×10^{-8}	0.0038	0
Beclomethasone	0.520±0.02	4.11×10^{-8}	0.0012	n.d.
Prednylidene	0.772±0.02	4.27×10^{-8}	0.0011	0
Triamcinolone	0.458±0.05	2.91×10^{-7}	0.0002	0
Cortisone	n.c.	n.c.	n.c.	0.002
Deflazacort	n.c.	n.c.	n.c.	n.d.
Dehydroepiandrosterone	n.c.	n.c.	n.c.	n.d.
Prednisone	n.c.	n.c.	n.c.	0.002

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Table 4 Different functional groups and their effects on the glucocorticoid (GC) and mineralocorticoid (MC) activity of a steroid ↑ Enhancement or ↓ attenuation of the glucocorticoid (upper part) or mineralocorticoid activity by functional groups (left column). Compared are compounds without the corresponding group (column second from the right) with those with the group (right column). Differences are significant ($p < 0.05$), except for (↑) or (↓) which show trends ($p > 0.05$).

GC activity				MC activity			
Functional group	Effect	Examples		Functional group	Effect	Examples	
11-keto	↓	Cortisol	Cortisone	11-keto	↓	Cortisol	Cortisone
	↓	Prednisolone	Prednisone		↓	Prednisolone	Prednisone
9α-fluoro	↑	Cortisol	9α-fluorocortisol	9α-fluoro	↑	Cortisol	9α-fluorocortisol
	↑	Prednisolone	Isoflupredone	6α-fluoro	↑	Cortisol	
					↑	Dexamethasone	6α-fluorocortisol Flumethasone
6α-fluoro	↑	Cortisol	6α-fluorocortisol	Δ1-dehydro	↓	Cortisol	Prednisolone
	↑	Dexamethasone	Flumethasone		(↓)	9α-fluorocortisol	Isoflupredone
Δ1-dehydro	↑	Cortisol	Prednisolone	6α-methyl	(↑)	Prednisolone	6α-methylprednisolone
	(↑)	9α-fluorocortisol	Isoflupredone	16α-methyl	↓	Isoflupredone	Dexamethasone
6α-methyl	↑	Prednisolone	6α-methylprednisolone	16β-methyl	↓	Isoflupredone	Betamethasone
16α-methyl	(↑)	Isoflupredone	Dexamethasone	16-methylene	↓	Prednisolone	Prednylidene
16β-methyl	(↑)	Isoflupredone	Betamethasone	21-acetyl	↓	Desacetyldeflazacort	Deflazacort
16-methylene	↑	Prednisolone	Prednylidene	16,17-methyl-oxazoline	↓	Prednisolone	Desacetyldeflazacort
16-hydroxy	↓	Isoflupredone	Triamcinolone				
21-acetyl	↓	Desacetyldeflazacort	Deflazacort				
16,17-methyl-oxazoline	(↑)	Prednisolone	Desacetyldeflazacort				

The GC potency of dexamethasone *in vivo* is reported to be slightly higher than (24, 35) or equal to (21, 23) that of betamethasone. We observed a moderately higher transactivation activity of betamethasone compared with dexamethasone. 9α-fluorocortisol, 6α-methylprednisolone, desacetyldeflazacort and prednisolone all showed similar EC_{50} values of approximately 2 to 7×10^{-9} M in our GR assay, in an order that corresponds well to their *in vivo* potencies (4). The comparison of the *in vitro* activities of deflazacort and desacetyldeflazacort with their *in vivo* GC potencies illustrates the activation of the prodrug deflazacort to desacetyldeflazacort *in vivo*.

Aldosterone, the physiological MC, is often described as possessing no GC activity at all. However, we found GR-mediated transactivation by aldosterone, but at concentrations relative to cortisol that are not even reached in primary hyperaldosteronism (36, 37).

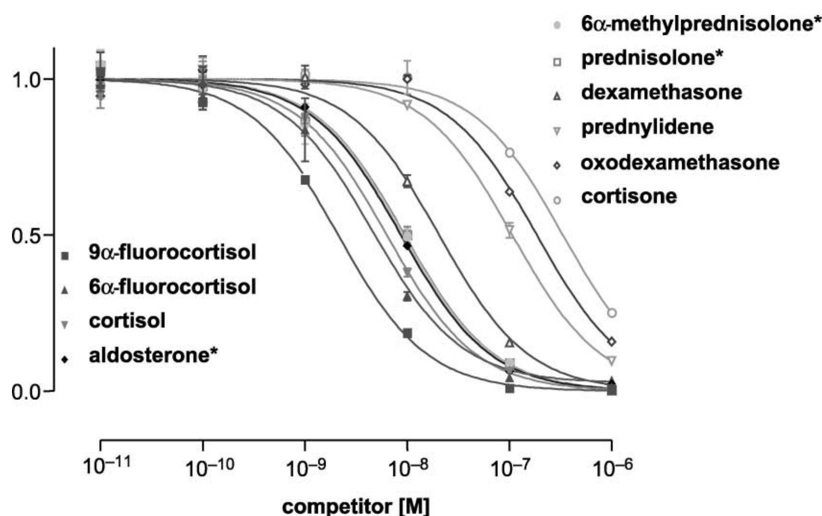

Figure 3 Displacement of 3H-aldosterone from the hMR. Calculated as the ratio of binding in the presence of competitor (B) to maximal binding of 3H-aldosterone (B_{max}) in the absence of competitors. Nonlinear regression curves for one site competition (duplicates). *The curves for aldosterone, 6α-methylprednisolone and prednisolone are almost identical.

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The low receptor binding of cortisone and prednisone is well known (26, 35). The biological activity of these substances depends on first pass activation by hepatic 11β-hydroxysteroid-dehydrogenase type 1 (11β-HSD1). When cortisone (or prednisone) is injected in an organ without 11β-HSD1, e.g. intra-articularly, it has no biological activity. This fits well with our results for cortisone and prednisone, which also prove that our assay does not contain relevant amounts of 11β-HSD1. Progesterone was previously shown to bind to the GR (26, 35) but is said to exhibit only slight agonistic activity (38) which was not detected with our assay.

The most noticeable incongruence is the high GC transactivation activity for prednylidene, given an *in vivo* potency comparable to that of prednisolone. As pharmacokinetics of prednylidene do not explain this discrepancy (39), prednylidene is a good candidate for further pharmacological investigations, e.g. concerning the relation between its transrepression and transactivation activity (17, 40, 41). The weak transactivation by triamcinolone corresponds to its relatively low receptor affinity (24), but contrasts with its high *in vivo* potency.

In summary, the influence of a 6α- and 16α-methyl substitution, 9α-fluorination, 16α-hydroxylation and the Δ1-dehydro-configuration on transactivation by synthetic GCs via the GR (Table 4) show good correspondence to *in vivo* data (4). In addition, 6α-fluorination, 16-methylene and 16,17-methyloxazoline groups increase GC potency *in vitro*.

Published comparative data on MC potencies are more scanty. Besides transactivation, MC activity in target tissues depends on prereceptor metabolism by 11β-hydroxysteroid-dehydrogenase type 2 (11β-HSD2), which is co-localized with the MR and inactivates 11-hydroxysteroids to their corresponding 11-oxo-derivatives (39). The very poor MR-mediated transactivation (Table 3) of cortisone is a well-known necessity for this gatekeeper effect of the 11β-HSD2, which is also true for prednisone.

The 6α-methyl group has been reported to diminish MC activity *in vivo* (4, 23). We could not find such an effect *in vitro*, neither in transactivation (Tables 3 and 4) nor in binding experiments (Fig. 3). Therefore the reduced *in vivo* MC activity of 6α-methylprednisolone compared with prednisolone may primarily be due to its enhanced GC activity (Tables 2 and 4) resulting in different equivalent dosages – 4 mg of 6α-methylprednisolone versus 5 mg of prednisolone – used for pharmacological GC therapy (4, 21, 23, 25).

The prodrug deflazacort exhibits a very low MC activity. At first sight, this seems to support the opinion that deflazacort causes less MC side effects than some of the older steroids. However, as Assandri *et al.* could show (42), deflazacort is rapidly metabolized to desacetyldeflazacort which does possess some MC activity in our assay.

Complementary to the transactivation assays, binding to the MR was analyzed (see Fig. 3 and Table 5). For most steroids tested, the binding affinity was compatible with the transactivation activity measured (Table 3). One remarkable discrepancy is the MR binding affinity of cortisol being very close to that of aldosterone, while cortisol proved to be the weaker mineralocorticoid in terms of transactivation. Similar binding of aldosterone and cortisol to the hMR, but more potent transactivation via the MR by aldosterone, was described by Rupprecht *et al.* (38) and Hellal-Lévy *et al.* (43). The latter group showed that although the affinities for the MR are nearly the same, aldosterone dissociates much slower than cortisol from this receptor, and they assumed different induction of conformation changes of the MR by these ligands. Similarly, almost identical MR binding of aldosterone, 6α-methylprednisolone and prednisolone contrasts with our transactivation experiments (Table 3) and *in vivo* data (4). In general, one can conclude that receptor binding is only a prerequisite of the much more complex process of transactivation, and that a correlation between binding affinities and transactivation properties cannot be assumed *a priori* for all steroids. A good example for this notion is progesterone (Table 3), which has been shown to possess high affinity to the hMR but causes only minor transactivation at the MR (33, 38) and acts as an antagonist *in vivo* (44).

The more *selective* GC transactivation activity of GCs with a 16α-methyl or 16β-methyl group and a Δ1-dehydro-configuration results from a significantly decreased activity via the MR and an enhanced activity via the GR (Table 4). The Δ1-dehydro-configuration in prednisolone both decreases MC transactivation and increases oxidation by 11β-HSD2 (39). Fluorination leads to enhanced transactivation (Tables 3 and 4) and attenuated oxidation by 11β-HSD2 (39). Therefore, the reduced MC activity *in vivo* of prednisolone compared with cortisol and the increased activity of fluorinated steroids is probably due both to pharmacokinetic (prereceptor metabolism) and pharmacodynamic (MC transactivation) reasons.

Table 5 Displacement of 3H-aldosterone from human mineralocorticoid receptor (hMR) (see Fig. 3). Given are the concentrations IC_{50} of the competitors that displace 50% of 3H-aldosterone.

Steroid	IC_{50} [M]
9α-fluorocortisol	2.12×10^{-9}
6α-fluorocortisol	4.60×10^{-9}
Cortisol	6.34×10^{-9}
Aldosterone	8.48×10^{-9}
6α-methylprednisolone	9.22×10^{-9}
Prednisolone	9.48×10^{-9}
Dexamethasone	2.06×10^{-8}
Prednylidene	1.07×10^{-7}
Oxodexamethasone	1.86×10^{-7}
Cortisone	3.35×10^{-7}

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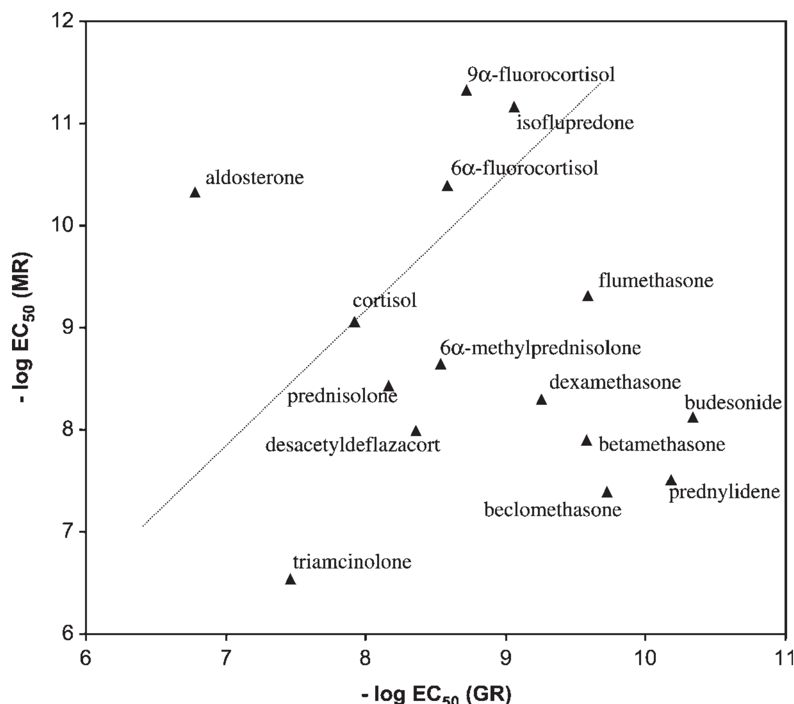

Figure 4 Selectivity of tested steroids. The GC potency increases from left to right and the MC potency increases from bottom to top. The diagonal line seperates typical gluco- from mineralo-corticoids. Selectivity increases with the perpendicular distance from that line: to the bottom right for glucocorticoids, to the top left for mineralocorticoids.

Because some catalytic activity of 11β-HSD2 has been demonstrated in CV-1 cells (45), an effect of this enzyme in our transactivation assay leading to a shift of EC_{50} values in either direction cannot be ruled out completely. This may contribute to the weaker effect of cortisol relative to aldosterone in the MR transactivation assay. However, because the 11β-HSD2 activity in the CV-1 cells affects the ligand concentration regardless of a transfection with vectors for GR or MR, the ratio of GC to MC potency in our assay is less sensitive to prereceptor metabolism. Therefore this ratio may be a useful parameter for receptor-mediated selectivity. This is illustrated in Fig. 4: highly selective GCs will be found in the lower right quadrant, highly selective MCs in the upper left quadrant. According to the EC_{50} ratio, the most selective glucocorticoids are prednylidene, budesonide, beclomethasone and betamethasone. The most selective mineralocorticoid is the natural hormone, aldosterone.

It is important to keep in mind that the luciferase assay is an *in vitro* system which differs from *in vivo* conditions in many aspects, e.g. altered prereceptor regulation by steroid metabolizing enzymes such as 11β-HSDs (39, 46–48), lacking integration of the reporter gene into chromatine (11) and different concentrations of heat shock proteins, GR (49) and other transcription factors (8–10, 14). An important influence of the cell type used in the transactivation assay has been shown (50). Nongenomic effects postulated both for cortisol and aldosterone (19, 51) are neither taken into account. Moreover, transrepression of genes (1, 17, 40) could not be evaluated by our approach. Therefore, additional testing with transrepression assays is required to detect possible dissociative glucocorticoid effects.

In spite of all constraints, the *in vitro* assay employed represents a suitable system to compare the specificity of natural and synthetic steroidal homones with regard to the human glucocorticoid and mineralocorticoid receptors. Comparing the influence of further functional groups on transactivation via the hGR and the hMR will help to understand the structure (52, 53) and functionality of the substrate binding sites in these receptors.

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