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Correlation between Chemical Structure, Receptor Binding, and Biological Activity of Some Novel, Highly Active, 16α,17α-Acetal-Substituted Glucocorticoids

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SUMMARY

The affinity for the glucocorticoid receptor in rat skeletal muscle of some glucocorticoids with a new type of 16α,17α-acetal substituent has been estimated and correlated to the glucocorticoid activities in three *in vivo* systems in rats. Budesonide (an approximately 1:1 mixture of the C(22) epimers of 11β,21-dihydroxy-16α,17α-[(22R,S)-propylmethylenedioxy]-pregna-1,4-diene-3,20-dione) and the isolated (22R)- and (22S)-epimers bound to the same binding site as the potent glucocorticoids dexamethasone (DEX) or triamcinolone 16α,17α-acetonide (TA), but with even higher affinity than DEX or TA, despite the lack of a 9α-fluoro atom in budesonide and its epimers. The (22R)-epimer was twice as active as the (22S)-epimer, 4 times more active than TA, and 14 times more active than DEX. The introduction of a 9α-fluoro atom slightly decreased the binding affinity of the (22R)-epimer of budesonide, in contrast to the positive effect of 9α-fluorination of, e.g., 16α,17α-acetonides. The negative influence of 9α-fluorination of the (22R)-epimer was partially reversed in the 6α,9α-difluorinated (22R)-epimer. Nevertheless, the fluorinated compounds were more active than DEX and TA (8 and 11 times more active than DEX, and 2 and 3 times more active than TA, in case of the 9α-fluoro- and 6α,9α-difluoro-derivatives of the (22R)-epimer, respectively). Budesonide is metabolized mainly to 16α-hydroxyprednisolone (11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione) and 6β-hydroxy-budesonide. Both metabolites were very weak competitors for the ligand-binding sites on the receptor (3% and 6% of the affinity of DEX, respectively). The affinity for the receptor *in vitro* was closely correlated to the topical glucocorticoid activity *in vivo* for the 12 steroids compared ($r = 0.98$; $R = 0.98$), which supports the contention that *in vitro* tests for receptor affinity are useful when screening for agonists among steroids with the present type of structures. The results on receptor-ligand interaction are in accordance with X-ray crystallographic data available for some steroids.

INTRODUCTION

Impressive insight has been gained into the receptor mechanism of action of hormonal steroids (for a review of glucocorticoid receptors, see ref. 1). Considerable interest has been devoted to structure-activity relationships of steroids. Steroid receptor proteins have pronounced ligand structural requirements, as shown, for example, by the correlation between receptor binding and 3-dimensional steroid structure, revealed by X-ray crystallography (see refs. 2–6 for a review).

With the aim of developing a glucocorticoid with high activity at the site of application and less activity in other organs and tissues, a series of compounds with a new type of asymmetrical 16α,17α-acetal substituent (Type B in Fig. 1) has been synthesized (7). The present study was carried out to determine whether the observed high local (topical) activity of some compounds belonging to this series (7, 8) is due to a more efficient interaction of these steroids with the glucocorticoid receptor. Such a study could also provide insight into the details of ligand-receptor interaction.

Rat skeletal muscle contains a cytosolic glucocorticoid receptor, the ligand-binding site of which has the same structural specificity requirements as that of the hepatic glucocorticoid receptor, and the receptor in muscle also has many of the physicochemical properties as that in

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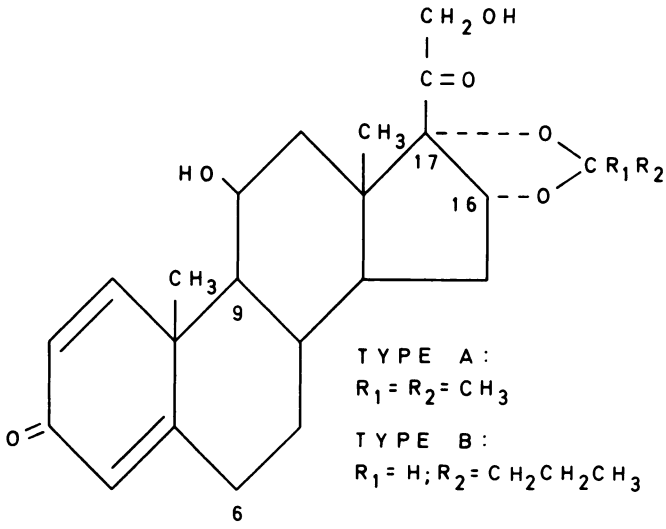

FIG. 1. *Structural formulae of some of the compounds in Table 1*
Note the types of 16α,17α-acetals referred to in the text as Type A and Type B. Two epimers exist of the asymmetrical Type B acetal, (22R)- and (22S)-epimers, respectively. Further details are given in footnote 4 and Table 1.

liver (9, 10). This receptor is subject to endocrine regulation (10), and is found also in murine (10),[3] porcine (11, 12), and human (13) skeletal muscle. Recent papers by Svec and Rudis (14) and by Manz *et al.* (15) also indicate that the ligand specificity of the glucocorticoid receptor does not vary much from tissue to tissue or from species to species. In marked contrast to the situation in rat hepatic cytosol, in which the glucocorticoid receptor is readily attacked by lysosomal proteinases (16), the receptor is quite stable in rat skeletal muscle cytosol (9). Lack of receptor stability is a potential source of error in the present types of experiments, and hence the muscle system should be advantageous for our purpose.

METHODS

Materials. [6,7-^{3}H]DEX[4] (specific activity 50.0 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.) and was purified by thin-layer chromatography to above 99% radiopurity just before use, since radioactive contaminants may cause serious disturbances in receptor research (17). Dr. J.-P. Raynaud, of Roussel-UCLAF (Romainville, France), kindly supplied RU 26988. Budesonide was synthesized, and resolved into its (22S)- and (22R)-epimers by liquid chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden), as described previously (7, 18, 19). The 9α-fluoro- and 6α,9α-difluoro-substituted (22R)-epimers of budesonide (S-1300 and S-1316, respectively) were prepared and their structures discussed as described elsewhere (18, 19). 6β-Hydroxy-budesonide was prepared using an unpublished procedure.[5] PA and TA were obtained from Lark, S.p.a. (Milano, Italy), and all other steroids were obtained from Sigma Chemical Company (St. Louis, Mo.). Insta-Gel was purchased from Packard Instrument Company (Downers Grove, Ill.). The water used was glass-distilled after deionization. All other chemicals were reagent-grade products from Merck A.G. (Darmstadt, West Germany) or Sigma Chemical Company.

Preparation of cytosol. Cytosol was prepared as the 105,000 × g_{av} supernatant after centrifugation of a 1:1 (w:v) homogenate of rat skeletal muscle tissue prepared as described previously (9, 10), using 8-week-old male Sprague-Dawley rats adrenalectomized 4–5 days prior to killing, since the level of unoccupied glucocorticoid receptor in muscle cytosol then increases (10). The anesthesia for adrenalectomy was induced by intramuscular injection of diazepam (Valium; Hoffmann-La Roche & Company AG, Basel, Switzerland; 2.5 mg/kg body weight) and fentanyl-fluanisone (Hypnorm-Vet; AB Leo, Helsingborg, Sweden; 0.2 ml/kg body weight; fentanyl 0.2 mg/ml and fluanisone 10 mg/ml). After operation, the anesthesia was partially reversed by i.m. injection of naloxone chloride (Nalonee; Endo Laboratories Inc., Garden City, N. Y.; 0.08 mg/kg body weight).

Ligand specificity studies. Portions of cytosol (0.2 ml) were incubated at 0° for 24 hr with 0.1-ml portions of [^{3}H]DEX (4.5 nM final concentration) in the presence and absence of competitors. This incubation period represents equilibrium incubation conditions for the binding of DEX to muscle cytosol (9). All RBAs were obtained using the same incubation conditions, which is important when comparing the activities of compounds, as the percentage of labeled steroid displaced by a competitor increases progressively with time in the situation when the competitor dissociates more slowly from the receptor than does the labeled ligand, and vice versa (20, 21). Longer incubation periods thus enhance the difference between efficient and inefficient competitors. The competitors were added to the [^{3}H]DEX (before the cytosol) in amounts that gave a 0.5- to a 128-fold molar "excess" over the labeled ligand (2-fold differences between close concentrations). The most active compounds were assayed also at lower concentrations (0.000976 to 4-fold "excess"). All competitors were added (0.010-ml portions) in ethanolic solution, and all incubations (including those in the absence of competitor) had the same ethanol concentration. Nonspecific binding was determined at the highest concentration of each competitor in the presence of an additional 1,000-fold excess of unlabeled DEX. All incubations were carried out in duplicate, started by the addition of cytosol, and terminated by the addition of a charcoal suspension to separate protein-bound from unbound steroid, as described previously (9, 10). Radioactivity was determined by liquid scintillation counting in a Wallac Rack-Beta counter (LKB, Bromma, Sweden), using the internal standard to sample channels ratio (IS-SCR) method for quench correction (22). The RBAs for the competitors were calculated from the concentrations of unlabeled DEX and competitor required to displace 50% of the [^{3}H]DEX from its binding site on the receptor. To this end, logit-log plots were used as described previously (9, 10).

In vivo glucocorticoid activities. Receptor binding per se does not demonstrate that a compound is an agonist. Thus, corticosteroids may bind avidly to the receptor and yet lack agonistic activity or be antagonists. We therefore investigated the in vivo effects of the new Type B acetal-containing compounds in two models for glucocorticoid activity. One of these determines the inhibition of an induced edema (topical anti-inflammatory activity) of the rat ear, as described elsewhere (8,

[3] L. Nyberg, E. Dahlberg, K. Lundström, P. Jonsson, and L.-E. Edqvist, submitted for publication.

[4] The abbreviations used are: DEX, dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione; fluanisone, 1-(4-fluorophenyl)-4-[4-(2-methoxyphenyl-19-1-piperazinyl]-1-butanone; RU 26988, 11β,17β-dihydroxy-17α-(1-propionyl)-androsta-1,4,6-trien-3-one; prednisolone, 11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione; F, cortisol, 11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione; B, corticosterone, 11β,21-dihydroxy-pregn-4-ene-3,20-dione; TA, triamcinolone 16α,17α-acetonide, 9α-fluoro-11β,21-dihydroxy-16α,17α-[(1-methylethylidene)bis(oxy)]-pregna-1,4-diene-3,20-dione; PA, prednacinolone 16α,17α-acetonide, 11β,21-dihydroxy-16α,17α-[(1-methylethylidene)bis(oxy)]-pregna-1,4-diene-3,20-dione; 16α, 16α-hydroxy-prednisolone, 11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione; budesonide, an approximately 1:1 mixture of the C(22) epimers of 11β,21-dihydroxy-16α,17α-[(22R,S)-propylmethylenedioxy]-pregna-1,4-diene-3,20-dione [denoted S-1322 (22R) and S-1321 (22S), respectively]; 6β, 6β-hydroxy-budesonide; S-1300, 9α-fluoro-11β,21-di-hydroxy-16α,17α-[(22R)-propylmethylenedioxy]-pregna-1,4-diene-3,20-dione; S-1316, 6α,9α-difluoro-11β,21-dihydroxy-16α,17α-[(22R)-propylmethylenedioxy]-pregna-1,4-diene-3,20-dione; ΔG, change in Gibbs' free energy; RBA, relative binding affinity.

[5] A. Thalén and L.-I. Wickström, manuscript in preparation.

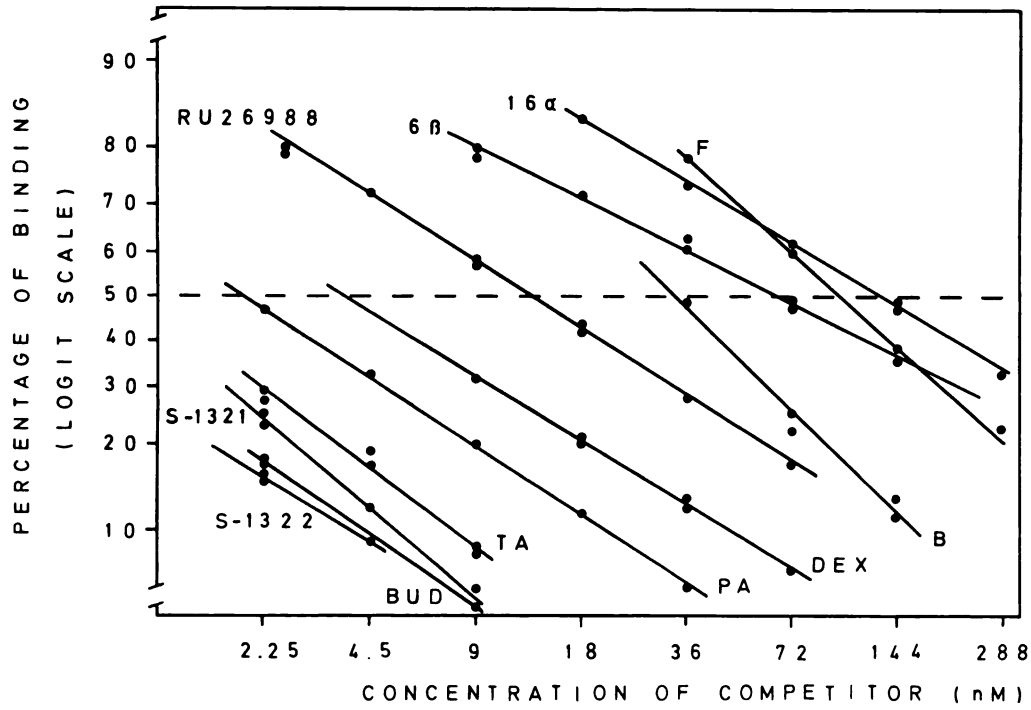

FIG. 2. *Logit-log plot of ligand specificity studies*

The competition studies were carried out as described under Methods using the logit-log plot for calculation of RBA values (Table 1). The concentration of the labeled ligand ([^{3}H]DEX) was 4.5 nM. Total binding in this experiment was 1.10 nM (SD 16 pM) and nonspecific binding was 113 pM (SD 11 pM). Where single points are shown, duplicates were within the diameter of ●. Linear regression was used to fit the lines to the data. The abbreviated names of the competitors are found along with RBAs in Table 1 (for details, see footnote 4).

23). Since glucocorticoids of the type analyzed are probably not biotransformed in skin (24), the potency in this model should depend mainly on the so-called "intrinsic activity," although percutaneous absorption may also affect the potency. In the other model, the compounds were given s.c. or p.o. to rats, and the extent of thymus involution, one of the most sensitive tests for systemic glucocorticoid activity in rats (8), was measured. Here, the potency should depend both on "intrinsic activity" and hepatic metabolism (24). In both models, affinity for receptors is certainly important as well, but it should play the same role in either model.

Statistical methods. Product-moment and Spearman's rank correlation coefficients were used as described by others (25).

RESULTS

All compounds tested showed some activity as competitors for the binding of DEX to the cytosolic glucocorticoid receptor in rat skeletal muscle (Figs. 2 and 3). The RBA values and the changes in Gibbs' free energies for the formation of steroid-receptor complexes (ΔG) are given together with pertinent data on the steroid structures in Table 1 (details in footnote 4). Gibbs' free energy changes for individual substituents are given in the text only.

Both cortisol (F) and corticosterone (B) (Fig. 2) were "weak" competitors for the DEX-binding sites; B, however, being more efficient than F (RBA values 0.11 and 0.04, respectively), in agreement with published data (e.g., ref. 26). The 17α-hydroxy group in F thus resulted in an unfavorable positive ΔG of 2.49 kJ/mole, in reasonable agreement with the 2.05 kJ/mole found by Wolff *et al.* (27).

Other "weak" competitors in Fig. 2 were 6β-hydroxybudesonide (RBA 0.06) and 16α-hydroxyprednisolone (RBA 0.03), which are the major metabolites of budesonide.[6] The presence of the 6β-hydroxy group gave an exceedingly unfavorable ΔG of 11.20 kJ/mole. This hampers binding to the receptor even more than does the oxidation of an 11β-hydroxy group to an 11-oxo group (ΔG 9.34 kJ/mole) (27).

Compounds in which the 16α- and 17α-hydroxy groups have been replaced by either of the two acetal structures shown in Fig. 1 were very efficient as competitors for the DEX-binding sites. Thus, prednacinolone 16α,17α-acetonide (PA) had an RBA of 2.21, and triamcinolone 16α,17α-acetonide (TA), which in addition to the Type A acetal of PA has a 9α-fluoro substituent, had an RBA value of 3.80 (Fig. 2; Table 1). Thus, when comparing TA and PA, a ΔG of −1.23 kJ/mole was calculated for the presence of the 9α-fluoro atom in TA, resulting in an increased binding strength. Wolff *et al.* (27) reported a mean ΔG value of −2.39 kJ/mole for 9α-fluorination of F and of prednisolone, none of which contains an acetal substituent, however (cf. below).

Compounds with the Type B acetal (Fig. 1), irrespective of 9α-fluorination, were even more active than those with a Type A acetal when tested as competitors for the DEX-binding sites (Figs. 2 and 3). Thus, budesonide had an RBA value of 7.84 (Fig. 2), corresponding to a ΔG of −2.88 kJ/mole for the change of acetal A (PA) to acetal B (budesonide); i.e., 6% of the total binding-force of budesonide is entirely due to the replacement of acetal A by acetal B. The RBA of budesonide was intermediate

[6] S. Edsbäcker, S. Jönsson, C. Lindberg, Å. Ryrfeldt, and A. Thalén, submitted for publication.

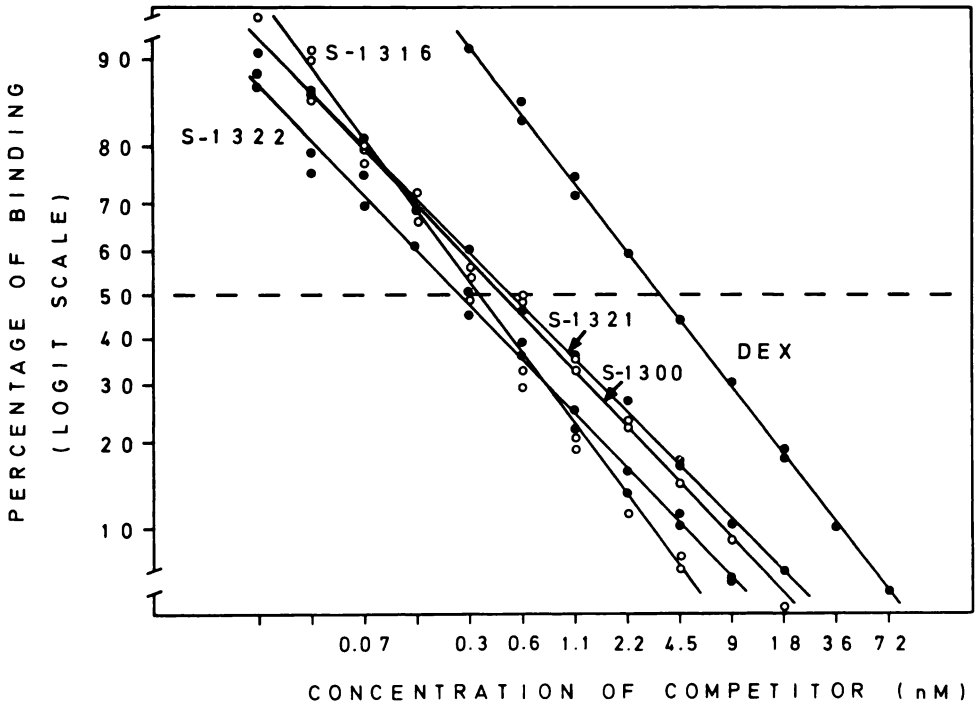

FIG. 3. *Logit-log plot of ligand specificity studies*

The competition studies were carried out as described under Methods using the logit-log plot for calculation of RBA values (Table 1). The concentration of the labeled ligand ([^{3}H]DEX) was 4.5 nM. Total binding in this experiment was 1.59 nM (SD 20 pM) and nonspecific binding was 81 pM (SD 8 pM). Where single points are shown, duplicates were within the diameter of ○ or ●. Linear regression was used to fit the lines to the data. The 9α-fluoro- and 6α,9α-difluoro-substituted derivatives of S-1322 (S-1300 and S-1316, respectively) are indicated by ○; DEX and the nonfluorinated compounds are indicated by ●. The abbreviated names of the competitors are given together with RBAs in Table 1 (for details, see footnote 4).

to the RBAs of the two C(22)-epimers (28), of which it constitutes an approximately 1:1 mixture, i.e., S-1321 (22S; RBA 4.25) and S-1322 (22R; RBA 11.17) (Fig. 2). Hence, not only is the hydrophobicity (29) of the acetal group important, but the configuration at the C(22) chiral center in the Type B acetal also plays an essential role in determining the magnitude of the binding-force between the ligand and the receptor. The (22S)- and

TABLE 1

RBAs, Gibbs' free energies (ΔG) for formation of ligand-receptor complexes, and some structural features of the compounds tested as competitors for the glucocorticoid receptor in rat skeletal muscle cytosol

Compound	1-Ene	9α-F	6α-F	16α[a]	17α[a]	RBA	ΔG
							kJ/mole
DEX	+	+	−	Me	OH	1	−42.6
S-1322 [(22R)-epimer of budesonide]	+	−	−		Acetal B	11.2	−48.1[b]
						(13.9)	(−48.6)[c]
S-1316	+	+	+		Acetal B	10.8	−48.0[c]
S-1300	+	+	−		Acetal B	8.0	−47.4[c]
Budesonide (22R,S)	+	−	−		Acetal B	7.8	−47.3[b]
S-1321 [(22S)-epimer of budesonide]	+	−	−		Acetal B	4.2	−45.9[b]
						(7.2)	(−47.1)[c]
TA	+	+	−		Acetal A	3.8	−45.7[b]
PA	+	−	−		Acetal A	2.2	−44.4[b]
RU 26988	+	−	−	−	1-Propenyl	0.3	−39.9[b]
Corticosterone (B)	−	−	−	−	−	0.1	−37.7[b]
6β-Hydroxy-budesonide (6β)	+	−	−		Acetal B	0.06	−36.2[b]
Cortisol (F)	−	−	−	−	OH	0.04	−35.2[b]
16α-Hydroxyprednisolone (16α)	+	−	−	OH	OH	0.03	−34.7[b]

[a] Figure 1 should be consulted for the structures of the acetals. Me denotes a methyl group. The abbreviated names are used in Figs. 2 and 3. The RBA values were calculated by linear regression from logit-log plots (Figs. 2 and 3) as described elsewhere (9, 10). The Gibbs' free energies (ΔG) were calculated using the expression $\Delta G = -RT\ln K_a$, where R and T have their usual meanings of gas constant and absolute temperature (273.15° K); K_a values were derived from the RBA values and a K_d for dexamethasone of 7 nM at 0° (9, 10). The ΔG values for individual substituents (given in the text) can be obtained by the expression $RT\ln(K_d$ substituted steroid$/K_d$ unsubstituted steroid).

[b] Data from Fig. 2.

[c] Data from Fig. 3.

(22R)-epimers of budesonide had ΔG values of −1.48 and −3.68 kJ/mole, respectively, for the difference in binding strength between the acetal group of PA (Type A) and the new type of acetal (Type B). If one compares the epimers, the difference in configuration at the chiral C(22) atom between the (22S)- and (22R)-epimers corresponds to a ΔG value of −2.19 kJ/mole (data from Fig. 2). In a more detailed experiment (Fig. 3), the (22R)-epimer was about as efficient as a competitor for the DEX-binding sites as in the experiment presented in Fig. 2 (RBA 13.87 versus 11.17), but the (22S)-epimer of budesonide was more active than that in Fig. 2 (RBA 7.24 versus 4.25). This discrepancy between the two assays must be due to the fact that too few concentrations were used in Fig. 2, but the data in Fig. 3 are more reliable for these compounds. On the basis of the data in Fig. 3, the difference in C(22)-configuration between the (22S)- and (22R)-epimers corresponds to a ΔG of −1.47 kJ/mole.

Since budesonide and its constituting epimers (a) were considerably more efficient ligands for the glucocorticoid receptor than, for example, TA and DEX, and (b) lack a 9α-fluoro atom, it was of interest to determine whether 9α-fluorination of budesonide would result in even more active compounds. The 9α-fluoro derivative of S-1322 (i.e., the (22R)-epimer) of budesonide (the most active one), was therefore synthesized, as well as the corresponding 6α,9α-difluoro compound.

When a 9α-fluoro group was introduced into the (22R)-epimer of budesonide (S-1322), resulting in compound S-1300, a ΔG value of 1.24 kJ/mole was obtained for the 9α-fluoro atom (cf. above). When a 6α-fluoro atom was introduced in addition to the 9α-fluoro substituent of S-1300 (giving compound S-1316), a ΔG value of −0.67 kJ/mole was obtained for the 6α-fluoro atom. The resulting ΔG value for the entire 6α,9α-difluoro substitution was 0.57 kJ/mole (S-1316 versus S-1322) (Fig. 3; Table 1). It is worthy of note that both the negative influence of 9α-fluorination on binding and the difference between S-1300 and S-1316 were slight, and also these compounds were unusually active competitors for the DEX-binding sites (RBAs 8.01 and 10.79, respectively).

The results of the *in vivo* investigations are given in Table 2. The compounds that bound most avidly to the glucocorticoid receptor were also the most potent inhibitors of rat ear edema (topical activity), whereas the inefficient competitors had weak topical activities. The new Type B acetal-containing steroids were approximately 5 times more potent inhibitors of edema formation than the corresponding Type A acetals PA and TA, and approximately 10 times more potent than DEX in this system. For the three (22R)-epimers, the difference was significant against both PA and TA and DEX. The (22S)-epimer of budesonide was significantly more potent than DEX, but not significantly more potent than PA or TA. The introduction of 9α-fluoro or 6α,9α-difluoro substituents did not have statistically significant effects on the topical anti-inflammatory activity. Thus, the Type A acetal-containing steroids PA (nonfluorinated) and TA (9α-fluorinated) had almost identical topical activity. Also, the differences between the Type B acetal-containing compounds S-1322 (nonfluorinated), S-1300 (9α-fluoro), and S-1316 (6α,9α-difluoro) did not reach statistical significance (Table 2).

TABLE 2

In vivo activities of the compounds

Compound	Edema inhibition[a]	95% Confidence interval	Thymus involution[a]	
			s.c. administration	p.o. administration
DEX	1		1	1
S-1322 [(22R)-epimer of budesonide]	13.0	7.4–24.1	1.70[b]	0.212
	(15.7[b])	(8.3–29.6)		
S-1316	15.6	9.3–27.8	ND[c]	ND
S-1300	9.35	5.6–15.7	ND	ND
Budesonide (22R,S)	9.26	3.6–23.3	0.847	0.138
S-1321 [(22S)-epimer of budesonide]	8.15[b]	3.7–20.4	0.254[b]	0.0894
TA	2.41[b]	0.9–5.6	1.19	0.422
PA	2.22[b]	0.9–4.6	0.424	0.26
Corticosterone (B)	0.0155	0.0044–0.0372	0.00576	0.0011
6β-Hydroxybudesonide (6β)	0.0619	0.0156–0.156	ND	ND
Cortisol (F)	0.0083	0.0033–0.0184	0.0212	0.00552
16 α-Hydroxyprednisolone (16α)	0.00463	0.00008–0.023	0.0178	0.00414

[a] Relative activities are used (potency relative to that of DEX) to facilitate comparisons. The data were obtained as described under Methods.

[b] Data taken from Brattsand *et al.* (8).

[c] ND, Not determined.

When thymus involution after subcutaneous or peroral administration was measured, the same principal findings were made regarding a correlation between receptor affinity and *in vivo* activity. However, here some noteworthy exceptions were found, e.g., that TA and DEX—despite RBA values (Table 1) and topical activities (Table 2) clearly lower than those of budesonide—were more potent in causing thymus involution, especially after p.o. administration (Table 2). The reason for this discrepancy is that TA (24) and probably also DEX are less efficiently inactivated by hepatic biotransformation than is budesonide (30). Hence it seems as if the systemic activity depends more on pharmacokinetical properties than on receptor affinity.

As anticipated, the closest correlation between receptor affinity and biological potency was obtained in the topical rat ear edema system (Fig. 4a). The linear correlation coefficients for nonlogarithmic and logarithmically transformed values were 0.967 and 0.985 when all values were included ($n = 12$), and 0.985 and 0.993 when omitting compounds not tested also systemically ($n = 9$) ($p < 0.001$ in all cases). The corresponding rank correlation coefficients between RBA and topical activity were 0.965 ($n = 12$) and 0.983 ($n = 9$) ($p < 0.001$ in both cases, irrespective of whether nonlogarithmic or logarithmic values were used, as the ranking order is unaffected by logarithmic transformation). It is worth noting that linear regression does not distinguish as clearly as rank correlation between the three test systems. Falsely high correlation coefficients were obtained with the former method for systemic administrations. Thus, the r values for RBA versus thymus involution after s.c. (Fig. 4b) and p.o. (Fig. 4c) administration were 0.718 ($p < 0.05$) and −0.059 (clearly not significant), and the corresponding r values for ln(RBA) versus ln(activity) were 0.878 ($p < 0.001$) and 0.786 ($p < 0.01$) after s.c. and p.o. administration. This is to be compared with the rank correla-

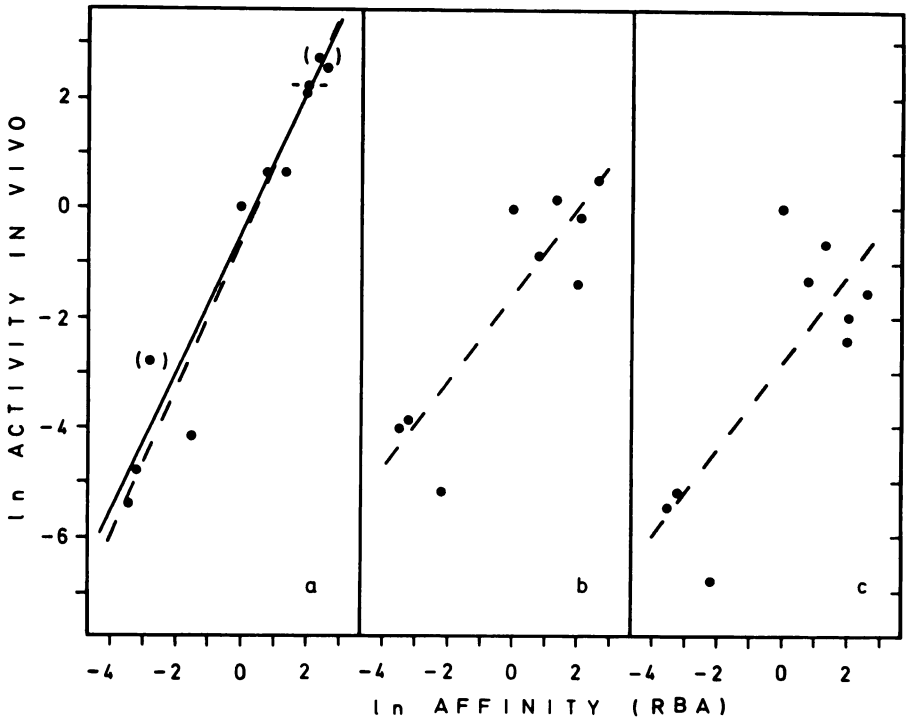

FIG. 4. *Correlation between RBAs and biological activities*

The values for RBA (Table 1) and *in vivo* activities (Table 2) are expressed relative to those of DEX. a, The correlation between RBA and rat ear edema inhibition; b, that between RBA and rat thymus involution after s.c. administration; c corresponds to b, but after p.o. administration. Natural logarithms were used due to the wide range of potencies. In a, one point (marked with *horizontal bars*) represents two compounds, one of which (S-1300) is excluded together with 6β-hydroxy-budesonide and S-1316 (in parentheses) from the *dashed line* in a, since these three compounds were not tested for systemic activity (b and c).

tions, which were 0.733 ($p < 0.05$) and 0.500 (nonsignificant) for s.c. and p.o. activity, respectively. The reason for this is that part of the linear correlation coefficient is influenced by the fact (apparent in Fig. 4) that there are two populations of observations, i.e., the values are distributed into two clusters, which are separate from each other. Rank correlation takes only the order of the values into account, and it is therefore not subject to such distribution-related phenomena. The results of these comparisons between the *in vitro* and *in vivo* activities give strong support to the theory that, for the present types of corticosteroids, receptor binding studies have a good applicability when screening for possible agonists.

DISCUSSION

Since RBAs are not absolute values, e.g., varying with incubation time, different RBAs are found in the literature for some of the compounds. Thus, Raynaud *et al.* (4) reported higher RBA values for B and F (0.67 and 0.31, respectively), and a lower RBA for TA (1.41), than those found in the present paper. Their RBA for TA was also lower than the RBA of 3.0 reported by Wolff *et al.* (27) and Bailly *et al.* (31). Moguilewsky and Raynaud (32) found RU 26988 to be more efficient (RBA 1.27) than we did. These discrepancies are predictable from the considerably shorter incubation period used by these authors (4 hr at 0°, whereas we incubated for 24 hr at 0°).

Differences between steroids in affinity for receptor binding sites are generally thought to be due to differences in dissociation rate constants, rather than association rate constants. Thus, different glucocorticoids associate at the same rate with receptors of rat thymocytes, murine L-cells or AtT-20 cells, whereas dissociation rates vary greatly with steroid structure (33–35). Caution is needed when interpreting such data (obtained under different conditions), since substantial differences have been reported from different laboratories for the dissociation rate of a given steroid (e.g., DEX) in different cell types (33–38). In line with this, we found that DEX-receptor complexes are formed at about the same rates in skeletal muscle cytosol from rat (9), pig (11), and man (13); others have found that DEX-receptor complexes associate at the same rate in different murine lymphoma cell clones (39). On the other hand, the dissociation rate constants differed between these clones (39).

In the absence of a 1-ene double bond, steroids with a 4-ene-3-one structure may take an A-ring conformation intermediate between two ideal (energy-minimized) conformations, viz., the 1α-sofa and the 1α,2β-half-chair conformations (3, 40). The introduction of a 1-ene double bond into such steroids flattens the A-ring (refs. 13 and 27 in ref. 3), and results in an increased receptor binding (ref. 27; ref. 12 in ref. 3). In line with this, all compounds in the present study with high RBA values had a 1,4-diene-3-one structure, and those without the 1-ene (B and F) were weak competitors. This was true also for topical *in vivo* activity.

The B- and C-rings of steroids, on the other hand, are thought to have relatively rigid conformations, and not to be much affected by a variety of substitutions (3). An

interesting finding in the present study is that the B-ring-substituted compound 6β-hydroxy-budesonide, a major metabolite of budesonide,[6] was a most inefficient competitor for the receptor. Also with respect to the B-ring, 9α-fluorination of the (22R)-epimer of budesonide did not have the positive effect, either *in vitro* or *in vivo*, that one would have expected from reported data on 9α-fluoro substitution and from the difference between TA and PA in this study (cf. above). To our surprise, 9α-fluorination and 6α,9α-difluorination of the (22R)-epimer even reduced the affinity for the receptor. Although both of these steroids were less active than their parent compound, both were highly potent ligands, clearly superior not only to DEX but also to PA and TA. Also, TA (9α-fluorinated) and PA (nonfluorinated) had virtually the same topical *in vivo* activity. It appears, therefore, that B-ring substitutions may require a more thorough study.

In 9α-fluorinated 3-one-4-ene glucocorticoids, the A-ring is bent underneath the least-squares plane of the other rings to a greater extent than in the fluorine-less parent compounds (ref. 110 in ref. 6). It is worthy of note that, in both the (22S)- and (22R)-epimers of the 3-one-1,4-diene budesonide, the A-ring is bent similarly (revealed by single-crystal X-ray crystallography) as in 9α-fluorinated glucocorticoids (28). Hence, in view of the X-ray-determined 3-dimensional structure of the (22R)-epimer and the structural effect of 9α-fluorination, the A-ring bending may well be about optimal in this steroid. Since the ΔG value for a 9α-fluoro atom reported by Wolff *et al.* (27) represents the mean value for 9α-fluorination of F and prednisolone (1-ene-F), that the effect of 9α-fluorination varies depending on whether or not a 1-ene structure is present cannot be excluded.

In contrast to the "classical" thinking with respect to the stability of the B- and C-rings (cf. above), it seems well established that different substitutions of the D-ring induce characteristic conformational changes of this ring (ref. 3; ref. 110 in ref. 6). A 17α-hydroxy substituent lowers the affinity of a steroid for the glucocorticoid receptor (ref. 27; ref. 12 in ref. 3). Similar data were found when comparing B and F in the present study. A 17α-methyl or -ethynyl group, however, favors efficient binding both to the progestin receptor (5) and to the glucocorticoid receptor (ref. 12 in ref. 3). Whereas a 16α-hydroxy group decreases the RBA of glucocorticoids, a 16α-methyl group increases receptor binding (27), conceivably because 16α-substitutions alter the positional flexibility of the 17β-side chain (ref. 40; ref. 13 in ref. 3; refs. 36 and 86 in ref. 6). The low RBA found for 16α-hydroxyprednisolone, a major metabolite of budesonide,[6] together with the relatively high RBA of prednisolone, is in accordance with these structural data. In DEX, the 16α-methyl group cannot alone compensate for the negative influence of the 17α-hydroxy group (ΔG values of −0.46 and +2.05 kJ/mole, respectively), but the 1-ene structure and the 9α-fluoro atom contribute to form a large binding force (ΔG values of −1.21 and −2.39 kJ/mole, respectively) (data from ref. 27). As is well known, DEX belongs to the most active glucocorticoids hitherto synthesized, at least with regard to systemic activity. However, several compounds with either of the two types of 16α,17α-acetals (Fig. 1) bound considerably better to the DEX-binding sites, and had also higher topical activities, than DEX itself.

Both affinity for receptor and *in vivo* glucocorticoid activity are sensitive to the presence and properties of the 17β-side-chain (ref. 41; ref. 12 in ref. 3). For example, the 20-one is very important. In six corticosteroids analyzed crystallographically, O(20) and O(21) were *cis* coplanar, and O(20) was oriented over the D-ring (ref. 110 in ref. 6). Since the same was found for O(20) and O(21) in both epimers of budesonide (28), it is not possible to explain the higher RBAs and biological potencies of budesonide and its epimers by a different orientation of O(20) in these latter steroids. It has been suggested that the side-chain conformation "preferred" by the glucocorticoid receptor binding-site is close to a C(16)-C(17)-C(20)-O(20) torsion angle, τ, of 270° (ref. 26 in ref. 3). Data on τ have been obtained by X-ray crystallography (ref. 36 in ref. 6) or force-field calculation (ref. 86 in ref. 6; ref. 26 in ref. 3). The (22S)- and (22R)-epimers of budesonide have τ values of −25.3 and −27.5°, respectively,[7] and τ values for B, F, TA, and DEX are −14, −31/−30, −21, and −23/−24° (refs. 36 and 86 in ref. 6), as determined by X-ray crystallography. We calculated rank and linear correlation coefficients for the correlation between these data on τ and the RBA values in the present study ($R = -0.09$ and $r = -0.320$, both clearly not significant). Hence, τ does not seem to play the large role in determining the ligand-receptor interaction suggested by Schmit and Rousseau (ref. 26 in ref. 3).

The present data indicate that the hydrophobicity (29) of a 16α,17α-acetal substituent significantly influences the affinity of acetal-substituted glucocorticoids to the receptor. This is in agreement with previous reports that ligand binding to the glucocorticoid receptor in rat hepatoma tissue culture cells (27) or in rat muscle (9) largely is of hydrophobic nature. The new type of acetal in budesonide not only increased the affinity to the glucocorticoid receptor (Table 1), but also the topical anti-inflammatory activity (Table 2) without a corresponding increase in the systemic glucocorticoid activity as thymus involution (Table 2). This discrepancy may perhaps be due to the lipophilic nature of budesonide, resulting in longer presence in the skin, as shown for other corticosteroids (ref. 67 in ref. 42). However, it does not depend on enhanced binding to corticosteroid-binding globulin. In accordance with other potent synthetic acetal-containing glucocorticoids, budesonide has very low binding to corticosteroid-binding globulin.[8] A much more probable explanation for the relatively low systemic activity of budesonide is its rapid inactivation by metabolism in the liver (24). The high topical activity and affinity for the receptor are nevertheless due in part to the hydrophobicity of the acetal group (cf. above). However, it has been reported that both hydrogen-bonding properties and over-all shape of the steroid molecule may influence the binding affinity (27). Interestingly, the (22S)- and (22R)-epimers of budesonide had different binding affinities, although both were more active than the Type A

[7] C. Svensson, unpublished results.

[8] S. Batra, A. Thalén, and R. Brattsand, unpublished results.

acetal-containing PA. It is worth noting that the two epimers of budesonide have slightly different polarities and shapes (19), which allow their separation by high-pressure liquid chromatography or liquid chromatography on Sephadex LH-20 (19), the (22S)-epimer being slightly less polar and a little larger than the (22R)-epimer (19). The different RBAs for the two epimers may thus be in agreement with the suggestion of Wolff *et al.* (27) that the over-all shape of the ligand is of importance, since the more active (22R)-epimer is globular and relatively compact, whereas the less active (22S)-epimer has a more extended shape (19). The most probable explanation, however, is that the stereochemistry at the C(22) chiral center plays a large role in the interaction between ligand and receptor binding site. Since the (C22)-propyl chain is equatorial to ring E in the (22R)-epimer, whereas it is axial or nearly axial to the E-ring in the (22S)-epimer, it may interfere with and shield the polar groups at C(16), C(17), C(20), and C(21). This could result in a decreased interaction between these polar groups and polar sites on the receptor. In view of the lower RBA of the (22S)-epimer, such polar sites on the receptor must necessarily carry positive partial charges. The lesser shielding of these polar groups on the steroid in the (22R)-epimer, together with its higher affinity, also necessitates that such polar sites in the binding site of the receptor be positively charged. Since from energetic considerations the steroid must be buried in a hydrophobic pocket which constitutes the binding site on the receptor (27), such an explanation seems less likely. However, the propyl substituent at C(22) could interact as such with a polar site (less adversely in (22R)- than in (22S)-configuration), or more probably with a hydrophobic site (more favorably in (22R)- than in (22S)-configuration), located within the ligand-binding region of the glucocorticoid receptor. This could explain the lower affinity of the (22S)-epimer as compared with that of the (22R)-epimer. The lower RBAs resulting from the symmetrical acetal in TA or PA would also be in agreement with such a hypothesis. Characterization of the purified glucocorticoid receptor by amino acid sequencing and high-resolution X-ray crystallography is greatly needed for a complete understanding of receptor-ligand interactions.

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