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17β-Carboxamide Steroids: Highly Effective Inhibitors of the Phytohaemagglutinin Mediated Blastogenesis of Normal Human Peripheral Lymphocytes

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Summary: Several novel 17β-carboxamide analogues of dexamethasone were synthesized. The common precursor, 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxylic acid, did not bind to the glucocorticoid receptors of rat liver and human spleen tumours. In addition, no inhibition of the mitogen-induced blastogenesis of cultured human peripheral lymphocytes was observed. The 17β-carboxamide analogues, however, bound with similar affinities to the glucocorticoid receptors of both tissues. They inhibited the mitogen-induced blastogenesis of peripheral lymphocytes, showing the same potency and same order of binding affinity as the natural glucocorticoids.

17β-Carboxamid-Steroide: Hochwirksame Inhibitoren der phytohämagglutinin-induzierten Blastogenese normaler peripherer menschlicher Lymphocyten

Zusammenfassung: Es wurden mehrere neuartige 17β-Carboxamid-Analoga des Dexamethasons synthetisiert. Die Ausgangsverbindung, 9-Fluor-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadien-17β-carbonsäure, zeigte keine Bindung an Rattenleber- oder menschliche Milztumor-Glucocorticoidrezeptoren. Ebenso konnte keine Inhibition der mitogen-induzierten Blastogenese normaler menschlicher Lymphocyten gezeigt werden. Im Gegensatz dazu banden die 17β-Carboxamid-Analoga mit ähnlicher Affinität an die Glucocorticoidrezeptoren beider Gewebe. Ihre Potenz, die mitogen-induzierte Blastogenese der Lymphocyten zu hemmen, war von der natürlicher Glucocorticoide nicht zu unterscheiden und korrespondierte mit der jeweiligen Affinität zum Rezeptor.

Introduction

Antagonists of steroid activity are useful probes for understanding both the role of steroids in vivo and their mechanism of action (1, 2). While it is known that many characteristic hormonal responses of steroids are contingent upon their binding to specific receptors in target tissue, a detailed understanding of the steps (receptor activation, translocation, interaction with cell chromatin, etc.) leading to the specific biological effects in the target tissues, is far from complete (3).

The existence of antagonists that compete for the steroid binding site of the receptor with high affinity demonstrate that the phenomena of binding and activity are at least partially independent. *Rousseau* et. al. (1) previously reported that 17β-carboxamide analogs of dexamethasone represent a new class of glucocorticoid antagonists. Their antiglucocorticoid effect on rat hepatoma tissue culture cells was dose dependent and the order of potency of the antagonists was consistent with their affinity for the rat hepatoma and rat liver glucocorticoid receptors.

Recently, we compared the inhibitory effects of synthetic and natural glucocorticoids as well as some of their 17β-carboxy analogues on the incorporation of radiolabeled thymidine into phytohaemagglutinin-stimulated human peripheral blood lymphocytes, and obtained the opposite result! The benzyl 17β-carboxamide analogue of dexamethasone (compound Ic) behaved like a glucocorticoid agonist and the order of potency of this agonist was consistent with its affinity for human spleen tumour and rat liver receptors (4–7).

In the present study we investigated a larger number of steroids (some of these are new steroids), and showed that 17β-carboxamide analogues of dexamethasone generally show a dose-dependent glucocorticoid effect on phytohaemagglutinin-stimulated human peripheral lymphocytes.

Materials and Methods

Hormones and chemicals

[1,2(N)-^{3}H]Dexamethasone (0.72 TBq/mmol) and [5′-^{3}H]thymidine (0.18 TBq/mmol) were generous gifts from Amersham International, Cardiff, England.

Dexamethasone (I) (9-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione) and betamethasone (II) (9-fluoro-16β-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione) were purchased from Sigma, München, F.R.G. All other chemicals were of reagent grade.

Analytical and preparative thin-layer chromatography was carried out using precoated silicic acid F_{254} plates (Merck, Darmstadt, F.R.G). Thin-layer plates were developed in the following solvent systems:

(A) chloroform/methanol 9 + 1 (by vol.);
(B) ethyl acetate/ethanol/NH_3 5 + 5 + 1 (by vol.).

The 17β-carboxylic acid analogues (Ia; IIa) (see tab. 1) were obtained by periodic acid oxidation. The following 17β-carboxamide analogues were synthesized according to *Formstecher* et al. (8):

N-benzyl 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (Ie; m.p. 258 °C);

N-benzyl 9-fluoro-16β-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (IIb; m.p. 250–252 °C);

2′-(*p*-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamido)glutaric acid di-tert-butyl ester (Ig; m.p. 215 °C);

N-propyl 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (Ic; 259–260 °C);

N-*p*-azidobenzyl 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (If; ir 2100 cm^{-1} (azido)).

The synthesis of N-(2-amino)-ethyl 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (Ib) and N-(5-amino)-pentyl 9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (Id) is described elsewhere (9).

Synthesis of [^{3}H]benzyl-9-fluoro-16α-methyl-11β,17-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide ([^{3}H]Ie)

54 nmol of [^{3}H]dexamethasone (92.5 MBq) in 2.5 ml of ethanol was evaporated to dryness in a round bottomed flask and redissolved in 1 ml methanol. Periodic acid solution (340 mg periodic acid, 97.5 ml H_2O, 0.5 ml of conc. HCl) (500 µl) was added and the oxidation was complete within 5 h. The organic solvent was removed under reduced pressure and the acid extracted three times with 5 ml ethyl acetate. The combined organic extracts were evaporated to dryness, and the remaining residue was again dissolved in 200 µl methanol and further purified on thin-layer plates in solvent system (B). Identity of the purified acid was confirmed by its R_f-values on thin-layer plates in solvent system (A) and (B).

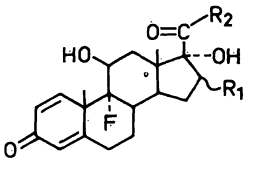
To 44.4 MBq (32 nmol) of the purified acid in 1 ml of ethyl acetate, 25 µl of a solution of dicyclohexylcarbodiimide (1 g/l) and 12.5 µl of a solution of benzylamine (1 g/l) in ethyl acetate were added and the mixture was kept for 36 h at room temperature. The carboxamide [^{3}H]Ie was purified on thin-layer plates in solvent system (A) and characterized as described above.

Preparation of homogenates

Human spleen tumour tissue was stored at −70 °C until use. After thawing at 0 °C in buffer consisting of 200 ml/l glycerol, 2 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 20 mmol/l Tris-HCl (pH 7.4) the tissue

Tab. 1. Steroid structures.

Compound	R_1	R_2
I	α-CH_3	$-CH_2OH$
Ia	α-CH_3	$-OH$
Ib	α-CH_3	$-NH-(CH_2)_2-NH_2$
Ic	α-CH_3	$-NH-(CH_2)_2-CH_3$
Id	α-CH_3	$-NH-(CH_2)_5-NH_2$
Ie	α-CH_3	$-NH-CH_2-C_6H_5$
If	α-CH_3	$-NH-CH_2-C_6H_4-N_3$
Ig	α-CH_3	$-NH-CH(CO-O-C(CH_3)_3)-(CH_2)_2-CO-O-C(CH_3)_3$
II	β-CH_3	$-CH_2OH$
IIa	β-CH_3	$-OH$
IIb	β-CH_3	$-NH-CH_2-C_6H_5$



was minced in 3 volumes of buffer and homogenized by 10 strokes in a *Potter-Elvehjem* homogenizer. The homogenate was centrifuged for 1 h at 105000 *g* and the supernatant taken as cytosol. Male Wistar rats (~ 150 g) were bilaterally adrenalectomized and maintained on normal saline for 4 days prior to sacrifice. The livers were perfused with cold buffer and processed as described above.

Protein concentrations were determined with the BioRad protein kit (BioRad, Richmond, USA).

Competition assay

The tubes were prepared as follows: to each tube 0.1 ml of [^{3}H]dexamethasone (in buffer) was pipetted, to give a final concentration of 32 nmol/l. Then aliquots of 0.1 ml of buffer containing the various competitors at 10 different concentrations (3.2–640 nmol/l) were added. Finally, 0.2 ml of cytosol was added to each tube and the tubes were incubated for 4 h at 0 °C. Incubation was terminated by the addition of 1 ml dextran-coated charcoal suspension. The final concentrations were 5 g/l of charcoal and 0.5 g/l of dextran T 500. After 10 min of incubation the tubes were centrifuged for 10 min at 5000 *g* and 1 ml of the supernatant was withdrawn and counted for radioactivity. All determinations were carried out in triplicates. The relative binding affinities of the steroids for cytosolic glucocorticoid receptors were determined as described by *Ojasoo & Raynaud* (10). The relative binding affinity of dexamethasone was taken to be equal to 100.

[^{3}H]Thymidine incorporation assay

Peripheral blood lymphocytes from healthy donors were isolated using a Ficoll Hypaque double gradient technique (11). The cells were washed three times in *Hanks* balanced salt solution and resuspended at a all concentration of 10^9/l cells in medium 199 (Flow Laboratories, Meckenheim, F.R.G.) supplemented with 50 ml/l heat-inactivated human serum (blood group AB), 2 mmol/l *L*-glutamine, 100 U/ml penicillin and 100 mg/l streptomycin. More than 95% of the lymphocytes were viable as determined by trypan blue exclusion. Culture medium (200 µl), containing 2 × 10^5 cells, was added to each well of round bottomed microtiter plates, followed by the addition of 10 µl of phytohaemagglutinin (from *Phaseolus vulgaris* Type V, Difco Laboratories, Detroit, Michigan, USA) (final concentration of phytohaemagglutinin 10 mg/l) and 10 µl of steroid solution (increasing concentrations dissolved in 0.15 mol/l NaCl solution). The plates were incubated for 72 h at 37 °C in a humidified atmosphere of 0.5 CO_2 and 0.95 air. Lymphocyte stimulation was assessed by the incorporation of [^{3}H]thymidine into the DNA during the last 4 h of culture (37 MBq/ml) (11–13).

Steroid metabolism in the cultures was examined by incubation [^{3}H]dexamethasone or carboxamide [^{3}H]Ie at a final concentration of 6.4 µmol/l with 2 ml of cultured peripheral blood lymphocytes as described above. Radioactivity was at least 1 MBq/l culture mixture. After 72 h the cells were centrifuged and the supernatant culture medium was saved. The pellet was washed with phosphate-buffered saline and homogenized in 1 ml of the same buffer by 10 strokes in a *Potter-Elvehjem* homogenizer. Both the culture medium and the homogenate were extracted twice with two volumes of methylene chloride. The organic phase was evaporated to dryness under a stream of nitrogen and the steroids were chromatographed in solvent system (A) together with non-radioactive standards. The radioactivity spots were scraped off the thin-layer plates and counted for radioactivity. Steroid metabolism in rat liver homogenates was examined as described (14).

Assay of receptor binding to DNA-cellulose

Cystosol fractions were incubated with 32 nmol/l [^{3}H]steroid alone or with a 1000-fold excess of the respective unlabeled steroid for 2 h at 4 °C. Subsequent activation of the steroid receptor complex was accomplished by heating the cytosols at 25 °C for 30 min.

The procedure of *Kalimi* et al. (15) was used to assay the amount of receptor binding to DNA-cellulose. Activated receptor complex (100 µl) was incubated with the pellet from 200 µl of a 25% slurry of DNA-cellulose. The suspension was gently mixed on a Vortex homogenizer and incubated for 45 min at 4 °C. The reaction was stopped by the addition of 2 ml of cold buffer and centrifuged for 10 min at 2000 *g*. The samples were then washed three times in cold buffer and the final pellet resuspended in 0.8 ml of the buffer. A 0.5 ml aliquot was taken for determination of radioactivity.

Results

Competition studies

The relative binding affinities of the steroids to the glucocorticoid receptors of human spleen tumours and rat liver are listed in table 2. The 17β-carboxylic acid compounds Ia and IIa did not compete significantly for [^{3}H]dexamethasone binding in human spleen or in rat liver tissue cytosols.

Tab. 2. Effect of substitutions in the steroid molecule on receptor binding and glucocorticoid activity.

Compound	Relative binding affinities[a]		Inhibition of [^{3}H]thymidine[b] incorporation (%) at 5×10^{-6} mol/l steroid
	Human spleen tumour leukocytes	Rat liver	
Dexamethasone			
(I)	100	100	100
Ia	0.1	0.1	0
Ib	0.9	0.4	10
Ic	12	9	70
Id	0.7	0.3	15
Ie	27	34	85
If	9	8	60
Ig	3.2	1.4	50
Betamethasone			
(II)	100	100	100
IIa	0.1	0.1	0
IIb	0.1	0.1	0
Hydrocortisone	50	40	100

[a] Human spleen tumour and rat liver cytosols were incubated with 32 nmol/l of [^{3}H]dexamethasone either alone or in the presence of various concentrations of competitors for 4 h at 0 °C. The unbound steroid was removed with dextran-coated charcoal. The relative binding affinity values listed are means of three determinations.

[b] Lymphocytes were isolated as described. Phytohaemagglutinin and increasing amounts of steroids were added followed by 72 h of incubation. Lymphocyte stimulation was assessed by the incorporation of [^{3}H]thymidine into the DNA during the last 4 h of culture. Similar results were found in peripheral blood lymphocytes of different donors. In this particular experiment the mean background was 10 min^{-1}, and the mean maximal incorporation corresponded to 336 min^{-1}.

Amidation partly restored the binding capacity with the exception of amide IIb. Representative competition curves are shown in figure 1. None of the fluorinated derivatives bound to corticosteroid binding globulin (data not shown).

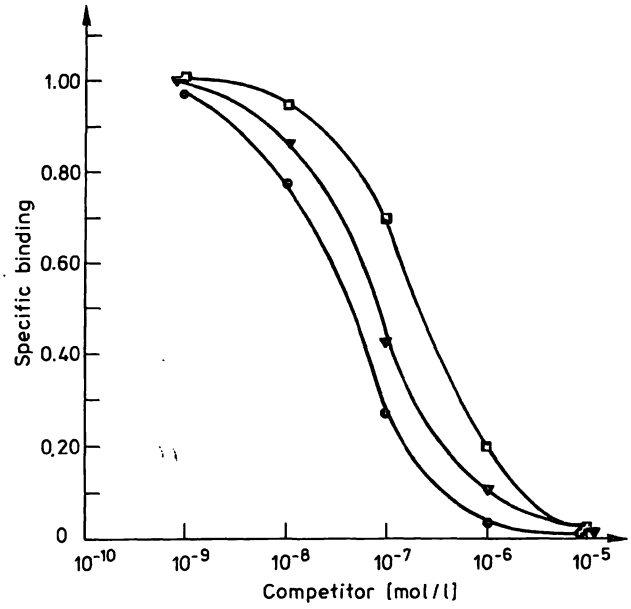


Fig. 1. Competition of various steroids for glucocorticoid receptors in human spleen tumour cytosol. Cytosol was incubated with 32 nmol/l [^{3}H]dexamethasone ± competitors for 4 h at 0 °C and specific binding determined. Total specific binding in the absence of competitor was taken as 100%. The symbols are: ○ dexamethasone (I), ▼ hydrocortisone, ◘ 17β-carboxamide (Ie).

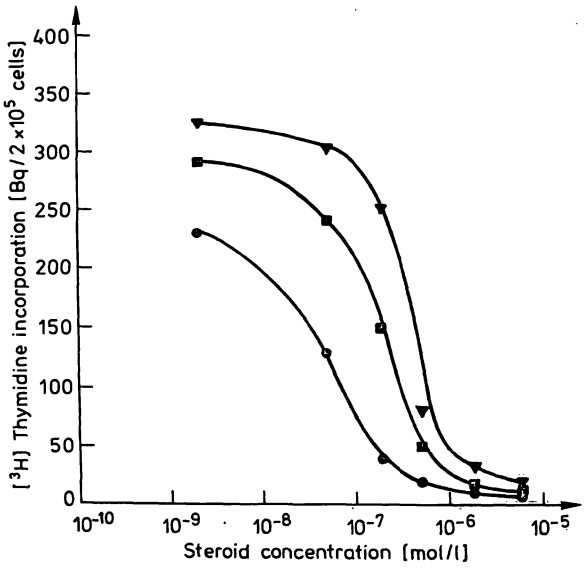


Fig. 2. Effect of various steroids on [^{3}H]thymidine incorporation in human peripheral blood lymphocytes. Lymphocytes were isolated as described. Phytohaemagglutinin and increasing concentrations of steroids were added and the cells incubated for 72 h at 37 °C. Stimulation was assessed by the incorporation of [^{3}H]thymidine into the DNA during the last 4 h of culture. The symbols are: ● dexamethasone (I), ▼ hydrocortisone, ◘ 17β-carboxamide (Ie).

Inhibition of phytohaemagglutinin-mediated blastogenesis of normal human peripheral blood lymphocytes

The inhibition of [^{3}H]thymidine incorporation in phytohaemagglutinin-stimulated peripheral blood lymphocytes by dexamethasone, betamethasone and their analogues is shown in table 2. The order of inhibition closely resembles that of the binding affinities to the spleen tumour receptors (fig. 1 and fig. 2), and is similar for different concentrations of phytohaemagglutinin and concanavalin A (i.e. for different levels of stimulation) (fig. 3a, b).

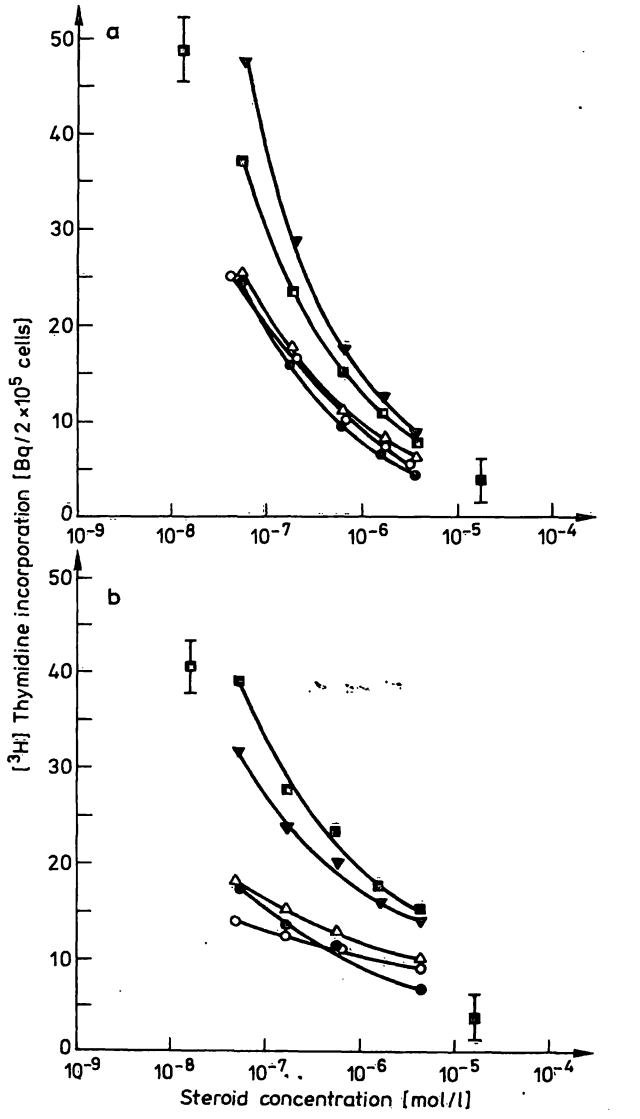


Fig. 3. Effect of various steroids on [^{3}H]thymidine incorporation in differently stimulated human peripheral blood lymphocytes. Lymphocytes were isolated as described and the cell count was adjusted to 2 × 10^5 cells per culture volume (200 µl). Phytohaemagglutinin (fig. 3a; final concentration 1.25 mg/l) or concanavalin A (fig. 3b; final concentration 2.5 mg/l solutions (10 µl) and steroid solutions (10 µl) of increasing concentrations were added and the cells incubated for 72 h at 37 °C. Stimulation was assessed by the incorporation of [^{3}H]thymidine into the DNA during the last 4 h of culture. The symbols are: ● dexamethasone (I), ○ betamethasone (II); △ desoxymethasone, ▼ hydrocortisone, ■ 17β-carboxamide (Ie).

The original cell number did not significantly change within the first 3 days of culture and there was no hint that the survival of peripheral blood lymphocytes was negatively affected by any of the steroids used in this study. In addition, no significant metabolic conversion of carboxamide [^{3}H]Ie by cultured lymphocytes or rat liver cytosols was observed.

DNA-cellulose binding of non-activated glucocorticoid-receptor complex

Cytosols were either labeled with [^{3}H]dexamethasone or carboxamide [^{3}H]Ie and heat activated (25 °C for 45 min). Bound radioactivity and DNA-cellulose binding were then determined (tab. 3). Under our experimental conditions, it appeared that only human spleen tumour glucocorticoid receptors were able to form activated carboxamide [^{3}H]Ie-receptor complexes, whereas in the presence of 10 mmol/l Na_2MoO_4 neither [^{3}H]dexamethasone nor carboxamide [^{3}H]Ie-receptor complexes were activated by heat treatment.

Tab. 3. DNA-Cellulose binding of non-activated and activated glucocorticoid-receptor complexes.
Human spleen tumour or rat liver cytosols were labelled with either [^{3}H]dexamethasone or carboxamide [^{3}H]Ie and heat activated for 45 min at 25 °C. Aliquots were assayed for DNA-cellulose binding and bound radioactivity prior to and after heat activation. The values listed are means of three determinations.

Compound	% bound DNA-cellulose[a]			
	Human spleen		Rat liver	
	Heat activation		Heat activation	
	prior	after	prior	after
[^{3}H]Dexamethasone	2.5	10.0	5.6	23.0
[^{3}H]Dexamethasone + 10 mmol/l Na_2MoO_4	2.2	2.1	2.0	1.8
[^{3}H]Ie carboxamide	1.6	10.0	3.0	3.1
[^{3}H]Ie carboxamide + 10 mmol/l Na_2MoO_4	1.8	2.0	1.8	2.1

[a] (DNA-cellulose binding) / (total cytoplasmic ^{3}H-steroid binding) × 100 (23).

Discussion

Antagonists of hormone action may exert their effect by interfering with the synthesis or metabolism of a specific or any of the proteins that are essential to the expression of that steroid's physiological response (2). Some antagonists act by direct competition for the hormone binding site on the receptor (1, 16, 17). In these cases it would be of inestimable value for the organic chemist to identify which structural features control agonist and antagonist responses.

X-ray crystal structure studies on dexamethasone and the possible glucocorticoid antagonist dexamethasone oxetanone (2) showed that both agonist and antagonist can accept a hydrogen bond to the oxygen atom at the C-20 position of the steroid skeleton, whereas only the agonist can also donate two hydrogen bonds to effect or stabilize a receptor interaction. Thus the lack of induction of tyrosine aminotransferase in cultured rat hepatoma cells by 17β-carboxamide steroids, as well as their potency as antagonists of the action of dexamethasone on these cells (1), might be explained by the lack of receptor stabilizing hydrogen bond donors in the carboxamide side-chain.

This hypothesis, however, is only applicable to rat liver (5) or rat liver derived cells, as the same derivatives may also behave like glucocorticoid agonists by inhibiting the mitogen-induced blastogenesis of human peripheral blood lymphocytes (tab. 2 and fig. 2). As no chemical alteration of carboxamide Ie could be detected during its incubation with cultured lymphocytes, the quite unlikely transformation of original antagonists to agonists, followed by an agonist-typical response (i.e. similarity of the order of magnitude of inhibition potency and binding affinity) of the lymphocytes (4, 7), can definitely be excluded. The divergent behaviour in the special case of carboxamide IIb is discussed elsewhere (7).

Structural details undoubtedly have a direct bearing upon receptor affinity and will directly or indirectly influence receptor activation, transport and nuclear interaction (18). It has been shown that activation increases the affinity of steroid-receptor complexes not only towards nuclei, but also towards various polyanions (19) and calf-thymus DNA immobilized on cellulose (15). We have therefore used a DNA-cellulose assay to further investigate whether the double nature of these carboxamide steroids might reflect differences in the activability of carboxamide-receptor complexes of both tissues. Our results indicate that carboxamide Ie, which binds to both receptors with high affinity, might bei either unable to form activable complexes with rat liver glucocorticoid receptors (tab. 3) or leads to activated but labile carboxamide-receptor complexes. The latter effect

could possibly explain the weak agonistic activity of some 17β-carboxamide steroids on tyrosine aminotransferase induction in cultured rat hepatoma cells (1).

The low amount of heat-activable spleen tumour receptors might be the consequence of the unfavourable protein-receptor ratio in these cytosols (livers of adrenalectomized rats contain ~ 900 fmol receptor per mg protein, human spleen tumours ~ 80 fmol receptors per mg protein (14)). Heat activation of all complexes could be inhibited by the presence of 10 mmol/l Na_2MoO_4 in the homogenisation buffer (20).

It was previously proposed (2) that the steroid A ring might be primarily responsible for initiating and maintaining hormone binding to the receptors while the D-ring might control expression of activity. This hypothesis, however, assumes that the hormone binding sites of glucocorticoid receptors of different species are at least similar (21). On the contrary, we maintain, on the basis of the literature (16, 17, 22) and our own results (7), that a generalized rationale of structure-activity relationships for glucocorticoid hormones is at the moment impossible.

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