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RU 38486: A POTENT ANTIGLUCOCORTICOID *IN VITRO* AND *IN VIVO*

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Summary—The antiglucocorticoid activity of RU 38486, was studied both *in vitro* and *in vivo*. *In vitro* studies, RU 38486 was characterized by a high affinity (3 times higher than that of dexamethasone) for the cytosolic glucocorticoid receptor in rat hepatoma tissue culture (HTC) cells. This high affinity was due to a very low dissociation rate of the complexes formed with the receptor. In whole cells it was a potent full antagonist of dexamethasone-induced tyrosine aminotransferase (TAT) activity: the IC50 was 6–7 times lower than the concentration of the dexamethasone used. It was devoid of any glucocorticoid activity up to a concentration of 10 μM. In *in vivo* studies using adrenalectomized rats, RU 38486 totally inhibited dexamethasone-induced hepatic tryptophan oxygenase (TO) activity. It is also the first pure antagonist of dexamethasone-induced hepatic TAT. However, doses as high as 5 mg/kg of body weight were required for a 50% inhibition of the effect of dexamethasone at 0.01 mg/kg. RU 38486 did not display any glucocorticoid effect on these two responses up to 50 mg/kg.

INTRODUCTION

A number of new steroids with potential antiglucocorticoid activity have been developed over the past years. Many analogs [1–3] have proved to possess this antagonistic effect, without agonistic activity, in the thymocytes, and in HTC cell cultures, the most currently used *in vitro* models [1, 4]. Some new compounds such as the 17β-carboxamide derivatives [5], dexamethasone mesylate [6] or oxetanone [7], $\Delta^{1,9(11)}$-deoxycortisol and Δ^{1}-11-oxa-11-deoxycortisol [8, 9] described more recently, belong to this class. Unfortunately, these compounds are not as effective *in vivo* and they generally act as partial antagonists only in selected tests and at very high doses [10–16].

In this paper we present the *in vitro* and *in vivo* antiglucocorticoid activity of RU 38486, which belongs to the new series of 11β-substituted 19-nor steroids. Its relative binding affinity for the glucocorticoid receptor and its association and dissociation rates were evaluated by comparison to those of dexamethasone. Its biological activity was determined on the basis of its effect on the induction of tyrosine aminotransferase (TAT) in HTC cells, and also *in vivo* on hepatic TAT and tryptophan oxygenase (TO) induction in adrenalectomized rats.

EXPERIMENTAL

Tritiated dexamethasone (20 Ci/mmol) was from Amersham; RU-38486 (17β-hydroxy-11β-[4-dimethylaminophenyl]-17α-propynyl-estra-4,5 diene-3-one) and 6–7 [^{3}H] RU 38486 (37 Ci/mmol) from Roussel Uclaf, Romainville, France; Tricine, DTT, sodium molybdate, dexamethasone from Sigma; pyridoxal-5′-phosphate (PLP) from Merck; sodium α-ketoglutarate from Boehringer; L-tyrosine from Fluka. Imenzo culture medium came from A.B.S. (NY, U.S.A.) and Swims's 77 from Gibco. The fetal and newborn calf serums are Seromed products. Before use, they were heated for a half hour at 56°C.

The HTC cells were cultured in monolayer or in suspension under conditions described by Thompson [4]. The cultures were left in suspension until confluence (10^6 cells/ml), then centrifuged at 1,200 *g* and washed with phosphate-buffered saline, pH 7.4 (PBS: 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl), to obtain cellular pellets (3 ml) which were stored at −20°C.

Preparation of cytosol

After thawing the cellular pellet, all further steps were carried out at 0–2°C. The cellular pellet was resuspended in the same volume of homogenizing buffer (20 mM tricine, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.8 at 0°C), homogenized with a Teflon-glass potter (10 strokes) then centrifuged at 35,000 *g* for 15 min. The supernatant thus obtained was centrifuged at 150,000 *g* for 90 min, yielding 4.5 ml of cytosol at about 15 mg of protein per ml, according to Lowry's method [17].

Binding in an acellular medium

Seventy-five μl cytosol was added to 175 μl of homogenizing buffer supplemented with 2 mM DTT, 2 mM EDTA, 10 mM sodium molybdate and containing 5 nM radioactive dexamethasone and varying concentrations of nonradioactive steroid (10^{-9} to 10^{-6} M). The total ethanol concentration in the final incubation medium was 1%. Duplicate samples were

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incubated for 2 h at 0°C. Incubation was stopped by the addition of 50 μl of dextran-coated charcoal (DCC: 10% Norit charcoal, 1% dextran T70). They were left for 10 min at 0°C, and then centrifuged at 5,000 g for 10 min. Radioactivity was measured in 150 μl of supernatant by liquid scintillation using a Packard model 3320 or 460 CD counter.

Association and dissociation of complexes in an acellular medium

Association. The cytosol was incubated with [^{3}H]dexamethasone (6 nM) or [^{3}H] RU 38486 (6 nM) in the presence or absence of non-radioactive dexamethasone (7.5 μM). Incubations were performed for various times (from 5 min to 4 h), at 0°C. The amounts of specific complexes were evaluated by the DCC method as described above.

Dissociation. Either radioactive dexamethasone (10 nM) or radioactive RU 38486 (10 nM) was added to 2 tubes of 500 μl of cytosol supplemented with 2 mM DTT, 2 mM EDTA, and 10 mM sodium molybdate. After incubations for 2 h at 0°C, the samples were treated with 100 μl dextran charcoal for 10 min, and then centrifuged for 10 min at 5,000 g. Non-radioactive dexamethasone (1 μm) or non-radioactive RU 38486 (1 μM) was added respectively to the different supernatants. Fifty μl fractions were removed from the 2 samples at different time intervals, treated with 200 μl of dextran charcoal (Dextran T70, 0.25%; charcoal 2.5%) and centrifuged. Supernatant radioactivity was measured by liquid scintillation.

Whole cell studies

The HTC cells were cultured in monolayer in 60 mm Petri dishes. One day before confluence, the culture medium was replaced by 3 ml of fresh medium containing different concentrations of RU 38486 with or without different concentrations of dexamethasone. The final ethanol concentration was 1%. After 14–16 h of incubation, the medium was eliminated, the cells were washed at 0°C with a PBS solution, harvested by scraping, and centrifuged for 5 min at 1,200 g. The cellular pellet was resuspended in 1 ml of sonication buffer at pH 7.6 (0.1 M potassium phosphate, 0.2 mM PLP, 0.5 mM α-ketoglutarate) and lysed with an MSE sonicator. Each sample (in duplicate) was assayed for its protein content according to Lowry's method[17], and for tyrosine amino-transferase (TAT) according to Diamondstone's method[18] modified by Valeriote[19]. Results are expressed in (nmoles) × (mg of proteins)$^{-1}$ × min^{-1}.

In vivo *studies*

Sprague–Dawley male rats weighing 160–180 g were adrenalectomized 4–7 days before use. RU 38486 was administered orally, suspended in CMCT (aqueous solution containing 0.25% carboxymethyl cellulose and 0.2% polysorbate 80) in a volume of 5 ml per kg of body weight. One hour later the animals received a single intraperitoneal injection of dexamethasone (10 μg/kg of body weight in CMCT). Animals were sacrificed 4 h after dexamethasone treatment. Livers were removed and homogenized and the homogenates were then centrifuged at 105,000 g for 90 min. TAT and TO were assayed according to Diamondstone[18] and Knox and Auerbach[20] respectively. Results are expressed in (nmoles) × (mg of proteins or g of tissue)$^{-1}$ × min^{-1} for TAT and TO respectively.

RESULTS

Figure 1 shows the relative binding affinities (RBA) of RU 38486 for the cytosolic glucocorticoid receptor. We observed that the affinity of RU 38486 was 2–3 times higher than that of dexamethasone for the specific glucocorticoid binding site. This result agrees with those already obtained with this compound on the rat thymus receptor [21]. It is noteworthy that RBAs in 2 and 20-h competition experiments had approximately the same values (data not shown). In order to explain this high RBA value we compared the association and dissociation rates of the RU 38486–glucocorticoid receptor complex with those of dexamethasone. As illustrated in Fig. 2A, RU 38486 is characterized by an association rate equal to that of dexamethasone, since, for equal values of the steroid concentrations in the same cytosol, the steroid–receptor complex amounts are equal, with the two compounds, at early incubation times (at which the dissociation of a complex is negligible). RU 38486 is also characterized by a very low dissociation rate of its complex from the glucocorticoid receptor: we can see (Fig. 2B) that the half-life of the RU 38486 complexes ($t_{\frac{1}{2}} \gg 24$ h) was much longer than that of dexamethasone ($t_{\frac{1}{2}} = 12$ h). Thus, the high affinity of

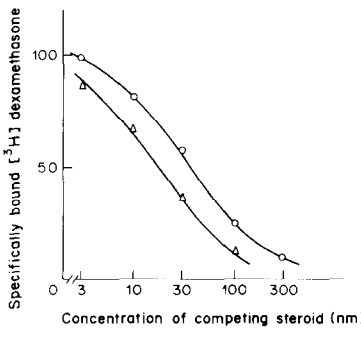

Fig. 1. Cell-free competition of radioactive dexamethasone binding at 2 h. Duplicate crude HTC cytosol glucocorticoid receptor solutions (10.1 nM) were incubated with various concentrations of dexamethasone (○) or RU 38486 (△). The average specifically bound [^{3}H]dexamethasone is plotted as a % of control binding without competitor. The 100% value corresponds to a tritiated dexamethasone–receptor complex concentration of 3.3 nM.

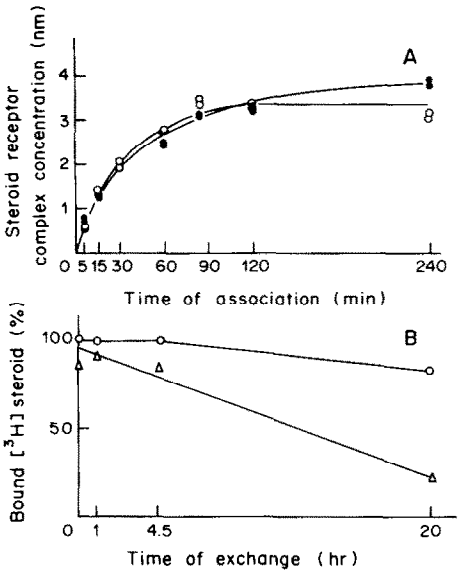

Fig. 2. Time course of association (A) and dissociation (B) reactions of dexamethasone and RU 38486 with HTC cell receptors. (A) The amount of specifically bound dexamethasone (○) or RU 38486 (●) is expressed as the concentration of the steroid–receptor complexes at various incubation times. The cytosolic glucocorticoid receptor concentration was 10.5 nM. (B) The amounts of specifically bound dexamethasone (△) or RU 38486 (○), at various times, are expressed as % of the total amount of complexes obtained at equilibrium (before addition of the radioinert steroids). The 100% values correspond to the concentrations of 6.3 nM dexamethasone–receptor complex or 7.6 nM RU 38486–receptor complex in the incubation solutions.

this compound was probably due to a very high stability of its complex with the receptor at 0°C.

The effects of dexamethasone and RU 38486 on HTC cells are reported in Table 1. We observed that dexamethasone induced TAT activity: the maximum reached at 100 nm was 8 times higher than the basal level. Under the same experimental conditions, RU 38486 was devoid of any glycocorticoid activity up to 10 μM.

With respect to its antagonist activity (Fig. 3), RU 38486 totally inhibited TAT induction in a dose-dependent manner regardless of the concentrations of dexamethasone. For a given concentration value of this glucocorticoid, the TAT activity was decreased by half with a concentration of RU 38486 that was 6–7 times lower than that of the dexamethasone. These results are in agreement with the RBAs of these compounds.

The study of the biological activities of this antagonist was carried out *in vivo* on hepatic TAT and TO activities in adrenalectomized rats. We observed (Fig. 4) that at a dose of 0.01 mg/kg, dexamethasone induced a 7.5-fold increase in TAT activity and a 4.5-fold increase in TO activity, whereas at doses of up to 50 mg/kg, RU 38486 did not display any glucocorticoid effect on these two responses.

Table 1. Specific activity* of TAT induced by dexamethasone and by RU 38486

Concentration (M)	10^{-9}	3.10^{-9}	10^{-8}	3.10^{-8}	10^{-7}	3.10^{-7}	10^{-6}	10^{-5}
Dexamethasone		15.2 ± 0.7	38.1 ± 1.8	56.2 ± 0.3	63.3 ± 5.1	65.0 ± 3.3		
RU 38 486	7.35 ± 1		7.4 ± 0.3		7.0 ± 0.6		7.85 ± 0.45	6.75 ± 0.7

*Expressed in nanomoles of substrate transformed per minute and per mg of protein. The basal level was 8.2 ± 0.5 nmol min^{-1} mg^{-1} (mean ± SD, $n = 4$).

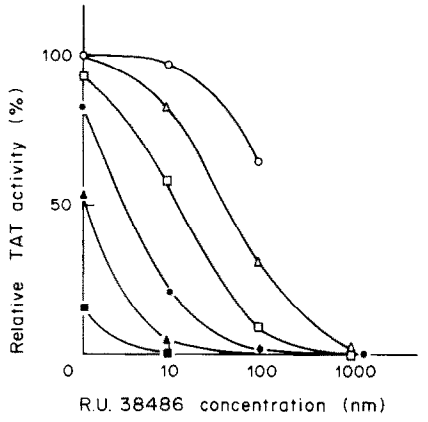

Fig. 3. Antagonistic activity of RU 38486 in whole HTC cells. Duplicate monolayer cultures of HTC cells were incubated with dexamethasone at 1000 nM (○), 300 nM (△), 100 nM (□), 30 nM (●), 10 nM (▲) and 3 nM (■) in the presence of various concentrations of RU 38486. The specific activity of TAT in cell extract is plotted as % of control without competitor. 100% (maximal induction) and 0% (basal level) correspond to TAT activities of 65 and 8.2 nmol/min × mg proteins, respectively.

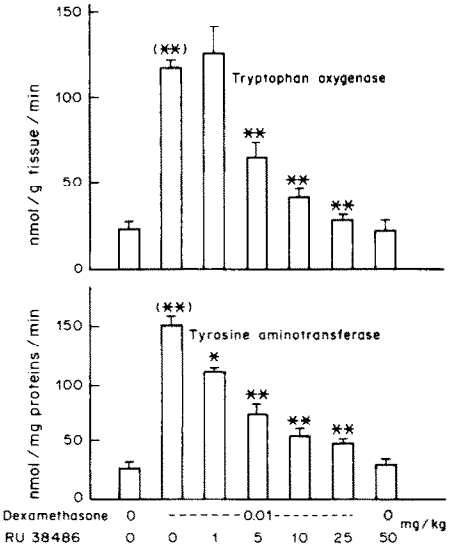

Fig. 4. Effect of RU 38486 on hepatic tryptophan oxygenase and tyrosine aminotransferase in adrenalectomized rats. All bars are means ± SEM of 9 values (3 rats per dose and 3 assays per rat). Statistical comparisons between groups were made using Dunnett's test [31]. *: $P < 0.05$ and **: $P < 0.01$, compared to the dexamethasone-treated group; (**): $P < 0.01$, compared to the control group.

When increased oral doses of RU 38486 were administered 1 h before dexamethasone, it exhibited a full antiglucocorticoid effect on these two enzymatic activities. Total inhibition was obtained at a dose of 25 mg/kg and a 50% effect was obtained at a dose of about 5 mg/kg.

DISCUSSION

At present, RU 38486 appears to be the most potent antiglucocorticoid known both *in vitro* and *in vivo*. Its originality lies in the fact that it exhibits a very strong affinity for the glucocorticoid receptor (10–100 times higher than the conventional antiglucocorticoids). Its interaction with the receptor is characterized by a very slow dissociation rate, even slower than that of dexamethasone.

In an *in vitro* system it exerted a full antagonist effect on TAT induction by dexamethasone. In HTC cells, its IC50 was 6–7 times lower than the concentration of dexamethasone used. These results are in close agreement with those obtained by several authors using various cellular models [22–28].

In vivo in ADX rats, RU 38486 completely inhibited the induction of TO and TAT by dexamethasone, but in these cases its ED50 was much higher than the dose of dexamethasone used. This high ED50 value, which could be due to an unfavorable tissue distribution or to a rapid metabolism of RU 38486, can be correlated with its low capacity for occupying the glucocorticoid specific binding sites *in vivo* [29]. We observed that, unlike the antiglucocorticoids known up to now, RU 38486 has a full antagonistic effect on the two enzymatic activities without exhibiting any glucocorticoid effect. Besides its antiglucocorticoid activity, RU 38486, now called RU 486, is also known for its potent antiprogestin property both in animals [29] and in humans [30]. In conclusion, RU 38486 constitutes a very useful tool for elucidating the mechanism of glucocorticoid action. Work is now in progress in our laboratory, using an HTC cell model, to determine the events involved in its potent antagonist action.

REFERENCES

1. Munck A. and Brink-Johnsen T.: Specific and non specific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells *in vitro*. *J. biol. Chem.* **243** (1968) 5556 5565.
2. Samuels H. H. and Tomkins G. M: Relation of steroid structure to enzyme induction in hepatoma tissue culture cells. *J. molec. Biol.* **52** (1970) 57–74.
3. Rousseau G. G. and Schmit J. P.: Structure–activity relationships for glucocorticoids—I: Determination of receptor binding and biological activity. *J. steroid Biochem.* **8** (1977) 911–919.
4. Thompson E. B.: Liver cells (HTC). In *Methods in Enzymology*. (Edited by W. B. Jakoby and I. H. Pastan). Academic Press, New York Vol. LVIII (1979), pp. 544–552.
5. Rousseau G. G., Kirchhoff J., Formstecher P. and Lustenberger P.: 17β-Carboxamide steroids are a new class of glucocorticoid antagonists. *Nature* **279** (1979) 158–160.
6. Simons S. S. and Thompson E. B.: Dexamethasone 21-mesylate: an affinity label of glucocorticoid receptors form rat hepatoma tissue culture cells. *Proc. natn. Acad. Sci. U.S.A.* **78** (1981) 3541–3545.
7. Lamontagne N., Mercier L., Pons M., Thompson E. B. and Simons S. S.: Glucocorticoid *vs* antiglucocorticoid activity: can a single functional group modification of glucocorticoid steroid always convey antiglucocorticoid activity? *Endocrinology* **114** (1984) 2252–2263.
8. Chrousos G. P., Cutler G. B. Jr, Simons S. S. Jr, Pons M., John L. S., Moriarty R. M. and Loriaux D. L.: Development of antiglucocorticoids with potential

clinical usefulness. In *Progress in Research and Clinical Application of Corticosteroids* (Edited by H. J. Lee and T. J. Fitzgerald). Heyden, Philadelphia (1982) pp. 152–176.
9. Chrousos G. P., Cutler G. B. Jr, Sauer M., Simons S. S. Jr and Loriaux D. L.: Development of glucocorticoid antagonists. *Pharmac. Ther.* **20** (1983) 263–281.
10. Cutler G. B., Barnes K. M., Sauer M. A. and Loriaux D. L.: 11-Deoxycortisol: a glucocorticoid antagonist *in vivo*. *Endocrinology* **104** (1979) 1839–1844.
11. Chrousos G. P., Barnes K. M., Sauer M. A., Loriaux D. L. and Cutler G. B.: $\Delta^{1,9(11)}$-11-Deoxycortisol: an improved glucocorticoid antagonist. *Endocrinology* **107** (1980) 472–477.
12. Chrousos G. P., Sauer M. A., Loriaux D. L. and Cutler G. B.: Δ^{1}-11-oxa-11-deoxycortisol: A new antiglucocorticoid with activity *in vivo*. *Steroids* **40** 425–431.
13. Guthrie G. P. and John W. J.: The *in vivo* glucocorticoid and antiglucocorticoid actions of medroxyprogesterone acetate. *Endocrinology* **107** (1980) 1393–1396.
14. Naylor P. H., Gilani S. S. H., Milholland R. J. and Rosen F.: Antiglucocorticoids: *in vivo* assay and evaluation of cortexolone, progesterone, and 6-β-bromoprogesterone. *Endocrinology* **107** (1980) 117–121.
15. Naylor P. H. and Rosen F.: Antiglucocorticoids: Differential Antagonism of In Vivo Responses to Dexamethasone. In *Hormone Antagonists* (Edited by M. K. Agarwal). Walter de Gruyter, Berlin (1982) pp. 365–380.
16. Duncan M. R. and Duncan G. R.: An *in vivo* study of the action of antiglucocorticoids on thymus weight ratio, antibody titre and the adrenal–pituitary–hypothalamus axis. *J. steroid Biochem.* **10** (1979) 245–259.
17. Lowry O. H., Rosenbrough N. I., Farz A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
18. Diamondstone I. J.: Assay of tyrosine transaminase activity by conversion of p. hydroxyphenylpyruvate to p. hydroxybenzaldehyde. *Anal. Biochem.* **16** (1966) 395–401.
19. Valeriote F. A., Auricchio F., Tomkins G. M. and Riley D.: Purification and properties of rat liver tyrosine amino transferase. *J. biol. Chem.* **244** (1969) 3618–3624.
20. Knox W. E. and Auerbach V. H.: The hormonal control of tryptophan peroxidase in the rat. *J. biol. Chem.* **214** (1955) 305–313.
21. Philibert D., Deraedt R. and Teutsch G.: RU 38486. A potent antiglucocorticoid *in vivo*. *Eighth International Congress of Pharmacology, Tokyo* (1981) Abstract 1463 p. 668.
22. Chobert M. N., Barouki R., Finidori J., Aggerbeck M., Hanoune J., Philibert D. and Deraedt R.: Antiglucocorticoid properties of RU 38486 in a differentiated hepatoma cell line. *Biochem. Pharmac.* **32** (1983) 3481–3483.
23. Jung-Testas I. and Baulieu E. E.: Anti-steroid action in cultured L 929 mouse fibroblasts. *J. steroid Biochem.* **20** (1984) 301–306.
24. Sakly R., Philibert D., Lutz-Bucher B. and Koch B.: Paradoxical involvement of glucocorticoid receptors in the aldosterone-induced impairment of ACTH secretion from perifused pituitary glands. *J. steroid Biochem.* **20** (1984) 1101–1104.
25. Moguilewsky M. and Philibert D.: RU 38486: potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. *J. steroid Biochem.* **20** (1984) 271–276.
26. Duval D., Durant S. and Homo-Delarche F.: Effect of antiglucocorticoids on dexamethasone-induced inhibition of uridine incorporation and cell lysis in isolated mouse thymocytes. *J. steroid Biochem.* **20** (1984) 283–287.
27. Bourgeois S., Pfahl M., and Baulieu E. E.: DNA binding properties of glucocorticoid receptors bound to the steroid antagonist RU-486. *EMBO J.* **3** (1984) 751–755.
28. Chasserot-Golaz S. and Beck G.: An approach to the mechanism of the potent antiglucocorticoid: 17β-hydroxy-11β-4-dimethylaminophenyl-17-propynyl-estra-4,9-dien-3-one. *J. steroid Biochem.* **21** (1984) 585–591.
29. Philibert D.: *RU 38486: An Original Multifaceted Antihormone* In Vivo (Edited by M. K. Agarwal). Walter de Gruyter. Berlin, New York (1984) pp. 77–101.
30. Herrmann W., Wyss R., Riondel A., Philibert D., Teutsch G., Sakiz E. and Baulieu E. E.: Effet d'un stéroïde antiprogestérone chez la femme: interruption du cycle menstruel et de la grossesse au début *C.r. hebd. Séanc. Acad. Sci. Paris.* **294** (1982) 933–938.
31. Dunnett C. W.: A multiple comparison procedure for comparing several treatments with a control. *Am. Stat. Assoc. Jl* **50** (1955) 1096–1121.