Biochemical Pharmacology, Vol. 32, No. 22, pp. 3481–3483, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

Antiglucocorticoid properties of RU 38486 in a differentiated hepatoma cell line

(*Received* 18 *March* 1983; *accepted* 2 *June* 1983)

Investigation of the mechanism of steroid hormone action using sex hormones and aldosterone antagonists has led to some important clinical applications [1, 2]. Some of the many synthetic analogs that have been used to characterize the biological effects of glucocorticoids *in vivo* and *in vitro* have proved to be potent antagonists [3–10]; most of those compounds also expressed agonist properties. A new synthetic steroid, RU 38486 (Fig. 1) was shown to have a high affinity for the glucocorticoid receptor and to display antiglucocorticoid properties *in vivo* [11, 12]. It was recently found that this drug also bound to the progestin receptor and inhibited progesterone effects [13, 14]. Both antiglucocorticoid and antiprogesterone potencies of RU 38486 have been found in humans thus allowing potential clinical applications [15]. Because of the dual properties of RU 38486 and of its potential use in therapy, more *in vitro* studies are needed in order to elucidate its effects at the cellular level.

We have recently shown that glucocorticoids induce the activity of a plasma membrane enzyme, gamma-glutamyltransferase (EC 2.3.2.2.) in a highly differentiated hepatoma cell line, Fao [16]. We report here the biological properties of RU 38486 in this new cell system, and demonstrate that it behaves as a potent glucocorticoid antagonist, devoid of agonist effects.

Materials and methods

[11β-(4-Dimethyl aminophenyl) 17β-hydroxy, 17α-(prop-1-ynyl) estra 4,9-dien-3-one] (Fig. 1), RU38486 was synthesized by the Centre de Recherche Roussel Uclaf (Roumainville, France). Dexamethasone and L-gamma-glutamyl-paranitroanilide, were purchased from Sigma and progesterone from Roussel Uclaf. Glycylglycine was obtained from the Calbiochem and bovine serum albumin from Miles.

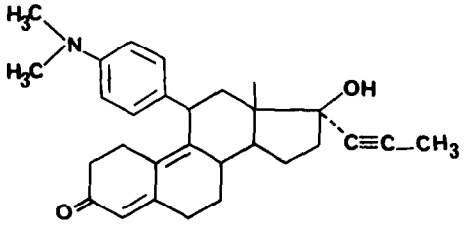
Cell culture The rat hepatoma clone Fao derived from line H_4IIEC_3 of Reuber H_{35} hepatoma was grown in monolayer culture in a Ham's F_{12} medium (Gibco) complemented with 5% fetal calf serum (Biopro-Seromed), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Flow laboratories). Cells were routinely cultured as described elsewhere [16].

Treatment of the cells Experiments were performed during the exponential phase of growth on duplicate monolayer cultures, seeded at a density of 1–2 × 10^5 cells/60 mm petri dish. In typical experiments, cells were exposed to fresh medium containing ethanol that never exceeded 0.5%, with or without 0.1 μM dexamethasone and increasing concentrations of RU 38486 or progesterone at 37° for 48 h.

Gamma-glutamyltransferase activity determination The culture medium was removed and the monolayer culture washed with phosphate buffered saline pH 7.4. The cells were detached with a scraper, centrifuged at 300 *g* for five minutes and resuspended in PBS (Gibco). For each experimental point, duplicate cultures were pooled and duplicate assays were run, just after cell preparation. Gamma-glutamyltransferase activity was determined according to the method of Orlowski and Meister [17] on 0.1 ml aliquots containing 100–300 μg of protein, as described previously [16] and expressed as m U/mg proteins. One mU corresponds to 1 nmole of product formed/min. Proteins were estimated by the Lowry's procedure [18].

Results and discussion

Gamma-glutamyltransferase is a specific glucocorticoid inducible plasma membrane enzyme in Fao cells. Dexamethasone maximally induces the enzyme at 0.1 μM; half maximal induction ocurred at 15 nM [16]. The inhibition of dexamethasone induction of gamma-glutamyltransferase activity by RU 38486 is depicted in Fig. 2. When cells were treated by increasing concentrations of RU 38486 in the presence of 0.1 μM dexamethasone during 48 h, the 2.3-fold induction by the glucocorticoids was prevented in a dose dependent manner. Complete inhibition was achieved at 1 μM RU 38486; half-maximal inhibition was attained at 56 nM RU 38486. The apparent inhibitory constant,



RU 38486

Fig. 1. Structure of RU 38486.

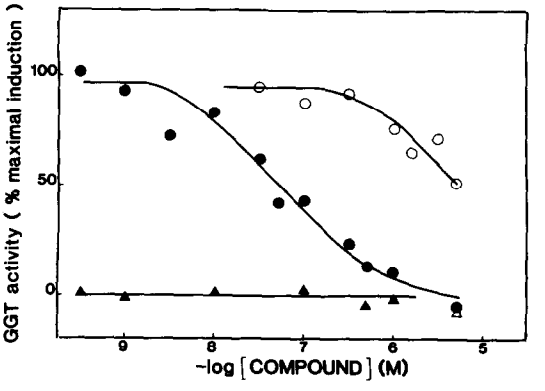


Fig. 2. Antiglucocorticoid effect of RU38486 and progesterone. Fao cells, in exponential growth, were exposed to varying concentrations of RU38486 (●, ▲) or progesterone (○, △) in the presence (●, ○) or absence (▲, △) of 0.1 μM dexamethasone during 48 h. Gamma-glutamyltransferase activity is expressed as percent of the activity obtained with 0.1 μM dexamethasone alone. The means of the basal and maximal activity were respectively 1.56 and 3.57 mU/mg proteins. Each experimental point is the mean of duplicate determinations of 2 (control and progesterone) to 4 (RU38486) different experiments.

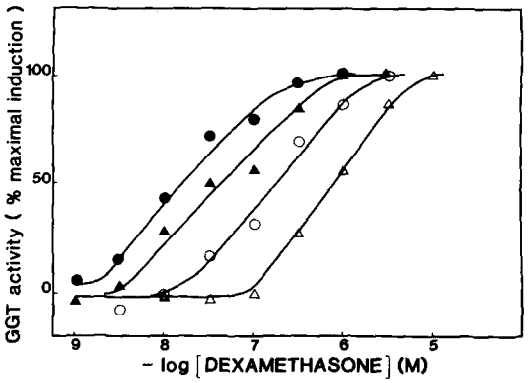


Fig. 3. Shift of the dexamethasone dose-response curve by increasing concentrations of RU 38486. Fao cells were treated during 48 h by increasing concentrations of dexamethasone without (●) or with 20 nM (▲), 0.3 μM (○) and 1 μM (△) RU 38486. Two separate experiments were averaged for each point, each assay being done in duplicate. The gammaglutamyltransferase activity is expressed as percent of maximal activity. The basal and the maximal activity were 1.47 mU/mg and 3.7 mU/mg protein, respectively.

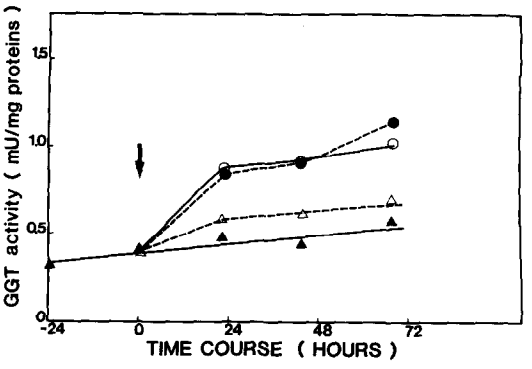


Fig. 4. Reversibility of the antiglucocorticoid effect of RU 38486 as a function of time. Fao cells, in exponential phase of growth, were incubated without or with 0.5 μM RU38486. After 24 h of pretreatment, monolayer cultures were extensively washed with fresh medium (arrow). RU 38486 pretreated cells were then treated with 0.1 μM dexamethasone alone (●) or with 0.1 μM dexamethasone and 0.5 μM RU 38486 (△). Control cells were treated with (○) or without (▲) 0.1 μM dexamethasone. At various time points, duplicate culture petri dishes were washed, cells were detached, pooled and resuspended in PBS for the determination of gamma-glutamyltransferase activity in duplicate. This experiment was repeated twice.

calculated according to Cheng and Prusoff [19], would be 7.3 nM; therefore RU 38486 appears to have a higher apparent affinity than dexamethasone in our system. In comparison, progesterone inhibited the induction with a much lower affinity, half-maximal inhibition being observed at 5 μM. The apparent inhibitory constant [19] of progesterone is at least 100-fold higher than that of RU 38486. When RU 38486 was used under the same conditions, but without dexamethasone, it did not enhance gamma-glutamyltransferase activity (Fig. 2). Therefore, this synthetic steroid molecule is devoid of agonist effect in our system. These results are in agreement with the direct binding studies of RU 38486 to the glucocorticoid receptor [11].

In order to investigate whether RU38486 is a competitive inhibitor of dexamethasone, we tested the effect of varying concentrations of RU 38486 upon the dose dependent dexamethasone effect. RU 38486 provoked a parallel rightwards shift in the dexamethasone dose-response curves (Fig. 3). The concentration of dexamethasone giving half maximal effect varied from 15 nM for dexamethasone alone to 0.7 μM in the presence of 1 μM RU 38486; all the curves reached the level of the maximal induction. This result is compatible with a competitive inhibition mechanism of RU 38486 in interfering with dexamethasone effect.

RU 38486 displayed very rapid inhibitory effects in cells preincubated with 0.1 μM dexamethasone during 48 h; a 35% inhibition of induced gamma-glutamyltransferase activity ocurred as early as 6 h after the addition of 0.5 μM RU38486 (data not shown). On the other hand, the reversibility of RU 38486 action was studied in cells preincubated with 0.5 μM antiglucocorticoid for 24 h and then washed extensively. As shown in Fig. 4, addition of 0.1 μM dexamethasone induced gamma-glutamyltransferase activity with the same time course and to the same extent in control and RU 38486 pretreated cells. When RU38486 was maintained in the cultures after dexamethasone addition, we observed, as expected, the marked inhibitory effect of the drug. Therefore, the antiglucocorticoid effect of RU 38486 is rapid, potent and reversible.

Among synthetic steroid molecules, RU 38486 represents a new class of 11β substituted steroid antihormones. This antagonist is the only one known to date which shows an affinity for the glucocorticoid receptor which is clearly larger than that of dexamethasone [11]. *In vivo*, it exerts a total antiglucocorticoid activity without agonist effect [11, 12]. In our *in vitro* system, namely the dexamethasone induction of gamma-glutamyltransferase in Fao cells, RU 38486 has the same antagonist properties, devoid of agonist effects. It is noteworhty that most of the antiglucocorticoids studied so far, such as cortexolone and 6 bromoprogesterone [6], medroxyprogesterone acetate [7] and dexamethasone 21 mesylate [10], are not pure antagonists. In contrast to cortisol 21 mesylate [9], which is an irreversible antiglucocorticoid *in vitro*, RU 38486 is rapidly reversible; such a property may be important for clinical applications. Finally, since RU 38486 has been reported to interact with the progesterone receptor, we have compared the effect of RU 38486 and progesterone in our system and found that RU 38486 was at least 100-fold more potent than progesterone in inhibiting the induction of GGT by dexamethasone. The effect of progesterone can be accounted for by its antiglucocorticoid properties at high concentrations. It is therefore very likely that RU 38486 acts in our system by competitively inhibiting glucocorticoid effects.

In conclusion, the dexamethasone induction of GGT activity in the highly differentiated Fao cells, which is similar to that observed in adult rat liver [16, 20] and probably due to an increase in specific mRNA synthesis [16], is a very convenient system both for the study of the molecular mechanisms of glucocorticoid action, and for the screening of antiglucocorticoid drugs.

Acknowledgements—This work was supported by the Institut National de la Santé et de la Recherche Médicale, The Délégation Générale à la Recherche Scientifique et Technique and the Centre National de la Recherche Scientifique. We wish to thank Dr. Mary C. Weiss and Dr. Jacqueline Levilliers, from the CNRS, Gif sur Yvette, France, for providing Fao cells and for excellent advice, Dr. L. Aggerbeck for his critical review and C. Petit and V. Poli for skillful secretarial assistance

Unité INSERM 99,
Hôpital Henri Mondor,
94010 Créteil, France

MARIE-NOËLE CHOBERT
ROBERT BAROUKI
JOËLLE FINIDORI
MARTINE AGGERBECK
JACQUES HANOUNE

Centre de Recherche Roussel Uclaf,
93230 Romainville, France

DANIEL PHILIBERT
ROGER DERAEDT

REFERENCES

1. J. H. Clark, E. J. Peck Jr, J. W. Hardin and H. Erikson, Receptors and Hormone Action (Eds. B. W. O'Malley and L. Birnbaumer Vol. 2, p. 1, Academic Press, New York. (1978).
2. G. M. Addison, N. W. Asmussen, P. Corvol, P. Kloppenborg, N. Norman, R. Schroder and J. I. S. Robertson Eds. *Aldosterone Antagonists in Clinical Medicine*, Excerpta Medica, Amsterdam (1978).
3. P. H. Naylor and F. Rosen, in *Hormone Antagonists* (Ed. M. K. Agarwal), p. 366. Elsevier/North Holland, Amsterdam (1982).
4. G. P. Chrousos, G. B. Cutler Jr, S. S. Simons Jr, M. Pons, D. Lynn-Loriaux, L. S. John, R. M. Moriarty, in *The Proceeding of the VIth Annual Clinical Pharmacy Symposium*, Heydun and Son Inc., Phila, PA, p. 152 (1982).
5. G. B. Cutler Jr, K. M. Barnes, M. A. Saver and D. Lynn-Loriaux, *Endocrinology* **104**, 1839 (1979).
6. P. H. Naylor, S. S. Gilani, R. J. Milholland, M. Ip and F. Rosen, *J. Steroid Biochem.* **14**, 1303 (1981).
7. P. G. Guthrie Jr and W. J. John, *Endocrinology* **107**, 1393 (1980).
8. G. G. Rousseau, J. Kirchhoff, P. Formstecher and P. Lustenberger, *Nature, Lond.* **279**, 158 (1979).
9. S. S. Simons Jr, E. B. Thompson and D. F. Johnson, *Proc. Natn. Acad. Sci.* U.S.A. **77**, 5167 (1980).
10. S. S. Simons Jr and E. B. Thompson, *Proc. Natn. Acad. Sci.* U.S.A. **78**, 3541 (1981).
11. D. Philibert, R. Deraedt and G. Teutsch, *VIII Int. Congress of Pharmacology, Tokyo*, Abst. 1463 (1981).
12. M. Moguilewsky, R. Deraedt, G. Teutsch and D. Philibert, *J. Steroid Biochem, 6th International Congress on Hormonal Steroids. Jerusalem*, Abst. 203 (1982).
13. D. Philibert, R. Deraedt, G. Teutsch, C. Tournemine and E. Sakiz, *64th Meeting of Endocrine Society*, San Francisco, Abst. 668 (1982).
14. D. Philibert, R. Deraedt, C. Tournemine, I. Mary and G. Teutsch, *J. Steroid Biochem., 6th International Congress on Hormonal Steroids, Jerusalem* Abst. 204 (1982).
15. W. Hermann, R. Wyss, A. Riondel, D. Philibert, G. Teutsch, E. Sakiz and E. E. Baulieu, *C.R. hebd. Séanc., Paris Acad. Sci.* **294**, 933 (1982).
16. R. Barouki, M. N. Chobert, M. C. Billon, J. Finidori, R. Tsapis and J. Hanoune, *Biochim. Biophys. Acta.* **721**, 11 (1982).
17. M. Orlowski and A. Meister, *J. Biol. Chem.* **240**, 338 (1965).
18. O. H. Lowry, M. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
19. Y. Cheng and W. H. Prusoff. *Biochem. Pharmac.* **22**, 3099 (1973).
20. M. C. Billon, G. Dupré and J. Hanoune, *Mol. Cell. Endocr.* **18**, 99 (1980).

Biochemical Pharmacology, Vol. 32, No. 22, pp. 3483–3485, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

The effect of cysteine and N-acetyl cysteine on rat liver glutathione (GSH)

(*Received* 11 *April* 1983; *accepted* 8 *June* 1983)

Cysteine and its derivatives are extensively used as mucolytics or as protective agents against acetaminophen (paracetamol) toxicity [1].

However, several reports in the literature indicate that moderate doses of cysteine can be toxic when added to tissue cultures [2], when injected to rats [3–5] or when given orally with the diet [6].

We reported [7] that intraperitoneal injections of large doses of N-acetyl cysteine (NAC) decreased GSH content of rat liver and that incubation of isolated hepatocytes in saline solution of Krebs and Henseleit [8] with cysteine (above 0.5 mM) decreased their GSH content [9]. However, in a recent study, Beatty and Reed [10] reported that incubation of hepatocytes with cysteine did not induce GSH depletion. This, together with the fact that NAC is used as a treatment of paracetamol overdose, prompted us to undertake a systemactic study of the effects of cysteine and NAC administered orally or intraperitoneally to control rats and to rats treated with high doses of paracetamol.

Experimental

Animals. Wistar rats (150–200 g body wt.) were fed *ad libitum* on a standard diet for rats and mice (Prada, Vara de Quart, Valencia, Spain). They always had free access to food and water. Although some previous studies on acetaminophen toxicity in rats have been carried out in fasted rats [11], we used well fed animals because fasting significantly decreases hepatic GSH content in rats [12].

Acetaminophen was injected intraperitoneally in a small volume of a 10% dimethyl sulfoxide solution in a physiological saline. Amino acids were dissolved in a small volume of physiological saline and administered orally or intraperitoneally. It was tested that injection of similar amounts of both solvents did not affect hepatic GSH content.

The dose of paracetamol injected was 0.5 g/kg body wt. (3.3 mmol/Kg). A similar dose had been previously used by other authors [11].

Chemicals. Paracetamol was a gift of the department of Pharmacy of the Faculty of Medicine (Valencia, Spain). Dimethyl sulfoxide was purchased from Sigma Chemical Company, St. Louis, MO., U.S.A. L-cysteine was from Merck (Darmstadt, Germany) and N-acetyl cysteine from Zambon Laboratories (Barcelona, Spain). All other chemicals were of the highest purity available.