Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 793-800

ANTI-INFLAMMATORY PYRAZOLO-STEROIDS: POTENT GLUCOCORTICOIDS CONTAINING BULKY A-RING SUBSTITUENTS AND NO C_3-CARBONYL.#

S.S. Simons, Jr.*+, E.B. Thompson‡ and D.F. Johnson+

+Laboratory of Chemistry, NIAMDD, and ‡Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Received December 15, 1978

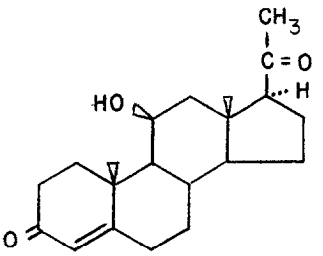
SUMMARY: Certain pyrazolo-steroids are extremely potent anti-inflammatory agents but are predicted to be inactive glucocorticoids on the basis of their structure. However, one representative compound is found to possess a high affinity for cytoplasmic glucocorticoid receptors. The biological activity of this steroid is greater than that predicted from its affinity for receptors. This may be due to an exceptionally slow rate of dissociation of the receptor-steroid complex, which would prevent an accurate determination of the equilibrium affinity constant.

Numerous structure-activity studies have led to the generalization that the pregn-4-ene-11β-ol-3,20-dione structure I contains the minimum chemical functionality required for a potent glucocorticoid (1,2). Less extensive data support the concept that bulky substituents on the steroid A-ring drastically reduce, or abolish, glucocorticoid activity (2,3). While the expression of biological activity of physiological levels of glucocorticoid steroids appears to require the formation of a cytoplasmic receptor-steroid complex (4), as is also observed with all other steroid hormones (5), many glucocorticoid induced effects are sufficiently complex that the receptor-mediated mechanism of action has not yet been specifically established for each effect. The anti-inflammatory action of glucocorticoids is an example of this situation. Most potent anti-inflammatory steroids are also potent glucocorticoids. However the use of hyperphysiological concentrations of glucocorticoids in anti-inflammatory therapy (1) raises the possibility that receptors may not be required to mediate the anti-inflammatory action. Furthermore, in our

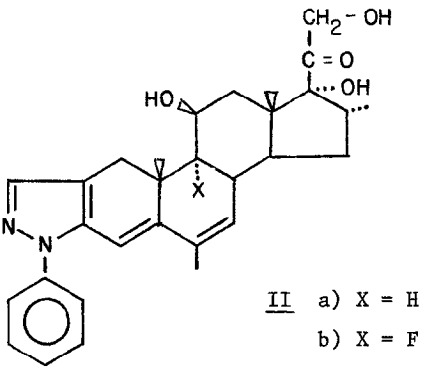
#Presented at the Worcester Foundation Symposium on Steroid Hormone Receptor Systems, October 18-20, 1978.
*Address correspondance to this author.
Abbreviations used: HTC, hepatoma tissue culture; TAT, tyrosine amino-transferase; cortisol, 4-pregnene-11β,17α,21-triol-3,20-dione; dexamethasone, 16α-methyl-9α-fluoro-1,4-pregnadiene-11β,17α,21-triol-3,20-dione.

0006-291X/79/030793-08$01.00/0

continuing study of glucocorticoid-receptor interactions (6), we were intrigued by an old (7), generally neglected (8), study of a series of steroids containing bulky A-ring substituents and no 3-keto group (e.g., II). These compounds



I



II a) X = H
b) X = F

would be predicted to have neither glucocorticoid activity nor affinity for glucocorticoid receptors, and yet they are among the most potent anti-inflammatory steroids known (7). In a systemic granuloma assay, IIa and IIb were respectively 551 and 2000 times more potent than cortisol (7). We now report that one of these atypical anti-inflammatory steroids (i.e., IIa) is also a potent glucocorticoid and binds to cytoplasmic glucocorticoid receptors. These results further define the mechanism of action of anti-inflammatory steroids and contribute to the growing body of glucocorticoid structure-activity relationships. The latter information not only will assist in the design of more potent glucocorticoids but also opens new approaches for affinity labelling of glucocorticoid binding molecules (9) since photo-labile aromatic functional groups (10) are now possible.

Results and Discussion

Studies with a line of rat hepatoma tissue culture (HTC) cells have determined that the administration of physiological levels of glucocorticoids induces a limited number of responses (11), one of which is an increase of the enzyme tyrosine aminotransferase (TAT) (12). Structure-activity and dose-response studies of gluconeogenic steroids have established that the induction of TAT in HTC cells is mediated by a cytoplasmic glucocorticoid-specific receptor (13,14). The half-maximal concentrations for induction of TAT activity

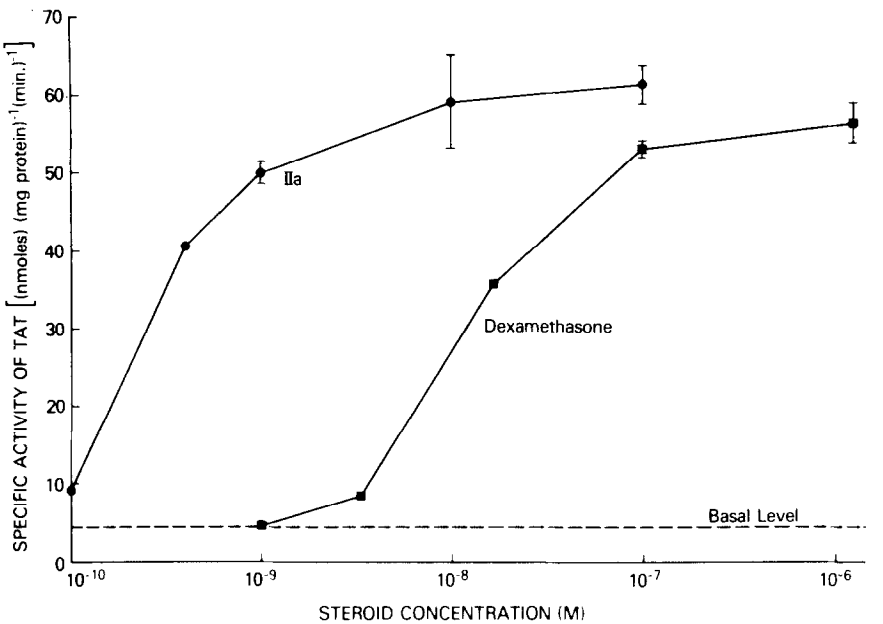
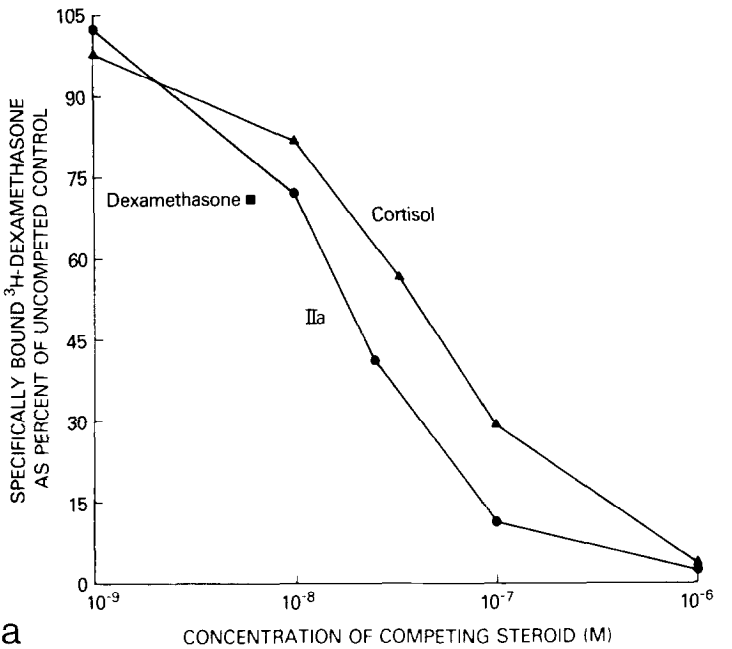
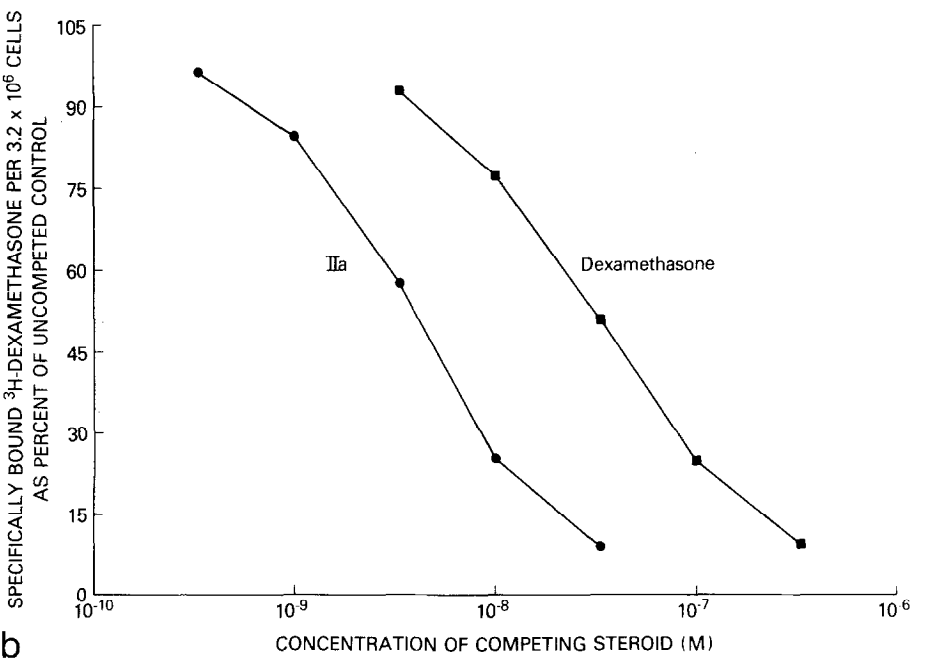


Fig. 1: Dose-response curves for the steroidal pyrazole IIa and dexamethasone: HTC cells were grown as monolayer cultures on 60mm petri dishes in improved minimal essential medium containing 5% fetal calf serum as described elsewhere (15). Fresh medium containing 1% EtOH + steroid was added and the cells were harvested after about 18 hrs. The specific enzyme activity of TAT in each dish was determined (15) and the average value for duplicate dishes is plotted against the steroid concentration of IIa (-●-) or dexamethasone (-■-) present during the 18 hr induction. The basal level of TAT activity is indicated by the dashed line. The range of each duplicate determination is shown by error bars when it exceeds the area of the data points.

by IIa and dexamethasone in HTC cells (Fig. 1) show that IIa is an optimal inducer (13) and is 35 ±9 times more potent than dexamethasone (two experiments) When steroid concentrations required to give identical levels of TAT induction are compared instead, IIa is 41 ± 8 times more potent than dexamethasone, which is 20 fold greater than that observed in a less well characterized system of mouse thymocytes (8b). Based on other experiments in HTC cells comparing the relative potency of dexamethasone and cortisol (data not shown), IIa is calculated to be 300-430 times more potent than cortisol. This degree of potency compares favorably to the factor of 551 seen in the anti-inflammatory assay (7). Steroid IIa is also is about 30 fold more potent than dexamethasone in causing lymphocytolysis in a line of human leukemic cells (16). Thus



a



b

Fig. 2: a.) Determination of relative affinities of IIa, dexamethasone and cortisol for HTC cell receptors in a cell-free competition assay: HTC cells were grown in spinner cultures, harvested (14), washed free of medium with phosphate buffered saline and stored as pellets at -20°. Steroid-free cytosol was prepared by homogenizing the cell pellets in an approximately equal volume

increased glucocorticoid activity has been achieved by eliminating the 3-keto group and adding a very bulky substituent to the A-ring of I.

The relative affinities of IIa and dexamethasone for HTC cell receptors were determined by two types of competition binding assays--the conventional cell-free assay (Fig. 2a) and a new whole HTC cell assay (Fig. 2b) with conditions similar to those of the TAT induction assay. In both competition assays, IIa exhibits a high affinity interaction with HTC cell receptors. However the conventional competition assay, which is routinely used to determine the affinity of steroid hormones for their receptors, does not faithfully reflect the situation in whole cells. Instead of showing an affinity for IIa that is much greater than that of dexamethasone, the cell-free results would suggest that IIa's affinity is actually less than that of dexamethasone.

Among the numerous possible explanations for the different affinity determinations (e.g., active transport of IIa or its conversion to a more

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of Buffer A containing 20mM N-tris-(hydroxymethyl)methylglycine, 2mM $CaCl_2$ and 1mM $MgCl_2$ (pH = 7.8 at 0°). The homogenate was centrifuged at 35,000xg at 0° for 15 min and then at 200,000xg at 0° for 60 min to give the crude cytosol at a protein concentration of about 18 mg/ml. Duplicate tubes were prepared by adding 75µl of crude cytosol to 175µl of 25 Ci/mmole [^{3}H]dexamethasone (Amersham; final conc. = 6.3×10^{-9} M), EtOH (final conc. = 1%), the competing unlabelled steroid and Buffer A. After 2 hrs at 0°, the amount of receptor-bound [^{3}H]dexamethasone was quantitated by the addition of 25µl of 10% dextran-treated activated charcoal in Buffer A, centrifugation at 2500xg at 0° for 10 min and counting 150µl of the supernatant in 10ml of Aquasol (New England Nuclear) at about 54% efficiency. Non-specific binding was determined as the bound [^{3}H]dexamethasone observed in the presence of 3×10^{-6} M [^{3}H]dexamethasone and was subtracted from the other values to give the amount of specifically bound [^{3}H]steroid. The amount of specific binding in the presence of IIa (—●—), cortisol (—▲—; Sigma) or dexamethasone (—■—) is expressed as % of the specific binding observed for 6.3×10^{-9} M [^{3}H]dexamethasone (i.e., 64,100 dpm/250µl assay volume). Each point represents the average of a duplicate determination. The relative affinity constants were determined by the method of Rodbard (17). 2b.) Determination of relative affinities of IIa and dexamethasone for HTC cell receptors in a whole cell competition assay: HTC cells in spinner culture at 5×10^5/ml were centrifuged at 400xg for 5 min at 18° and resuspended in 0.1 x original volume of growth medium containing 0.1% $NaHCO_3$. In a modification of a procedure developed for leukemia cells (16), 0.96ml of the resuspended HTC cells was added to 30µl of 2.3×10^{-7} M [^{3}H] dexamethasone and 10µl EtOH ± competing steroid. After incubation at 37° for 30 min with occasional agitation, 3 ml of 22° PBS (0.137M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 and 15.2mM Na_2HPO_4 adjusted to pH 7.7) was added to stop the binding reaction. Centrifugation rapidly followed by 3x3 ml of 22° PBS washes and resuspension in 1ml of PBS gave a solution of cells of which 0.8ml were counted in 10ml of Aquasol at 43% efficiency and 0.1ml was diluted 1:10 for hemocytometer counts. The number of cells in each tube were the same (± 10%). The average specifically bound [^{3}H]dexamethasone of duplicate tubes was plotted and analyzed as in Fig. 2a.

potent steroid in whole cells, degradation of IIa in cell extracts), we favor a kinetic explanation. The affinities of potent glucocorticoids such as dexamethasone and cortisol are largely determined by differences in off-rates (18). Thus, with a $T_{1/2}$ for dissociation for dexamethasone equal to 96 hrs at 0° (18), competition assays like those of Fig. 2 usually do not achieve equilibrium. If the off-rate of IIa is slower than that of dexamethasone, then the apparent affinity of IIa for receptor, and proximity of the competition assay to equilibrium would increase in going from 2 hrs at 0° (Fig. 2a) to 30 min at 37° (Fig. 2b). When the incubation times of the cell-free competition at 0° were extended to 22 hrs, there was no change in the relative inhibition by IIa vs dexamethasone (Fig. 3). Thus if this kinetic hypothesis is correct, even 22 hrs at 0° is not long enough to detect differences in the off rates.

The agreement between the potency of IIa vs dexamethasone for TAT induction (IIa = 35 to 41 x dexamethasone after 18 hrs at 37°) and their affinities for receptors in whole cells (IIa = 8.93 $\pm$ 0.06 x dexamethasone in two experiments after 30 min at 37°) is only fair. The factor of four difference between relative potencies and affinities could again arise from non-equilibrium in the whole cell competition assay, due to the above postulated differences in receptor-steroid dissociation rates. However, should future experiments confirm this difference, it may be that the affinity of steroids for receptors is not always the limiting factor determining steroid biological activity.

Steroidal pyrazoles such as IIa have been described as extremely potent anti-inflammatory steroids (7). We have now shown that IIa is also a potent glucocorticoid in a well defined system. The observed high affinity of IIa for the HTC cell receptors indicates that the current concepts of glucocorticoid structure-activity (1-3) should be revised. The close correlation of biological activity of IIa in the systemic granuloma and TAT induction assays suggests that the latter assay, using HTC cells, might be a much more convenient and rapid screening test for anti-inflammatory steroids than the presently used, more cumbersome assays. Furthermore, the close correlation between the

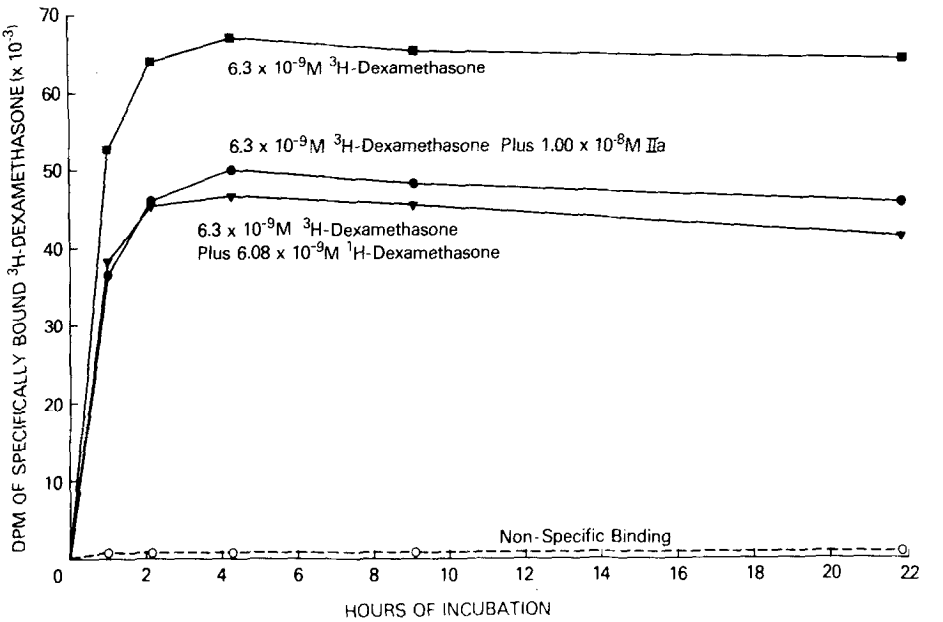

Fig. 3: Kinetics of binding in the cell-free competition assay: As described for Fig. 2a, a competition assay was constructed using 6.3 x 10^{-9} M [^{3}H]dexamethasone and 1% EtOH (-■-) ± 1.00 x 10^{-8} M IIa (-●-) or 6.08 x 10^{-9} M [^{3}H]dexamethasone (-▼-). Work-up at various times and determination of the specifically bound [^{3}H]dexamethasone were accomplished as described in Fig. 2a. Each point represents the average of a duplicate determination.

two assays supports the hypothesis that both glucocorticoid induced responses are mediated by specific steroid receptors.

Acknowledgements

We thank Dr. William Ripka (Laboratory of Chemistry, NIAMDD; on leave from DuPont) for bringing compound IIa to our attention, Merck Sharpe and Dohme Research Laboratories for a gift of IIa, Dr. John Hannah (Merck Sharpe and Dohme) for several helpful discussions, and Mrs. Billie Wagner for expert technical assistance.

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