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MOLECULAR MODIFICATIONS OF ANTI-ALDOSTERONE COMPOUNDS: EFFECTS ON AFFINITY OF SPIROLACTONES FOR RENAL ALDOSTERONE RECEPTORS*

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Abstract—The currently accepted hypothesis of the anti-mineralocorticoid action of spirolactones is that of competition for specific aldosterone receptors in target tissues. Binding of aldosterone to cytoplasmic receptors was studied by incubating adrenalectomized rat kidney slices with ^{3}H-aldosterone in the presence of 10-fold non-radioactive dexamethasone to prevent binding of tracer to glucocorticoid receptors. From the ability of a series of 24 spirolactone analogues to compete for ^{3}H-aldosterone binding sites under these conditions, the relative affinities for this receptor have been determined. Affinity for the receptor is decreased by B ring unsaturation at the C-6/C-7 position, by γ-lactone unsaturation, or by γ-lactone ring opening with the formation of the water-soluble K^+ salt. Affinity is markedly increased by esterification, or thioesterification, at the C-7 position in the B ring. Various 19-nor spirolactones show greater, equivalent or lesser affinity vis-a-vis their parent compounds. These structure–affinity relationships should define one of the determinants of pharmacologic activity.

CURRENT concepts of the mechanism of action of aldosterone include an initial step of binding to specific receptor-proteins in the cytoplasm of target tissue cells.[1] The aldosterone antagonist SC 14266 has been shown to compete for specific cytoplasmic aldosterone binding sites in renal cytosol at concentration ratios which produced anti-mineralocorticoid responses *in vivo*.[2,3] Accordingly, it appears likely that the antagonist effect of the spirolactone series of compounds is mediated by their ability to block aldosterone from specific mineralocorticoid receptors.

The method currently in use for measuring the anti-mineralocorticoid activity of various potential aldosterone antagonists is by bioassay after either parenteral or intragastric administration of the test compound. For a large number of test compounds, such assays are inevitably multi-factorial in that they involve variations between test compounds in absorption, plasma binding and rate of metabolism. If,

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as appears probable, the anti-mineralocorticoid action of a spirolactone is mediated by competing with aldosterone for mineralocorticoid receptors, then the affinity of any given compound for this receptor is an absolute factor in its potential usefulness. Recently, it has been demonstrated that ^{3}H-aldosterone binds to two classes of sites in adrenalectomized rat kidney cytosol;[4] subsequently evidence has been adduced that these sites represent physiological mineralocorticoid (type I) and glucocorticoid (type II) receptors.[5,6] In the studies to be detailed in this paper, the relative affinities of 24 different spirolactone compounds were determined for the aldosterone receptors (type I) in rat kidney cytoplasm.

MATERIALS AND METHODS

1,2-^{3}H-Aldosterone (^{3}HA), 52 Ci/m-mole, was purchased from New England Nuclear Corp. Unlabeled dexamethasone (DM) was kindly provided by Merck, Sharp & Dohme. The spirolactone analogues were generously supplied by G. D. Searle & Co. Unlabeled aldosterone (A grade) was purchased from CalBiochem Corp. Conventional reagents used were Mallinckrodt Reagent quality.

Male, Sprague–Dawley rats (150–200 g), adrenalectomized at least 5 days, were used in all experiments. Both before and after adrenalectomy, the animals were fed standard Purina lab chow; after adrenalectomy, 0·9 per cent saline was substituted for tap water as the drinking solution. Rats were killed by exsanguination via cardiac puncture under ether anesthesia, and the kidneys perfused *in situ* by the intra-aortic infusion of 20 ml ice-cold incubating solution ($Na^+ = 133$, $K^+ = 6$, $Mg^{2+} = 0{\cdot}5$, $Ca^{2+} = 1$, $Cl^- = 134$, Tris HCl = 5, glucose = 5 and $H_2PO_4^- = 6$; all in m-moles; pH 7·4). The perfused kidneys were removed, decapsulated, halved and placed in ice-cold incubating solution to await slicing. Kidney slices of 275 μm thickness were made using a McIlwain tissue chopper; in each experiment kidney slices from four rats were pooled, and the pool was then divided into 24 aliquots for incubation with steroids.

In the first series of experiments, 13 of the 24 spirolactones were studied in detail. In such experiments, 24 flasks containing kidney slices and steroids were incubated for 30 min at 25° in a waterbath (New Brunswick) with continuous agitation (150 rev/min). All flasks contained 2×10^{-9} M ^{3}HA plus 2×10^{-8} M DM. Addition of 10-fold concentration of unlabeled DM effectively limits the binding of ^{3}HA to type I receptors.[5,6] In each experiment, three aliquots of slices were incubated with these steroids alone to determine the "100 per cent" level of binding of ^{3}HA to the mineralocorticoid receptors under these conditions. In three additional flasks, 100-fold unlabeled aldosterone (2×10^{-7} M) was added to determine the level of nonspecific (low affinity, high capacity) binding. The level of nonspecific binding thus determined ranged from 7 to 11 per cent, with a mean of 10 per cent of total bound. This control series of incubations was included in each experiment to correct for nonspecific binding and ensure that the levels of specific binding were comparable between experiments. To half the remaining flasks, three concentrations of one antagonist were added and to the remainder, three concentrations of another antagonist. The concentrations of antagonist used were chosen, after preliminary experiments, in an attempt to bracket the point of 50 per cent inhibition of ^{3}HA binding to mineralocorticoid receptors.

The second series of experiments consisted of a screening procedure for the remaining 11 of the 24 aldosterone antagonists. The same concentrations of ^{3}HA and DM, and the same conditions of incubation, were used as for the first series of experiments. A 10-fold concentration (2×10^{-8} M) of each of the 11 potential antagonists was used, in a single point analysis, to estimate their ability to block the specific binding of ^{3}HA. For each compound, determinations were made in duplicate.

In both series of experiments, at the end of the period of incubation, the slices were drained under suction, rinsed with iced incubating solution, and homogenized in 2·5 ml of 0·25 M sucrose–3 mM $CaCl_2$ with eight strokes of a Potter Elvehjem homogenizer. This, and all subsequent steps, were carried out at 0–4°. After centrifugation for 30 min at 30,000 *g*, 1 ml of the supernatant was passed through 3·6 ml of G-50 fine Sephadex in a 5-ml pipet, to separate free ^{3}HA from that protein-bound. The bound ^{3}HA is unmetabolized as determined by CH_2Cl_2 extraction and chromatography.[7] Aliquots of the void volume, containing only protein-bound ^{3}HA, were taken for radioassay[7] and determination of protein concentration by the method of Warburg and Christian.[8] The radioassays were performed by adding 0·5-ml aliquots to 14·5 ml of phosphor[7] and counted for 10 min in a Mark I-Nuclear Chicago liquid scintillation spectrometer. Corrections for background and quenching (by the external standard method) were applied to each assay.

RESULTS AND DISCUSSION

A summary of all of the results is given in Table 1. Included in the table are the results of parallel experiments in which unlabeled aldosterone was used at four concentrations in order to define the affinities of the spirolactones vis-a-vis aldosterone. The equilibrium (dissociation) constant for the ^{3}HA–receptor complex at 25° is 2×10^{-9} M.[9] The concentration of ^{3}HA used in both series of experiments was chosen so that ~ half of the available receptors would be occupied even in the absence of competitor. This nonsaturating concentration provides a maximum ratio between binding to specific receptors and nonspecific binding (high capacity, low affinity); the latter increases linearly with concentration. In the present experiments, the absolute quantities bound varied from 3·5 to 4·5 $\times 10^{-14}$ moles of ^{3}HA/mg of cytosol protein. In an earlier study, we found that N_{max} was $\sim 9 \times 10^{-14}$ moles/mg protein.[9]

Quantitative estimates of relative affinities were obtained by graphic estimation of the concentration required for 50 per cent displacement of ^{3}H-aldosterone from specific binding sites.

Alterations in the structure of the γ-lactone ring *per se* resulted in a marked reduction in affinity for the aldosterone receptor. The effect of opening the γ-lactone ring, with formation of the water-soluble K^+ salt, is shown in Fig. 1. SC 9376 (canrenone) has 10 times the affinity for the mineralocorticoid receptors than its water-soluble congener SC 14266 (K^+canrenoate). The difference between the second pair of congeners, SC 26304 and SC 27169, is even more striking; in this case ring opening lowers affinity to 3–4 per cent that of the parent compound. In addition, unsaturation of the γ-lactone ring results in a marked loss of affinity for the aldosterone receptors. In Fig. 2 are shown two pairs of congeners, studied over the same range of concentrations. SC 9376 has ~ 25 times the affinity for type I receptors as does its unsa-

TABLE 1. PERCENTAGE BINDING OF TRITIATED ALDOSTERONE*

Competitor	Concentration of competitor (M)							
	$1{\cdot}6 \times 10^{-9}$	8×10^{-9}	2×10^{-8}	4×10^{-8}	2×10^{-7}	10^{-6}	5×10^{-6}	$2{\cdot}5 \times 10^{-5}$
Aldosterone	79	44	31	24	10			
SC 26304	81	49		25				
SC 19886	83	61		32				
SC 23133	93	66		38				
SC 8109		67		43	17			
SC 9420		80		46	22			
SC 10584		81		48	24			
SC 5233				54	35	20		
SC 9376				76	41	25		
SC 10039				93	57	28		
SC 27410				83	79	61		
SC 4988				81	69	54		
SC 27169					51	28	18	
SC 14266						54	30	15
SC 9928			42					
SC 24813			48					
SC 26519			49					
SC 10915			56					
SC 15983			61					
SC 11016			64					
SC 25951			79					
SC 12590			87					
SC 23273			89					
SC 9417			92					
SC 11835			95					

* Effects of unlabeled aldosterone and 24 anti-aldosterone compounds upon the binding of tritiated aldosterone (^{3}HA). All flasks contained 2×10^{-9} M ^{3}HA and 2×10^{-8} M unlabeled dexamethasone (DM). In each experiment, kidney slices from four rats (eight kidneys) were pooled and divided into 24 aliquots. Binding of ^{3}HA in the presence of 10-fold DM is taken as 100 per cent; the figures in the table represent the percentage binding in the presence of competitor. For the compounds tested at three dose levels, the figures given are an average of three determinations (i.e. n = 3); for the single-point determinations, n = 2. The absolute level of bound ^{3}HA varied from $3{\cdot}5$ to $4{\cdot}5 \times 10^{-14}$ moles/mg of cytosol protein.

turated 4-ene-3-one analog SC 27410; similarly, SC 5233 has ~25 times the affinity of its 4,6-diene-3-one unsaturated analog SC 4988.

The second focus of interest was on the effects of changes in affinity seen in 19-nor analogs (i.e. removal of the methyl group at C-10). The presence or absence of the methyl group at C-10 affected affinity in a variable fashion. The comparative affinities of three pairs of compounds, differing (within each pair) only by the presence or absence of the C-10 methyl group, are shown in Fig. 3. Between SC 9420 and its 19-nor analog, SC 10584, there was no detectable difference in ability to compete for ^{3}HA binding sites. Absence of the C-10 methyl (SC 8109) doubled the affinity of the 19-nor analog SC 5233; conversely, a third 19-nor analog (SC 10039) had only half the affinity of its parent compound, SC 9376. In contrast to the effects of ring opening or unsaturation, the results of C-10 demethylation were variable and presumably dependent on relationships between the structure of the parent compound and the type I sites which have not yet been defined.

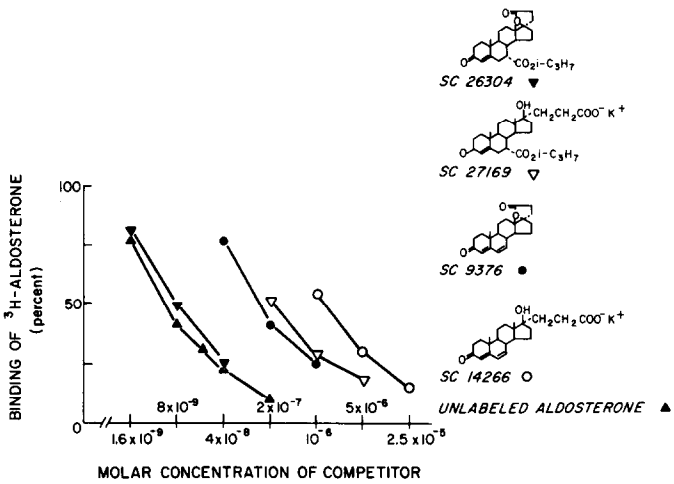

FIG. 1. Effect of opening of the γ-lactone ring and formation of the water-soluble K^+ salt upon spirolactone affinity for the mineralocorticoid receptor. Open symbols denote water-soluble spirolactones, solid symbols closed ring congeners. For the sake of comparison, the effect of various levels of unlabeled aldosterone is shown by upright triangles. Results are expressed as per cent binding, where 100 per cent is the mean value obtained when rat kidney slices (prepared from a pool of eight kidneys from four rats and divided into 24 aliquots) were incubated for 30 min at 25° with ^{3}H-aldosterone 2×10^{-9} M and unlabeled dexamethasone 2×10^{-8} M. The same conditions and the same concentrations of ^{3}H-aldosterone and dexamethasone were used for all the points shown in the figure, together with varying concentrations of competitor. For each point n = 3.

The series of compounds tested included a substantial number with various substitutions in the C-6 and/or C-7 positions in the B ring. Figure 4 shows the effects of changes at the C-6 and C-7 positions. SC 5233 has an affinity one tenth that of aldosterone for the mineralocorticoid binding sites. SC 9376, the unsaturated congener, has half the affinity of SC 5233. On the other hand, the effect of a cyclopropyl linkage between C-6 and 7 (SC 23133) is to double the affinity for the aldosterone binding

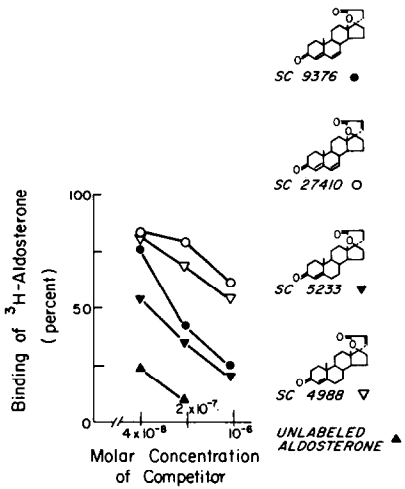

FIG. 2. Effect of unsaturation of the γ-lactone ring upon spirolactone affinity for the mineralocorticoid receptor. Open symbols denote ring-unsaturated compounds, solid symbols saturated congeners. Experimental details are identical to those described for the results shown in Fig. 1.

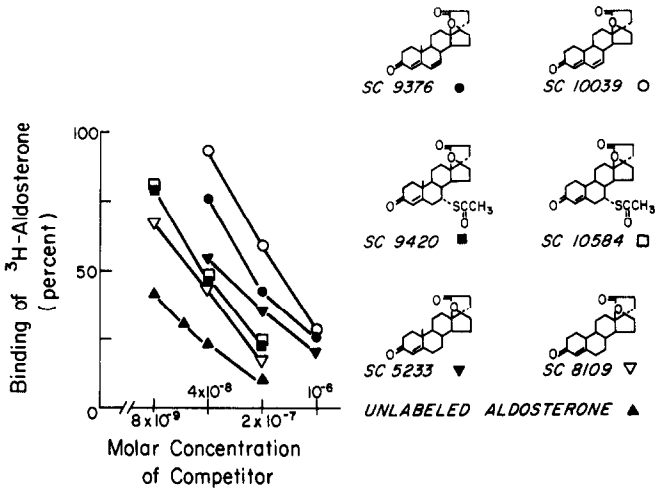

FIG. 3. Effect of formation of 19-nor derivative upon spirolactone affinity for the mineralocorticoid receptor. Open symbols denote 19-nor derivatives, solid symbols the parent compounds. Experimental details are identical to those described for Fig. 1.

sites. When substitutions at the C-6 position alone are considered, a similar effect upon affinity can be seen (Fig. 4). Although a direct comparison can only be approximate, the effect of adding a C-6 methyl group (SC 9928) is to increase affinity ~ 5-fold over the parent compound, SC 5233.

SC 5233 can again be considered the parent compound for the series of C-7 α-substitutions that were included in the series of compounds studied. In Fig. 5 are shown the effects of C-7 substitutions upon the affinity for mineralocorticoid receptors. Thioacetylation at C-7 (SC 9420) doubles the affinity vis-a-vis SC 5233. Although caution is needed in extrapolating from a single point analysis, it would appear that

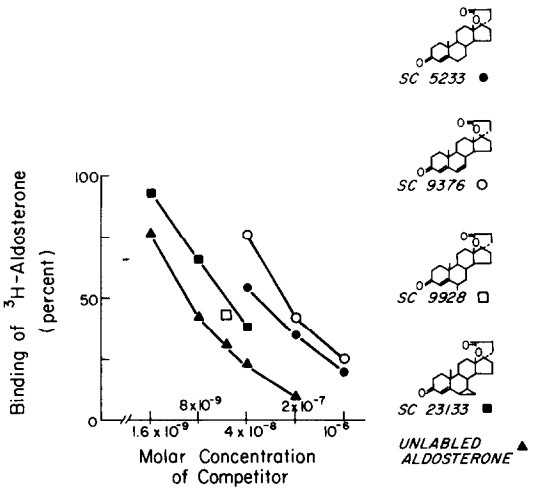

FIG. 4. Effect of B ring substitutions at the C-6 and C-6/C-7 positions upon spirolactone affinity for the mineralocorticoid receptor. The different symbols used denote different competitors; each point represents a mean of three experiments save that for SC 9928, for which n = 2. Other experimental details are identical to those described for Fig. 1.

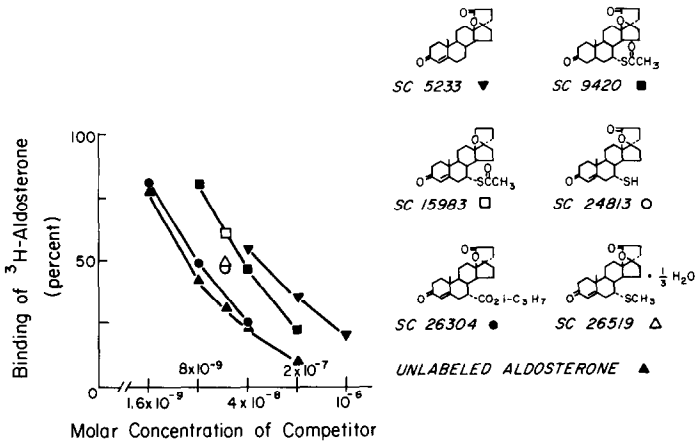

FIG. 5. Effect of B ring substitutions at the C-7 position upon spirolactone affinity for the mineralocorticoid receptor. The different symbols used denote different competitors; each point represents a mean of three experiments, save those for SC 24813 and SC 26519, for which n = 2. Other experimental details are identical to those described for Fig. 1.

C-7 sulfhydrylation (SC 24813), or thiomethylation (SC 26519) results in a further doubling in affinity. The most potent compound tested (SC 26304) bears a carboxyl as an isopropyl ester at the C-7 position, and has an affinity for the mineralocorticoid receptor ~ 70 per cent that of aldosterone itself.

One alteration in the γ-lactone ring structure which may be without significant effect upon affinity—in contrast to ring opening or unsaturation, detailed above—is that of elimination of the carboxyl group. The apparently equivalent affinity, by single point analysis, of SC 15983 (Fig. 5) with the dose–response curve of its oxygenated congener (SC 9420), suggests that the keto function in this position is not required for activity. Further desoxy compounds need to be studied to evaluate this suggestion.

An additional systematic substitution considered was on the basis of SC 5233 as the parent compound (Fig. 6). Fluorination at the 9-α position, together with the addition of a keto group at C-11 (SC 10915), appears to enhance the activity marginally. A 9–11 acetal linkage, on the other hand, probably reduced the affinity considerably (SC 12590). These compounds, however, were tested at but a single concentration level.

There remain six compounds which do not fit into any of the categories of substitution detailed so far. For the most part, these compounds represent rather radical departures from the basic spirolactone, or in some cases even steroidal, structure. Based on single point analysis, four compounds—SC 25951, SC 23273, SC 9417 and SC 11835—appear to have a relatively low affinity for the mineralocorticoid receptor (Fig. 7), and a fifth, SC 11016, an affinity approximately equal to that of SC 5233. The final compound, the etiojervane SC 19886, however, had an affinity of ~ 35 per cent that of aldosterone for the type I receptors; of the series of compounds tested, it is second only to SC 26304 in ability to compete for aldosterone binding sites.

Other factors must be considered in addition to the intrinsic affinity of a given antagonist for mineralocorticoid receptors in assessing potential clinical utility. The

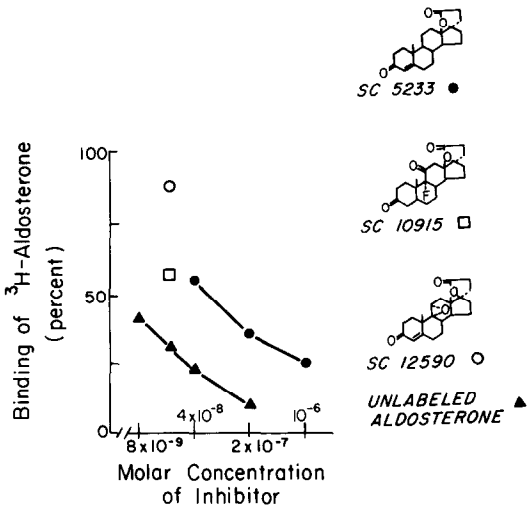

FIG. 6. Effect of C-9/C-11 substitutions upon spirolactone affinity for the mineralocorticoid receptor. For the points denoted by open symbols, n = 2; for those denoted by solid symbols, n = 3. Other experimental details are identical to those described for Fig. 1.

first of these concerns the obvious differences between the oral or parenteral administration of a compound *in vivo* and the incubation of the same compound *in vitro* with kidney slices. Variations in absorption via the oral route may significantly alter the effective potency ratios. The rapidity and extent of metabolism by the liver or kidney may differ between compounds, and the metabolites may conceivably be more, less or equivalently active. Moreover, the extent to which the compound, or its active metabolites, are bound in plasma will probably affect the ability to compete with circulating aldosterone for receptor sites. A second consideration in extrapolat-

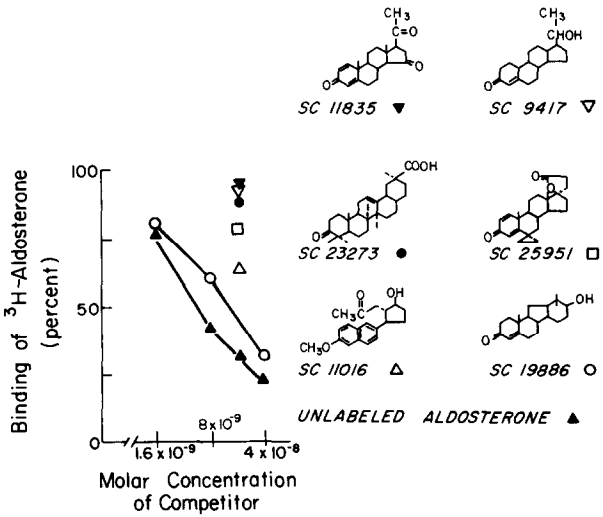

FIG. 7. Relative affinities of a series of anti-aldosterone compounds for the mineralocorticoid receptor. For each point n = 2 save those for SC 19886 and unlabeled aldosterone, for which n = 3. Other experimental details are identical to those described for Fig. 1.

ing from receptor affinity to potential clinical utility as an aldosterone antagonist is the possibility that any given test compound is in itself a partial mineralocorticoid agonist. A compound with a moderate affinity for the mineralocorticoid receptor, but absolutely without mineralocortocoid effect, may be more useful than one with a higher affinity but some salt-retaining action. Accordingly, determination of the affinity of an anti-aldosterone compound is not intended to replace testing *in vivo*. If the receptor theory is correct, however, it provides an accurate measure of one determinant with respect to potential clinical utility, and allows the investigations *in vivo* to be concentrated upon those compounds with the highest intrinsic affinity for the mineralocorticoid receptor.

In addition, studies of the sort detailed in this paper may prove to be of value in two other areas. The first of these is the synthesis of new anti-mineralocorticoid compounds. If correlations can be drawn between changes in structure and changes in affinity of the antagonist for the mineralocorticoid receptor, then it is possible to draw logical, if incomplete, guidelines for the development of more powerful anti-mineralocorticoid agents. Secondly, such structure–function studies may contribute to the eventual understanding of the process of receptor–steroid interaction. The affinity of the renal cytosol receptor for aldosterone [equilibrium (dissociation) constant K_{diss} 37° 5×10^{-10} M] implies very tight binding of the steroid to the receptor protein.[5] Given the groups on the aldosterone molecule capable of forming intermolecular bonds, a reasonable inference from this very high affinity is that the aldosterone molecule is deeply buried within the receptor protein. That the presumed multiple sites of interaction between steroid and protein may all be necessary for tight binding is suggested by the ~40-fold difference in the receptor affinity for corticosterone and desoxycorticosterone,[5] which differ only by the presence or absence of a hydroxyl function at the C-11 position. A more detailed examination of the structure–affinity relationships within the spirolactone series may contribute to our understanding of the mechanisms that determine, and the events that follow, the physiological specific binding of aldosterone to the mineralocorticoid receptor.

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