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DIFFERENCES IN THE DETERMINANTS OF EPLERENONE, SPIRONOLACTONE AND ALDOSTERONE BINDING TO THE MINERALOCORTICOID RECEPTOR*

Fraser M Rogerson,† Yizhou Yao,† Brian J Smith‡ and Peter J Fuller†

†*Prince Henry's Institute of Medical Research, Clayton and* ‡*Walter and Eliza Hall Institute, Parkville, Victoria, Australia*

SUMMARY

1. The importance of mineralocorticoid receptor (MR) antagonists in the treatment of cardiovascular disease has been emphasised by two recent clinical trials, one using spironolactone and the other using a new selective MR antagonist, namely eplerenone.

2. Eplerenone has a very low affinity for the glucocorticoid receptor (GR). Determinants of binding specificity of eplerenone to the MR were investigated using chimeras created between the ligand-binding domains (LBD) of the MR and the GR. These chimeras had been used previously to investigate aldosterone and spironolactone binding specificity to the MR.

3. Eplerenone competed strongly for [^{3}H]-dexamethasone binding to a MR/GR chimera containing amino acids 804–874 of the MR and weakly to a chimera containing amino acids 672–803 of the MR. Within the 804–874 region, eplerenone competed for [^{3}H]-dexamethasone binding to a chimera containing amino acids 820–844 of the MR, although the calculated affinity was approximately 10-fold lower than for binding to the full-length MR LBD. Similar results were obtained using another MR antagonist, namely spironolactone. Modelling of eplerenone binding to the MR LBD, based on the GR LBD crystal structure, suggests that amino acids 820–844 affect the overall shape of the ligand-binding pocket and that eplerenone acts as an MR antagonist because it fails to stabilize the active conformation of the receptor.

4. In contrast with results with the MR antagonists eplerenone and spironolactone, amino acids 820–844 are sufficient in themselves to confer high-affinity aldosterone binding to the MR, suggesting that the binding determinants of the two antagonists are similar to each other but differ from those of aldosterone.

Key words: aldosterone antagonists, aldosterone, corticosteroid receptors, mineralocorticoid receptor.

INTRODUCTION

In 1999, there was a resurgence of interest in mineralocorticoids with the publication of the Randomized Aldactone Evaluation Study (RALES).[1] In this clinical trial, the mineralocorticoid receptor (MR) antagonist spironolactone was added to the treatment regimen of patients with moderate to severe heart failure (New York Heart Association class III and IV). This resulted, over a 3 year period, in a 30% decrease in mortality and a 35% decrease in hospitalization. Spironolactone is also an antagonist of the androgen receptor and one major side-effect of the spironolactone treatment was a 10% increase in the rate of gynaecomastia in men. Eplerenone is an MR antagonist with a very low affinity for the MR compared with spironolactone *in vitro*, but with a comparable bioavailability *in vivo*.[2] Importantly, and in contrast with spironolactone, eplerenone has very low affinity for other steroid hormone receptors, thus reducing the risk of gynaecomastia and other side-effects. The efficacy of eplerenone as a treament for heart failure was evaluated in the Eplerenone Post-Acute Myocardial Infarcation Heart Failure Efficacy and Survival Study (EPHESUS).[3] The EPHESUS trial differed from the RALES trial in that patient selection was limited to those with left ventricular dysfunction post-myocardial infarction and the great majority were on optimal therapy. Eplerenone produced a 15% decrease in mortality over a 2 year period and, importantly, did not increase the incidence of gynaecomastia. Eplerenone is a low-affinity MR antagonist and needs to be taken in high doses. There is a need for high-affinity, specific MR antagonists. Development of such drugs will be facilitated by a better understanding of how MR antagonists bind to the receptor protein.

Aldosterone, eplerenone and spironolactone (Fig. 1a) bind to the MR with much higher affinity than they do to the related glucocorticoid receptor (GR). Both the MR and GR belong to the steroid hormone receptor (SHR) family of ligand-dependent transcription factors. All SHR can be divided into three major domains: the N-terminal domain, the DNA-binding domain and the ligand-binding domain (LBD). As its name suggests, the LBD binds steroid ligands and we have previously used chimeras created between the MR and GR LBD to investigate the specificity of aldosterone[4] and spironolactone[5] binding to the MR. For an antimineralocorticoid to be of clinical usefulness, it is essential that it binds with much higher affinity to the MR than the GR. In the present study, we have used the same MR/GR chimeras to determine the region(s) in the MR LBD that enables eplerenone to bind to the MR but not the GR.

Correspondence: Professor Peter Fuller, Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia. Email: peter.fuller@phimr.monash.edu.au

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METHODS

Expression constructs

In previous work examining aldosterone binding to the MR LBD, we created a series of MR : GR chimeras encompassing the entire LBD. The creation of the these chimeras is described elsewhere.[4] In the present study, we examined four chimeras in which a section of the GR LBD was replaced with the corresponding MR sequence (Fig. 1b). Each chimera is designated by the identity of each particular section, where 'M' indicates MR sequence and 'G' indicates GR sequence. In all cases, the LBD was placed into an expression vector containing the N-terminal domain and the DNA-binding domain of the GR.

A second round of chimeras, concentrating on amino acids 804–874 of the MR, was created by overlap extension polymerase chain reaction (PCR) using pRShGR and the GMGG chimera as templates. Both plasmids were linearized using *Kpn*I before PCR. In all cases, PCR was performed using *Pfu* polymerase (Stratagene, La Jolla, CA, USA). The final PCR products were digested with *Xho*I and *Bgl*II and ligated into pSP72 (Stratagene), then fully sequenced. The chimera sequence was subsequently removed from the vector by digestion with *Xho*I and *BamHI* and ligated into GR-pcDNA3.1[5] digested with *Xho*I and *BamHI*. The primers used for PCR are as follows (restriction enzyme sites are underlined). GR1: 5′-CTA TTC AAG CCC CAG CAT-3′ (GR + 1176–1193 forward); GR2: 5′-AGG GAT CCT TTC TTA GTA AGG CAG TCA-3′ (GR + 2464–2490 reverse, incorporating the *BamHI* site); MR815F: 5′-GAG ATC GTA CAG ACA ATC AAG TGC AAA C-3′ (MR + 2664–2673 : GR + 1972–1989); MR815R: 5′-TTG ATT GTC TGT ACG ATC TCC AGC TCA A-3′ (GR + 1972–1981 : MR + 2656–2673); MR844F: 5′-AGT CTG CCA TGT ACG ACC AAT GT-3′ (MR + 2741–2747 : GR + 2046–61); MR844R: 5′-TTG GTC GTA CAT GGC AGA CTG AT-3′ (GR + 2046–58 : MR + 2738–2747); MR847F: 5′-TCT ACC CTG CAT GTA TGA ACT ATG CCA G-3′ (GR + 2037–51 : MR + 2754–2766); MR847R: 5′-TTC ATA CAT GCA GGG TAG AGT CAT TCT-3′; (MR + 2754–2757 : GR + 2029–51); MR820–844F: 5′-GAG ATC ATA TAA ACA TAC GAA CAG CCA A-3′ (GR + 1962–1971 : MR + 2674–2691); MR820–844R: 5′-TCG TAT GTT TAT ATG ATC TCC ACC CCA G-3′ (MR + 2674–2683 : GR + 1954–1971).

MR(804–815)

The 5′ end was amplified from the GMGG template using the primers GR1 and MR815R. The 3′ end was amplified from the pRGhGR template using the primers MR815F and GR2. The two PCR products were joined together by overlap extension PCR using the primers GR1 and GR2.

MR(804–844)-GR

The 5′ end was amplified from the GMGG template using the primers GR1 and MR844R. The 3′ end was amplified from the pRShGR template using the primers MR844F and GR2. The two PCR products were joined together by overlap extension PCR using the primers GR1 and GR2.

MR(820–844)-GR

The 5′ end was amplified from the pRShGR template using the primers GR1 and MR820–844R. The 3′ end was amplified from the MR(808–844)-GR chimera template using the primers MR820–844F and GR2. The two PCR products were joined together by overlap extension PCR using the primers GR1 and GR2.

MR(847–874)-GGMM

The 5′ end was amplified from the pRShGR template using the primers GR1 and MR847R. The 3′ end was amplified from the pRShGR template using the primers MR847F and GR2. The two PCR products were joined together by overlap extension PCR using the primers GR1 and GR2.

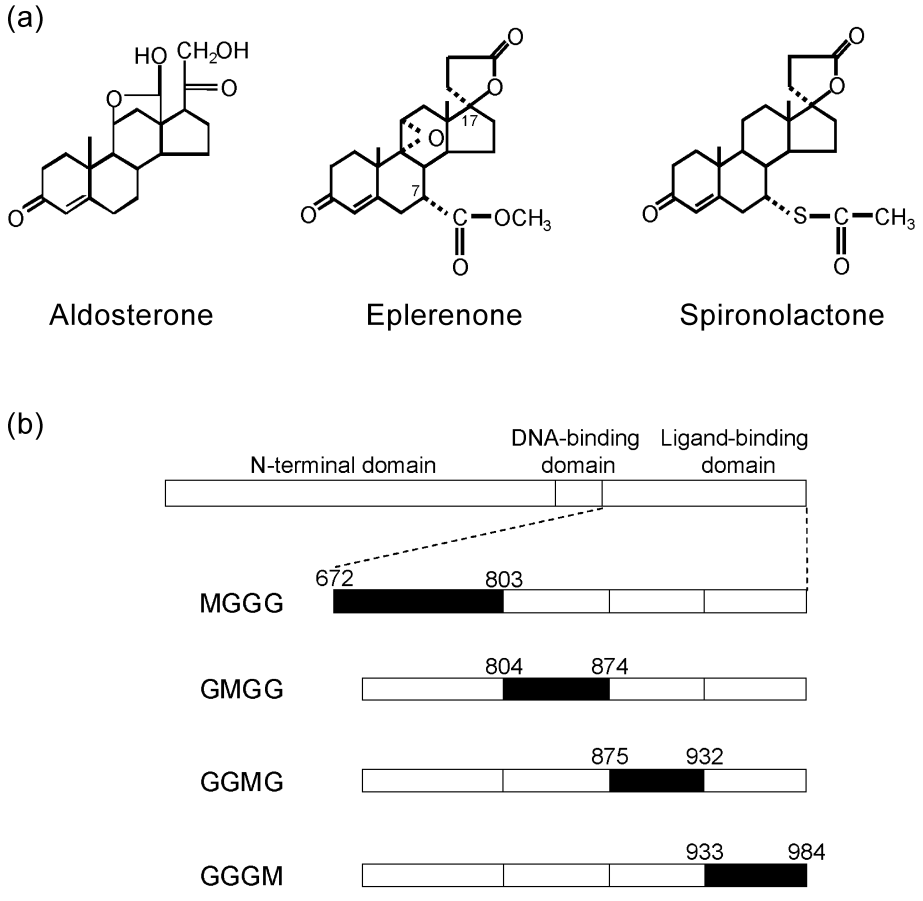

Fig. 1 (a) Structures of aldosterone, eplerenone and spironolactone. (b) Representation of the mineralocorticoid receptor (MR) : glucocorticoid receptor (GR) chimeras used in the present study (see text for details).

Ligand-binding competition assays

Ligand-binding assays were performed in transfected COS-1 cells using [1,2,4,6,7-^{3}H]-dexamethasone (specific activity 2.6–4.1 TBq/mmol; Amersham Biosciences, Amersham, UK). Cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mmol/L glutamine, non-essential amino acids, 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). Cells were trypsinized and replated in six-well plates at a density of 2×10^5 cells/well. After 20–24 h, cells were transfected with the expression plasmid using 'Fugene™ 6' (Roche Molecular Biochemicals, Indianapolis, IN, USA) and then incubated overnight in DMEM + 2% FBS. The medium was replaced with DMEM 1 h before the ligand-binding assay. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), then [^{3}H]-steroid ± eplerenone was added in DMEM. Non-specific binding was assessed by adding a 500-fold excess of non-radioactive dexamethasone. Cells were incubated at 37°C for 1 h. Cells were then washed three times with ice-cold PBS and then lysed using 1 mol/L NaOH. The suspension was added to scintillant and radioactivity was measured in a Packard 2500 TR liquid scintillation counter (Packard, Meriden, CT, USA). Statistical significance was measured by the Games–Howell analysis of variance test (heterogeneous variances) using the GB-STAT software (Dynamic Microsystems, Houston, TX, USA). The IC_{50} values were determined by plotting percentage binding against $\log_{10}$ (molar concentration) and apparent K_i values were then calculated using the Cheng–Prusoff equation.[6]

Modelling of the MR LBD structure

A model of the MR LBD bound to eplerenone was created based on the crystal structure of the GR LBD bound to dexamethasone[7] using the same procedure as described previously.[5]

RESULTS

Eplerenone binding to the MR : GR chimeras

Eplerenone binding was examined by its ability to compete for the binding of [^{3}H]-dexamethasone to the chimeras MGGG (MR amino acids 672–803), GMGG (MR amino acids 804–874), GGMG (MR amino acids 875–932) and GGGM (MR amino acids 933–984). [^{3}H]-Dexamethasone was used because it binds to all the chimeras, whereas [^{3}H]-aldosterone only binds to the GMGG

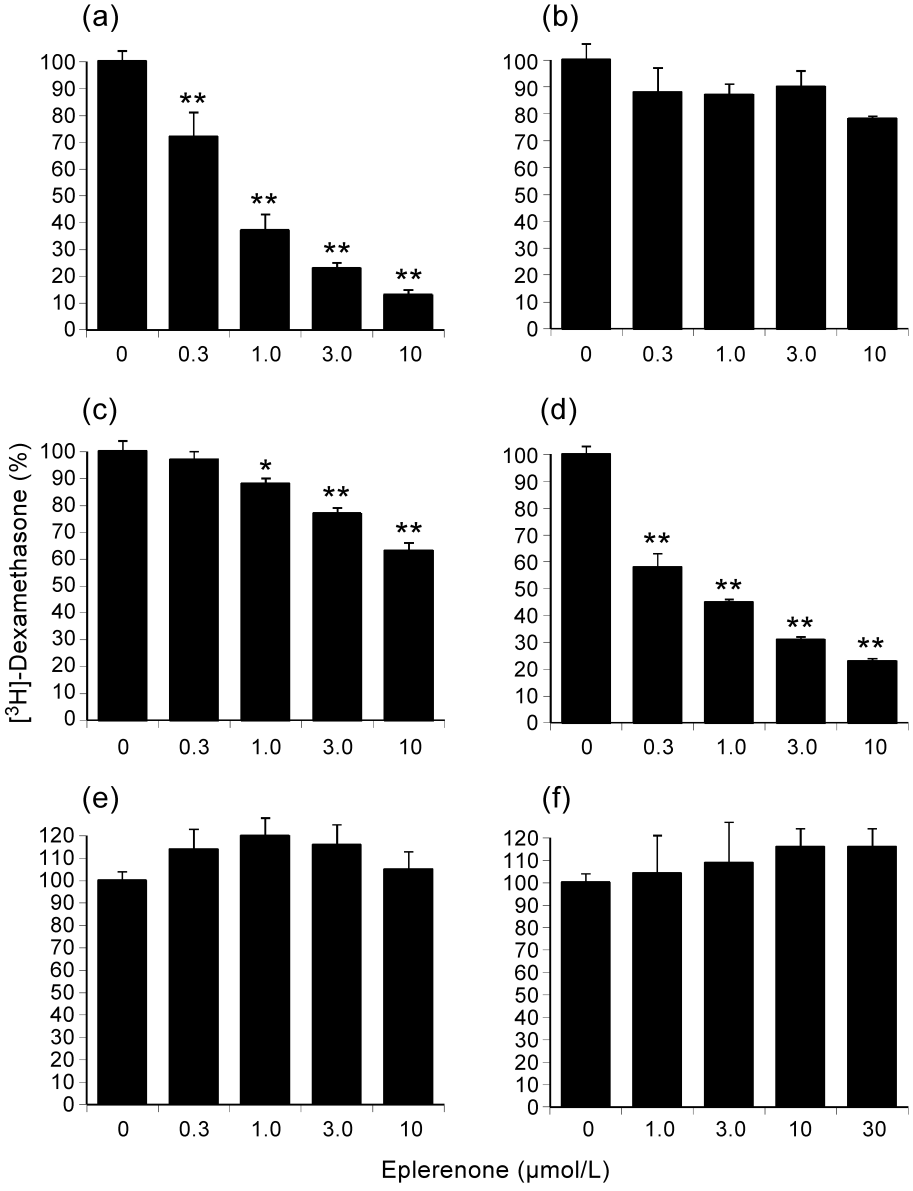

Fig. 2 Eplerenone competition for [^{3}H]-dexamethasone binding to (a) the mineralocorticoid receptor (MR) ligand-binding domain (LBD), (b) glucocorticoid receptor (GR) and the chimeras (c) MGGG, (d) GMGG, (e) GGMG and (f) GGGM. Ligand-binding studies were performed in COS-1 transformed monkey kidney fibroblast cells using a concentration of 20 nmol/L [^{3}H]-dexamethasone for all constructs except GGGM, where 50 nmol/L [^{3}H]-dexamethasone was used. Non-specific binding was assessed using a 500-fold excess of dexamethasone. Results are expressed as a percentage of the binding in the absence of eplerenone and show the mean±SEM from the results of two independent experiments ($n = 6$). *$P < 0.05$, **$P < 0.01$ compared with an assay containing no eplerenone.

chimera.[4] The binding experiments were performed in transfected COS-1 cells that lack endogenous MR and GR.

The affinities (K_d) of [^{3}H]-dexamethasone binding to the GR LBD, MR LBD, MGGG, GMGG, GGMG and GGGM chimeras were determined previously.[5] On the basis of the results, the eplerenone competition experiments were performed using 20 nmol/L [^{3}H]-dexamethasone for the GR, the MR LBD and the MGGG, GMGG and GGMG chimeras and 50 nmol/L [^{3}H]-dexamethasone for the GGGM chimera. Eplerenone was added at concentrations of 0.3, 1, 3 and 10 μmol/L, except for GGGM, where concentrations of 1, 3, 10 and 30 μmol/L were used. Figure 2 shows the competition curves for the MR LBD, the GR LBD and the four chimeras and Table 1 gives the derived, apparent K_i values for eplerenone. As expected, at the concentrations used, eplerenone is able to compete strongly with [^{3}H]-dexamethasone for binding to the MR LBD, but only very weakly with [^{3}H]-dexamethasone for binding to the GR LBD. Eplerenone competes strongly for [^{3}H]-dexamethasone binding to the GMGG chimera: the K_i of eplerenone binding to this chimera is very similar to that for binding to the full-length MR LBD. Eplerenone also binds weakly, but significantly, to the MGGG chimera and does not bind to the GGMG and GGGM chimeras at the concentrations used.

Table 1 Apparent K_i values of eplerenone binding to the mineralocorticoid receptor : glucocorticoid receptor chimeras, as assessed by competition for [^{3}H]-dexamethasone binding to transiently transfected COS-1 cells

Construct	Dexamethasone K_d (nmol/L)[5]	Eplerenone K_i (apparent; μmol/L)
MR LBD	8	0.2
GR LBD	11	–
MGGG	21	20
GMGG	15	0.3
GGMG	10	–
GGGM	54	–
MR(820–824)	4	2.4

The values were calculated from the results of two independent experiments, each of $n = 3$. The previously calculated values for the K_d of [^{3}H]-dexamethasone binding[5] used for the calculation of the apparent K_i values are shown.

–, insufficient binding to calculate the K_i; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; LBD, ligand-binding domain.

The GMGG chimera contains amino acids 804–874 of the MR, a region also critical for the binding of aldosterone[4] and spironolactone.[5] A second series of chimeras was created encompassing this region: MR(804–815)-GR, MR(820–844)-GR and MR(847–874)-GR. The binding of both eplerenone and spironolactone to these chimeras was examined by competition for [^{3}H]-dexamethasone binding. The K_d values for [^{3}H]-dexamethasone binding to the three chimeras were 52 ± 8 nmol/L ($n = 2$) for MR(804–815)-GR, 4 ± 1 nmol/L ($n = 2$) for MR(820–844)-GR and 58 ± 5 nmol/L ($n = 2$) for MR(847–874)-GR. On the basis of these results, the competition binding assays of MR(820–844)-GR were performed using 20 nmol/L [^{3}H]-dexamethasone and the assays of MR(804–815)-GR and MR(847–874)-GR were performed using 50 nmol/L [^{3}H]-dexamethasone. Figure 3 shows the competition curves for MR(820–844)-GR chimeras. The MR(804–815)-GR chimera did not bind either eplerenone or spironolactone at the concentrations used (data not shown) and the MR(847–874)-GR chimera was not expressed at high enough levels to give useful results. Both eplerenone and spironolactone bound to the MR(820–844)-GR chimera, with apparent K_i values of 2.4 μmol/L and 16 nmol/L, respectively.

MR LBD model

The MR LBD model based on the GR LBD crystal structure is very similar to a previous model we created of the MR LBD[5] based on the progesterone receptor LBD crystal structure. Figure 4 shows two cut-away views of the binding pocket, comparing the binding of aldosterone (left) and eplerenone (right). The binding pocket consists predominantly of hydrophobic amino acids. Aldosterone makes three hydrogen bonds with the protein structure in the

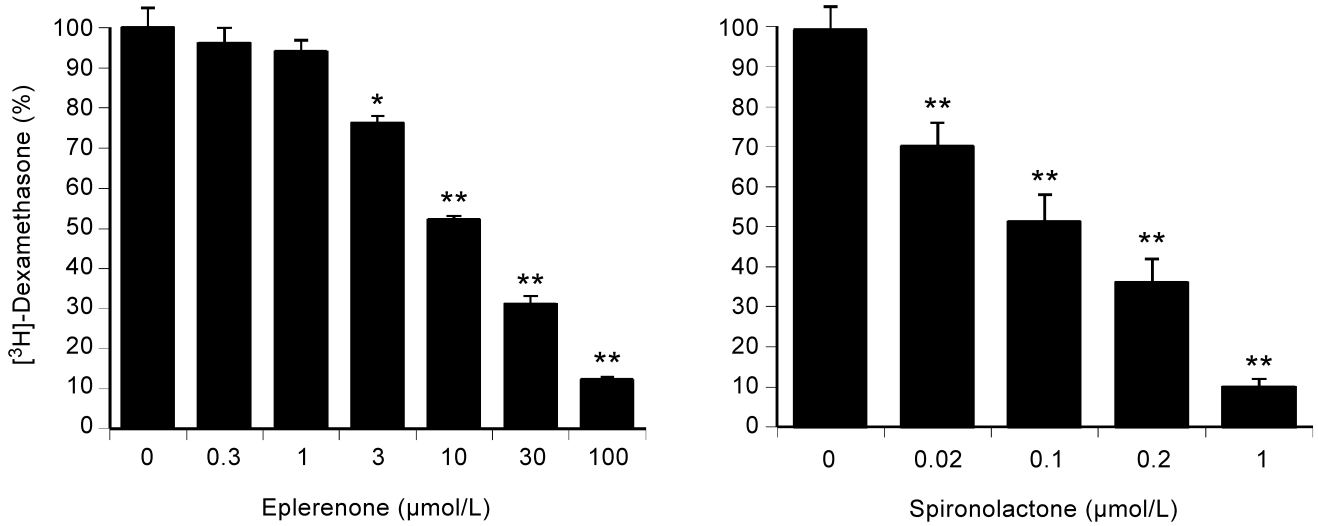

Fig. 3 Eplerenone and spironolactone competition for [^{3}H]-dexamethasone binding to the MR(820–844)-GR chimera. Ligand-binding studies were performed in COS-1 transformed monkey kidney fibroblast cells using a concentration of 20 nmol/L [^{3}H]-dexamethasone. Non-specific binding was assessed using a 500-fold excess of dexamethasone. Results are expressed as a percentage of the binding in the absence of competing ligand and show the mean ± SEM from the results of two independent experiments ($n = 6$). *$P < 0.05$, **$P < 0.01$ compared with an assay containing no competing ligand.

model: the C_3 ketone interacts with glutamine(776) and arginine(817) and the C_{21} hydroxyl interacts with asparagine(770). In the model of eplerenone binding, the hydrogen bond contacts between the C_3 ketone and glutamine(776) and arginine(817) are maintained, but there is no hydrogen bond between the ligand and asparagine(770). As was observed for spironolactone,[5] the C_7 side chain of eplerenone fits in between helices 3 and 7, making hydrophobic contacts with leucine(769), leucine(848) and methionine(852) (data not shown).

DISCUSSION

In the present study, we have examined eplerenone binding to the MR using chimeras created between the MR and GR LBD and by molecular modelling based on the GR LBD crystal structure. The two techniques provide us with valuable insights into the nature of ligand binding to the MR. Amino acids 804–874 of the MR are critical for the binding of eplerenone. The same region is critical for the binding of aldosterone[4] and spironolactone.[5] Within this region, differences emerge between the binding determinants of aldosterone and, collectively, the two antagonists. The apparent K_i value of eplerenone binding to MR(820–844)-GR is an order of magnitude less than that to the full-length MR LBD (2.4 *vs* 0.2 μmol/L, respectively). The apparent K_i value of spironolactone binding to MR(820–844)-GR was 16 nmol/L. Using data from our previous study,[5] the apparent K_i value for spironolactone binding to the full-length MR LBD was calculated to be 1 nmol/L; therefore, as was found for eplerenone, the affinity of spironolactone binding to MR(820–844)-GR is an order of magnitude lower than that to the full-length MR LBD. In contrast, the K_d values for aldosterone binding to MR(820–844)-GR and the MR LBD are almost identical (1.3 and 0.9 nmol/L, respectively[8]) and, in a transactivation assay, aldosterone activated MR(820–844)-GR and the full-length MR LBD with equal potency.[8] This suggests that the binding determinants of eplerenone and spironolactone binding are very similar, but differ somewhat from the determinants of aldosterone binding. Although not sufficient in itself to confer high-affinity binding of eplerenone and spironolactone, the (820–844) region is nevertheless a critical determinant of binding of the two antagonists. In a model of MR LBD structure we published previously,[5,8] based on the progesterone receptor LBD crystal structure, amino acids 820–844 lie on the surface of the structure: only one residue, phenylalanine(829), forms part of the ligand-binding pocket and is conserved between the MR and GR. In the present model, based on the GR LBD crystal structure, amino acids 820–844 of the MR are in the same position on the surface of the protein (data not shown). Therefore, the modelling suggests that the amino acids within this region that are critical for MR binding specificity do not make direct contact with the ligand. It would appear that this region affects the overall shape of the binding pocket, which, given the differing structures, affinities and biological activities of the three ligands examined, is perhaps not surprising. The similarity between the results using eplerenone and spironolactone is also perhaps not surprising. As shown in Fig. 1, the structures of eplerenone and spironolactone are similar: the two ligands contain a lactone ring at the C_{17} position and have similar C_7 side chains.

The other important sequences remain to be determined and must also lie within the 804–874 region. The MR(804–815) chimera did not bind eplerenone at the concentrations used. This suggests that the other determinants of binding specificity lie between amino acids 847 and 874, but, unfortunately, it was not possible to obtain useful data from the MR(847–874)-GR chimera. Amino acids 847–874 of the MR encompass helix 7, which forms part of the binding pocket. However, all the residues in helix 7 that make direct contact with the ligand are conserved between the MR and GR,[5] again suggesting that binding specificity is determined by amino acids that do not form part of the binding pocket.

Molecular modelling also provides insights into why eplerenone acts as an MR antagonist. The most obvious difference between the models of aldosterone and eplerenone binding to the MR LBD is that only the former makes a hydrogen bond with asparagine(770) in helix 3 of the structure. Studies of an activating mutation of the MR[9] suggest that asparagine(770) is critical for the agonist activity of MR ligands. The interactions between agonist ligands and this asparagine act to stabilize the active conformation of the receptor, presumably by stabilizing the position of helix 3. The S810L activating mutation[9] effectively bypasses the need for this ligand–receptor interaction by providing a direct protein–protein interaction between leucine(810) on helix 5 and alanine(773) on helix 3. As result of these conformational changes induced by the S810L mutation, spironolactone acts as an agonist.[9] The model suggests that eplerenone does not make contact with asparagine(770) and, therefore, cannot stabilize the active conformation of the receptor and is a likely explanation of why eplerenone acts as an antagonist. These interactions are consistent

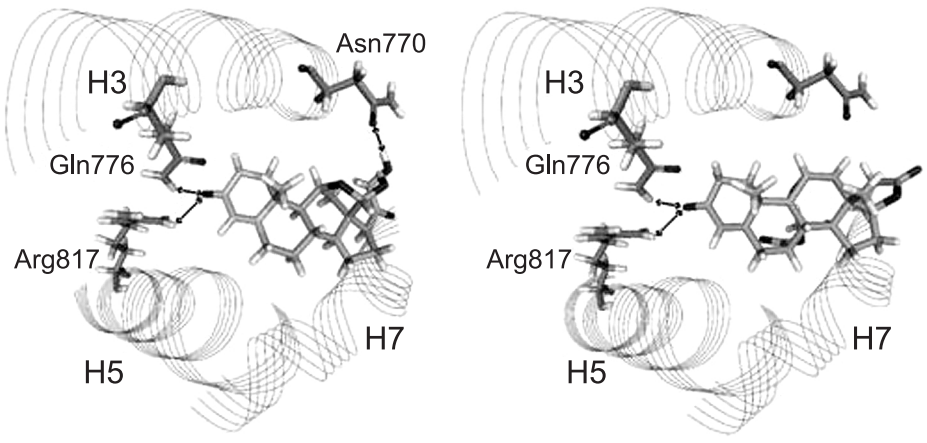

Fig. 4 Model of the mineralocorticoid receptor ligand-binding domain (LBD) based on the crystal structure of the glucocorticoid receptor LBD, showing cut-away views of aldosterone (left) and eplerenone (right) in the ligand-binding pocket.

with those we observed when we modelled spironolactone with the MR.[5]

In summary, we have shown that differences occur between the binding determinants of the MR antagonists eplerenone and spironolactone and the natural agonist aldosterone. We have identified a critical region for the binding of all three ligands. Further characterization of this region should provide important insights into binding specificity in the MR.

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