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Development of Specific Bioluminescent *In Vitro* Assays for Selecting Potential Antimineralocorticoids

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Efficient antimineralocorticoid selection requires a reliable, discriminating and easy assay for monitoring biological activity of not only the specific receptor, but also closely related receptors such as glucocorticoid and progestin. These related activities should be as low as possible to obtain specific antimineralocorticoid compounds. In this paper, we describe two cellular models used for easy and specific measurement of mineralocorticoid and progestin activities. These models involve the induction of firefly luciferase under hormonal control mediated by a chimeric receptor. The first model comprises transiently transfected MCF-7 cells, whereas the second uses stably transfected HeLa cells. Glucocorticoid activity was assayed with the classic tyrosine-aminotransferase induction method in HTC cells. Six compounds of a new family of 11β-substituted-17-spirolactone steroids were thus studied and compared to control compounds. Five of them showed antimineralocorticoid activity and one was active at a concentration lower than that of mespirenone.

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INTRODUCTION

Mineralocorticoids are involved in the regulation of electrolyte balance, and disorders of their synthesis may cause hypertension by increasing sodium and water retention. Antimineralocorticoids are therefore used in the treatment of diseases with sodium/potassium imbalance. Because antimineralocorticoids such as spironolactone do not only interact with the renal mineralocorticoid receptor (MR), the use of such drugs, especially at high doses and/or for long-term treatment, becomes limited due to endocrine side effects [1, 2]. Data in the literature show the existence of cross-reactions with receptors for other steroid hormones, for example progestins and glucocorticoids [3, 4]. These cross-reactions reflect the very similar chemical structure of these hormones, leading to a relatively high affinity of these hormones for other receptors. Recently, a second type of possible cross-talk was revealed for the receptors themselves. These receptors show marked sequence identity and have indistinguishable DNA binding sites [4]. These similarities underly the recognition of the same response element [4–6] and transcription activation of the same genes.

Therefore, as reported by Sutanto and de Kloet [2], "ideal" aldosterone antagonists are those that interact with MR with high affinity, but show no affinity for other steroid receptors. Development of such potent and specific antagonists require several specific test-systems, such as evaluation of the affinity of test-compounds for the MR, evaluation of their mineralocorticoid and/or antimineralocorticoid activities and evaluation of their secondary (anti)progestin and (anti)glucocorticoid activities. The objectives of this paper are firstly to describe quick and easy *in vitro* test-systems that reliably measure mineralocorticoid (and progestin) activities for screening new synthetic compounds, and secondly to describe the *in vitro* biological activities of a series of six test-compounds developed for potential antimineralocorticoid activity [7–9]. Given the various levels of cross-talk between hormones and receptors, and in order to perform a Structure-Activity Relationship (SAR) study on a series of molecules able to specifically bind the MR, we chose for a quick and easy "first" screening study, to test the specificity and activity of compounds rather than to test their closeness to a typical physiological

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behavior (a physiological behavior is often multifaceted, varying with the nature of the response studied, the target organ, the animal species). Also, we decided to create a highly specific cellular study model with a "minimal receptor" responsible for the most important functions: ligand binding, nuclear localization, dimerization, transactivation of a reporter gene. Furthermore, in order to evaluate the intrinsic MR mediated responses without any specific influence from a target tissue or cell [10] (e.g. steroid metabolism), the cell lines used in this study were chosen from those known not to be targets for mineralocorticoids. The use of this model, therefore, will require a second screening study and a physiological study to be performed on natural target organs or cells or with standard pharmacological techniques [11, 12], but only with the most interesting compounds.

Molecular cloning of mineralocorticoid, progestin and glucocorticoid receptors cDNAs enabled the identification of structural and functional domains in the receptor proteins [4, 13–16]. Apart from the DNA-binding domains (DBD or C region) where the marked sequence identities led receptors to recognize the same response elements on genes, the C-terminus (D, E, F regions) was shown to be responsible not only for hormone binding, but also for nuclear localization and for interactions with other proteins such as heat shock proteins, transcription factors and the receptor itself in the dimerization process. The N-terminus (A/B region) of the protein, although containing a region involved in transcriptional activation, has a function less well understood: in transient transfection experiments [17] where the MR amino-terminus was entirely deleted, it was shown that the corresponding ΔMR had activity not significantly different from that of the wild-type MR, suggesting that this A/B domain is essentially neutral in this assay. These data led us to develop a cellular model to study mineralocorticoid activity, where the chimeric "minimal receptor" is able to specifically bind the ligand, and to stimulate or inhibit a response that cannot be otherwise stimulated by the various endogenous receptors. Such a receptor was constituted by the C-terminus domain (D, E, F regions) of MR, and the heterologous DBD of the highly specific yeast transcription factor GAL4. This DBD is able to stimulate heterologous transcription in mammalian cells [18], and avoids the use of the natural MR-DBD responsible for several natural side effects. This principle was also applied to the development of a model responding to progestins.

Biological responses obtained with these models reflect the natural hormonal or antihormonal activities of control-compounds. Surprisingly, we also showed that this *in vitro* test developed to study mineralocorticoid activity seems to discriminate between mineralocorticoid and glucocorticoid hormone molecules. Finally, we described the biological behavior of six steroidal 17-spirolactones substituted in the 11β position, developed for their potential antimineralocorticoid activity [7–9]. The relationship between the nature of the 11β arm of these compounds and their affinity for cytosolic MR and glucocorticoid receptor (GR) has been discussed recently [19]. Their antimineralocorticoid action observed in the present study confirmed the hypothesis of a "hydrophobic pocket" located in the MR region, corresponding to the 11β position. However, progestin and antiglucocorticoid activities [the latter being measured by evaluation of tyrosine-aminotransferase (TAT) induction in HTC cells] exhibited by these molecules requires their specificities to be improved further.

EXPERIMENTAL

Chemicals, materials, cell culture

All the tested cell lines were cultured in Dulbecco's modified essential medium (DMEM) with phenol red, supplemented with 5% fetal calf serum (FCS) and maintained in a 5% CO_2 atmosphere, 95% humidity, at 37°C. Aldosterone, estradiol, progesterone, dexamethasone, testosterone and luciferin were purchased from Sigma Chemical (St Louis, MO). Neomycin (geneticin, G418) was from Gibco/BRL (Sarl). RU 486 and R5020 were gifts from Dr D. Philibert at Roussel Uclaf (Romainville, France). Spironolactone and mespirenone (Δ^1-15β,16β-methylene-spironolactone) were gifts from Schering Aktiengesellschaft (Berlin and Bergkamen, Germany). A single photon-counting camera (ARGUS-100) from Hamamatsu Photonics (Hamamatsu, France) was used to select stable G418 resistant clones. Detection of luciferase activity on cell-free extracts was done using an LKB Wallac 1251 luminometer (Sundyberg, Sweden).

Plasmid constructions

DNA constructions were performed using standard procedures [20]. The 670–984 MR fragment (amino acids of the D, E, F regions) was obtained by PCR amplification of the corresponding 945 bp fragment of the hMR cDNA, using primers in which restrictions sites (XhoI–KpnI) were added. The plasmid pGal-MR was constructed by inserting this XhoI–KpnI fragment into the expression vector pGal4 (1–147)ER region F [21]. The expression vector pGal4-hPR(E) has been described previously [22, 23]. These plasmids encoding for chimeric mineralocorticoid or progesterone receptors are used to stimulate specific chimeric genes: as shown in previous studies [18] the yeast GAL4 protein can be expressed in mammalian cells, and stimulate transcription after binding to a gene promoter containing a GAL4-binding site (17M: a 17 bp sequence, 5′-CGGAGTACTGTCCTCCG-3′). The plasmid p(17M)5-βGlob-Luc was constructed by ligation of the SalI–BglII fragment of (17M)5-βGlob (which contains 5 repeats 17M and the β-globin promoter) upstream of the luciferase gene [24] used as a reporter

gene [25]. All constructions were checked by restriction enzyme analysis. The MR DNA fragment amplified by PCR was sequenced according to the Sanger method.

Transient and stable transfections

Transient transfection experiments were performed in MCF-7 cells by the previously described calcium phosphate procedure [20]. The stable transfected HGPL cells presented in this paper were obtained by cotransfection of HeLa cells with plasmid pAG-60 [26] for resistance to neomycin (or G418), reporter plasmid p(17M)5-βGlob-Luc and expression plasmid pGal-hPR(E). Cotransfection was carried out by the calcium phosphate precipitation technique. Transfected cells were then selected according to their neomycin resistance (usually 1 to 2 days after transfection): cells were cultured for 3 weeks in routine medium containing 1 mg/ml of G418 and then cultured in the routine medium alone. A second selection of the various G418 resistant clones was carried out by measuring the inducible luciferase as described previously [27]: cells were incubated for 24 h with medium containing various effector concentrations, while controls received only 0.2% EtOH and the luciferase was then assayed in whole cells using the single photon counting camera. After localization, the luminescent clones were harvested, spread out and grown in T25 culture flasks.

Luciferase assay in whole cell- and in cell free-conditions

Selection of positive stable transfected cells was performed using a photon-counting camera coupled with an imaging analysis system. After incubation of the clones with hormones, the medium was replaced by a medium containing 0.2 mM of the substrate luciferin. Luminescence values were read after 5 to 10 min integration. The luminescent clones were localized as described previously [27].

The luciferase assays in cell free conditions were performed in 6-well multidishes to screen the hormone activities with the cellular models described above. At the end of the cell incubation with test-compounds, cells were washed with 2 × 2.5 ml of cold luminescence buffer (15 mM potassium phosphate, 8 mM $MgCl_2$, pH 7.4), and scraped in 1 ml of the same buffer containing 2 mM ATP harvested and disrupted by sonication. Luciferase activity was determined on an aliquot fraction (150 μl plus 50 μl of 3% Triton X-100 solution) luminescence was measured (integration time 15 s), after injection of 100 μl solution containing 6×10^{-4} M luciferin and 6×10^{-4} M coenzyme A. Protein assays were performed according to Lowry's method [28] on an aliquot fraction (0.1 ml) of the cellular homogenate. Results were expressed as arbitrary luminescence units per mg of proteins.

TAT assay

TAT induction was performed by incubating HTC cells in duplicate on 6-well culture tissue plates with serum-free medium, containing 1% ethanol (final concentration) with or without steroids at concentrations as described in the figures. After 16–19 h incubation, the cells were scraped, harvested and disrupted by sonication. The specific TAT enzyme activities and protein contents in the cell lysates were determined as described previously [29, 30].

RESULTS

Steroid specificity of the chimeric Gal-MR

After transient cotransfection in MCF-7 cells, with pGal-MR and p(17M)5-βGlob-Luc, and incubation with steroids, the response was evaluated by measuring the luciferase activity. In the absence of added compound, the basal luciferase activity was very low. After exposure to the agonist mineralocorticoid (aldosterone), luciferase was expressed in a dose-dependent manner (Fig. 1). The maximum response obtained at 10 nM aldosterone showed a >100-fold increase in luciferase induction.

The biological effects of two potent aldosterone antagonists, spironolactone and mespirenone [31–33], were then analyzed (Fig. 1). Their antimineralocorticoid activity was investigated by measuring their capacity to block aldosterone-induced luciferase activity. At 10 nM concentration, they significantly decreased luciferase activity induced by 10 nM aldosterone. At concentrations of 1 μM, they were able to block the expression of aldosterone-induced luciferase activity. Evaluation of their inhibitory activities in the presence

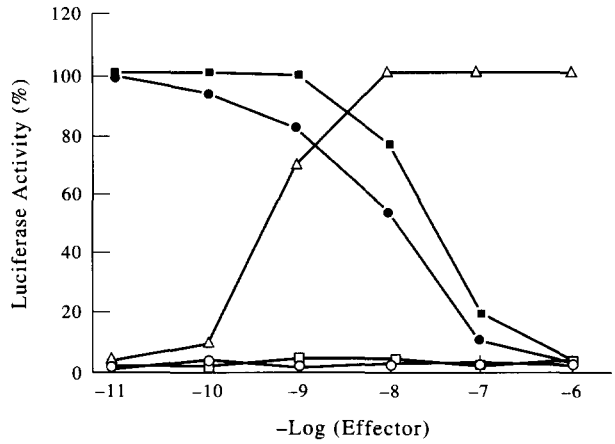

Fig. 1. Dose-dependent stimulation by aldosterone of luciferase induction in transiently transfected MCF-7 cells. Transfected cells were incubated for 20 h with various aldosterone concentrations (△), with various mespirenone concentrations in the presence (●) or in the absence of 10 nM aldosterone (○), or with various spironolactone concentrations in the presence (■) or in the absence of 10 nM aldosterone (□). Maximum induction was obtained with 10 nM aldosterone. Luminescence values were expressed as a mean percentage of maximum activity obtained in three experiments performed in duplicate. The 0% value was the luminometer background value obtained in the absence of cells.

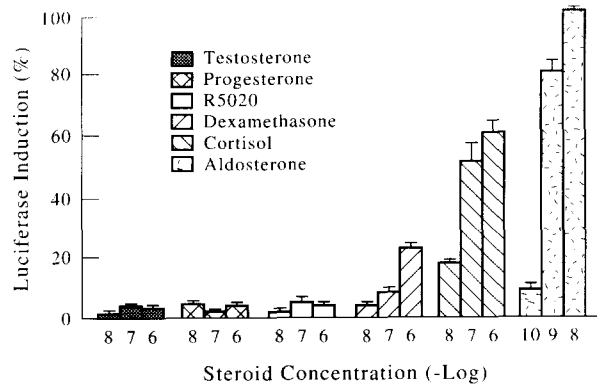

Fig. 2. Specificity of the mineralocorticoid response. Luciferase activity was determined as described for Fig. 1. Transfected cells were incubated with test-compounds at indicated concentrations. Agonistic effects were expressed as a mean percentage of maximum response obtained with aldosterone. The 0% value was the luminometer background value obtained in the absence of cells. Standard deviations were calculated from three experiments performed in duplicate.

of 10 nM aldosterone show that spironolactone (IC_{50} = 40–50 nM) was inhibitory at a 2.7-fold greater concentration than mespirenone (IC_{50} = 15–20 nM). As seen in Fig. 1, they did not show any agonist activity, since no induction of luciferase activity was observed in the absence of aldosterone.

Concerning the other classes of hormones, Fig. 2 shows that progesterone, R5020 and testosterone, which are known to have no mineralocorticoid activity, did not induce luciferase activity. Glucocorticoids, such as dexamethasone and cortisol, are full agonists for MR in cell culture and bind with recombinant hMR *in vitro* with a relatively high affinity (more than 20% that of aldosterone) [34–36]. In the present cellular model, we observed that these steroids induced luciferase activity in a dose-dependent manner, with high concentrations of dexamethasone required to stimulate luciferase induction. As shown in Fig. 2, the response to 100 nM cortisol was 60% of that obtained with 10 nM aldosterone, while dexamethasone required even higher concentrations, since at 1 μM luciferase activity induced by dexamethasone was only 20–30% of the maximum level obtained with aldosterone. The same results were obtained for incubations in the presence of carbenoxolone, an inhibitor of the 11β-hydroxysteroid dehydrogenase (data not shown).

Steroid specificity of the chimeric Gal-PR

As shown in Fig. 3, in progesterone responsive HeLa-derived HGPL cells, treatment with the synthetic agonist R5020 resulted in dose-dependent induction of firefly luciferase. The maximum response was reached at 100 nM R5020 and the presence of R5020 induced a more than 100-fold increase in luciferase induction. At all tested concentrations (1–1000 nM), RU486, a synthetic progesterone antagonist [37], was unable to stimulate luciferase induction in the HGPL cells but can inhibit the R5020-induced response: the effects of 10 nM R5020 were completely inhibited by 10 nM RU486, showing that in this model RU486 was a full antagonist. As shown in Table 1, hormones of the other classes (testosterone, cortisol, aldosterone) did not induce luciferase, thus showing the specificity of this cell line for progestin activity.

Mineralocorticoid activity of 11β-substituted steroids

The mineralocorticoid or antimineralocorticoid activity of a series of six new steroidal 11β-substituted spirolactone derivatives was tested in the transiently transfected MCF-7 cell model. Their syntheses and relative binding affinities for MR were recently described elsewhere [19]. For all of these compounds, mineralocorticoid activity was investigated by measuring the capacity of these compounds to induce luciferase activity. In any case, no stimulation was observed, showing that they do not have any measurable mineralocorticoid activity. They were able to fully inhibit luciferase induction by 10 nM aldosterone in a dose-dependent manner (Fig. 4). Their biological efficiencies evaluated by their IC_{50}s at 10 nM aldosterone concentration are given in Table 2. The compounds were able to inhibit aldosterone-induced luciferase activity by half in the 4 to 100 nM concentration range. As expected from another study [19], one of them (FH 61), which has no affinity for the rabbit MR, had no effect. Among the others, the 11β-allenyl-3-oxo-19 nor-17-pregna-4,9 diene-21,17-carbolactone derivative (FH 151) inhibited luciferase activity at lower concentrations than noted with potent antimineralocorticoids mespirenone or spironolactone.

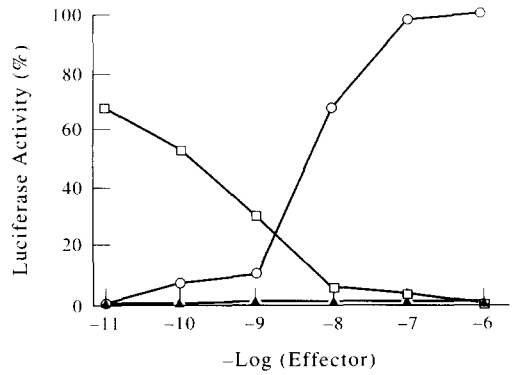

Fig. 3. Dose-dependent stimulation by R5020 of luciferase induction in HGPL cells. Stably transfected cells were incubated for 20 h with various R5020 concentrations (○) or with various RU486 concentration in the absence (▲) or in the presence of 10 nM R5020 (□). Maximum induction was obtained with 100 nM R5020. Luminescence values were expressed as a mean percentage of maximum activity obtained in three experiments performed in duplicate. The 0% value was the luminometer background value obtained in the absence of cells.

Table 1. Effects of various steroids on chimeric PR in HGPL cells

Compound	Progestin activity			
	EC_{50}	% of activation	IC_{50}	% of inhibition
Aldosterone	—	ND	~1000 nM	~50
Dexamethasone	6–7 nM	30	~1000 nM	~50
Cortisol	—	ND	~1000 nM	~50
Testosterone	—	ND	~1000 nM	~50
Progesterone	—	100		
R5020	5 nM	100		
RU486	—	ND	1 nM	100
Mespirenone	—	ND	>1000 nM	30

The effective (EC_{50}) and inhibitory (IC_{50}) concentrations giving 50% of the maximum response were determined in two separate experiments. % of inhibition was the value determined in the presence of 10 nM R5020 and 1000 nM of effector. % of activation was the value determined in the presence of 1000 nM effector alone. The 100% activation value was obtained in the presence of 1000 nM R5020. ND: not detectable.

Progestin activities of test-compounds

We analyzed the chimeric progestogenic response of compounds displaying antimineralocorticoid activity. Their progestin potencies (EC_{50}s) are presented in Table 2. FH61, which had no antimineralocorticoid effect, showed progestin activity of <1%. Compounds which displayed antimineralocorticoid activity also had progestogenic potencies (35–80%) with EC_{50}s of about 2–20 nM. Since these compounds are only partial agonists they partially antagonize the luciferase activity induced by 10 nM R5020, with IC_{50}s about 5–100 nM (data not shown). By comparison, mespirenone, an efficient aldosterone antagonist, displayed no progestin activity and only inhibited 20–30% of progesterone-induced luciferase.

Glucocorticoid activities of test-compounds

The glucocorticoid activities of the test-compounds were evaluated by classical induction of TAT in HTC cells. As shown in Table 2, two (FH151 and FH165) showed partial glucocorticoid activity (50 and 40%, respectively) and corresponding antiglucocorticoid activity, while three others (FH157, FH164 and AR126) were mainly antiglucocorticoid. The last compound (FH61) was inactive.

DISCUSSION

One of the objectives of this study was to develop cellular models for the specific measurement of hormonal activities, even when these hormones show natural cross-reactivity in other receptor systems. Recent developments in molecular biology [4, 13–16] have made it possible for us to build various chimeric "minimal receptors" adapted to studies on ligand functions and specificities. It is important to note that not all of the natural receptor is taken into account in this type of construction, particularly the N-terminal region, the function of which is still poorly understood. As discussed below, this may be responsible for discrepancies between these results, and results obtained using an entire receptor.

The results confirmed the functionality of these chimeric receptors as hormone-dependent activators of transcription. They showed that these mineralocorticoid- or progestin-specific cellular systems, gave specific responses to hormonal stimuli. Control

Table 2. Effects of test compounds on chimeric MR in transiently transfected MCF-7 cells, on chimeric PR in HGPL cells and on TAT induction in HTC cells

Compound	Antimineralocorticoid activity		Antiglucocorticoid activity		Progestin activity	
	IC_{50}	% of inhibition	IC_{50}	% of inhibition	EC_{50}	% of activation
AR 126	100 nM	80	170 nM	100	NT	NT
FH 61	—	ND	—	ND	—	<1
FH 151	4–5 nM	100	40 nM	50	3 nM	35
FH 157	30–40 nM	100	100 nM	80	10–20 nM	55
FH 164	30–40 nM	100	80–100 nM	80	4–5 nM	45
FH 165	20 nM	100	70 nM	50–60	2–3 nM	70–80

% of inhibition was determined in the presence of 10 nM of either aldosterone or dexamethasone for antimineralocorticoid or antiglucocorticoid activities, respectively and was expressed for 1000 nM of test-compounds. % of activation, EC_{50} and IC_{50} were determined as described in legend of Table 1. The 100% activation of the progestin activity was obtained in the presence of 1000 nM R5020. ND: not detectable. NT: not tested.

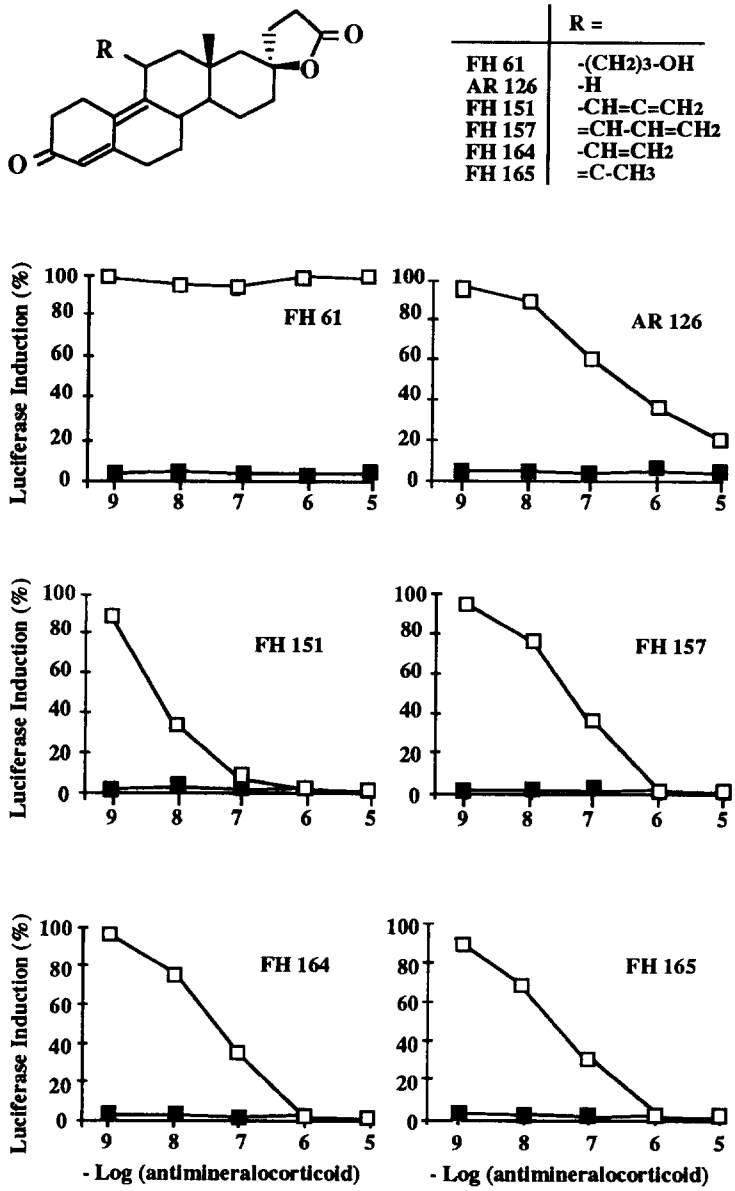

Fig. 4. Antimineralocorticoid and mineralocorticoid effects of test-compounds. Transfected cells were incubated with test-compounds at indicated concentrations. Maximum activity was obtained with 10 nM aldosterone. Inhibition of aldosterone induced luciferase (□) and luciferase activity in the absence of aldosterone (■) were plotted as a function of test-compound concentrations. The results were expressed as described for Fig. 1.

compounds, known for their agonist or antagonist activity in terms of *in vivo* cellular responses, stimulated or inhibited the induction of luciferase activity within these cellular models as expected. Steroids such as testosterone, progesterone, promegestone (R5020) and mifepristone (RU486), which have no mineralocorticoid effects, showed no agonistic activity. Progesterone, which is known as a mineralocorticoid antagonist [38], showed antimineralocorticoid activity in this study. While they are fully agonist in the presence of an entire recombinant receptor [4, 35], glucocorticoids such as cortisol and dexamethasone showed only partial mineralocorticoid activities at concentrations of 10–100 nM in this model. This "aldosterone selectivity" is not due to 11β-hydroxysteroid dehydrogenase (11-HSD) activity in MCF-7 cells, since we checked that (a) no conversion of tritiated cortisol to tritiated cortisone was observed in a cellular homogenate in the presence of $NAD(P)^+$, (b) carbenoxolone did not modify the mineralocorticoid activity of these glucocorticoids, and (c) in assays of glucocorticoid activity, in transiently transfected MCF-7 cells, a classic glucocorticoid response was obtained with either cortisol, corticosterone or dexamethasone, showing that there is probably little, if any, metabolism of these steroids. Therefore, this discrepancy may result from the fact that the chimeric

receptor GAL-MR does not possess the A/B region of the natural receptor, as already observed by Cato *et al.* [39] using a truncated receptor. A study is now under investigation to understand this discriminant property associated with the MR amino-terminus region.

The HGPL cell model showed the expected specificity for (anti)progestin activities, since R5020 and progesterone were the only molecules to induce the response while RU486 was a pure antagonist. The cellular model was used to study the potential antimineralocorticoid effect of 11β-substituted steroids of the 3-oxo-Δ4-17-spirolactone series. Their affinities for the rabbit MR had been established previously [19]. Five of the compounds (AR 126, FH151, FH157, FH164 and FH165) gave inhibition of aldosterone-induced luciferase for IC_{50}s in the 4–100 nM range in relation to their affinities. These results confirm that the presence of the spirolactone function is correlated with the antimineralocorticoid activity for this series of compounds as for the classic antimineralocorticoid series. However, the nature of the 11β-substitution was shown to be important in the efficiency of the test-compounds: the 11β-allenyl derivative (FH151), which was the best ligand for the cytosolic rabbit MR, was effective as an antimineralocorticoid at a 10-fold lower concentration than that of the other steroidal spirolactones of the series with a different 11β-substitution, and at a 2-fold lower concentration than that of the potent antimineralocorticoid, mespirenone. Such an enhancement could be attributed to the π potential of the 11β-allenic moiety and the orientation of this side chain as determined by precise X-ray determination of the crystal and molecular structure of this compound (Rambaud, Declercq and Auzou: unpublished data). At high concentrations, however, this compound showed some agonist/antagonist activities in terms of glucocorticoid and progestin responses. Further modifications in the chemical structures of these compounds are therefore necessary to find a ligand from this series with high specific antimineralocorticoid activity and low secondary effects mediated by the other receptors. The preparation of new 11β-derivatives is currently underway.

Using gene transfer experiments, we have shown that chimeric receptors can be used to screen potent antimineralocorticoid molecules. A stable transfectant cell line was used to assay progestin activity, but difficulties were encountered in attempting to obtain stable transfectants expressing luciferase under the control of mineralocorticoids or glucocorticoids, in that expression rapidly became hormone-independent. At the present time, we are attempting to achieve induction of a stable response in such transfectants.

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