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Selective aldosterone synthase inhibitors reduce aldosterone formation *in vitro* and *in vivo*

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ABSTRACT

Aldosterone plays a crucial role in salt and water homeostasis but in case of pathologically increased plasma aldosterone levels it is also involved in the development and the progression of severe cardiovascular diseases like heart failure and myocardial fibrosis. For the treatment of these diseases we propose inhibition of the aldosterone forming enzyme CYP11B2 as a new pharmacological strategy. We recently developed *in vitro* highly potent and selective inhibitors of human CYP11B2, but the evidence of their *in vivo* activity is still missing. For this purpose, rat aldosterone synthase gene was cloned and expressed in V79MZ cells to establish a new screening assay for the identification of "rat-active" substances. Compound **7** from the class of heteroaryl substituted 3,4-dihydro-1*H*-quinolin-2-ones showed a moderate inhibitory effect (65% at 2 μM) on rat CYP11B2 *in vitro*. Furthermore, it diminished the conversion of deoxycorticosterone to aldosterone in rat adrenals and significantly reduced plasma aldosterone levels *in vivo*.

1. Introduction

The responsible enzyme for the formation of the major mineralocorticoid aldosterone is aldosterone synthase (CYP11B2). This mitochondrial cytochrome P450 enzyme is localized mainly in the *Zona glomerulosa* in the adrenal cortex and catalyses the terminal three steps in the biosynthesis of aldosterone [1]. This hormone plays a key role in salt and water homeostasis by binding to epithelial mineralocorticoid receptors (MR), regulating sodium and water retention as well as potassium secretion. As a consequence of this it is involved in the regulation of blood volume and blood pressure. Aldosterone release is mainly triggered by angiotensin-II via the renin–angiotensin–aldosterone-system (RAAS) and the extracellular potassium concentration. Pathologically elevated plasma aldosterone levels are associated with severe cardiovascular diseases such as hypertension, heart failure and myocardial fibrosis [2–4]. In these disease states reduction of aldosterone action is indicated. The pharmacological therapies include angiotensin-converting enzyme (ACE) inhibitors or angiotensin-II receptor blockers to prevent the stimulation of aldosterone biosynthesis. However, the limiting factor of these treatments is an iterate increase of plasma aldosterone levels, also referred to as aldosterone escape [5]. Recently, MR antagonists like Spironolactone and Eplerenone decreased in two clinical trials (RALES and EPHESUS) hospitalization and mortality in patients after myocardial infarction and in heart failure patients [6,7]. However, with the use of MR blockers severe side effects such as gynaecomastia or dysmenorrhoea are associated due to their steroidal structure and as a consequence of this affinity to steroid receptors. Another complication is the increased hospitalization rate due to hyperkalemia [8]. Moreover, it was reported that the application of Spironolactone leads to an increase of urinary and plasma aldosterone levels in rats [9]. These facts suggest that a long-term treatment with aldosterone antagonists is not a satisfying therapeutic approach. A novel strategy for the treatment of patients with heart failure, myocardial fibrosis and certain forms of hyperaldosteronism is the reduction of aldosterone biosynthesis by inhibition of CYP11B2. Non-steroidal compounds are to be preferred in order to minimize the risk of side effects caused by interaction with steroid receptors or other steroidogenic enzymes. This approach has been propagated by our group since 1994 [10]. In the last decade great efforts were undertaken to develop lead structures from hits discovered by compound library screening [11–14]. Furthermore, a screening system was established to evaluate the potency and the selectivity of the compounds obtained by structural optimization [15–17]. Recently, we succeeded in developing aldosterone synthase inhibitors with improved activity and selectivity against other steroidogenic enzymes such as CYP11B1, CYP17 and CYP19 as well as hepatic cytochrome P450 enzymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) [18–20]. At this stage of preclinical development an *in vivo* proof of concept (POC) should

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be performed demonstrating that the administration of CYP11B2 inhibitors results in decreased plasma aldosterone concentrations. Consequently, some of these compounds were further tested in rats for their aldosterone-lowering activity. However, they were inactive. Subsequent *in vitro* studies showed that they were not able to inhibit rat aldosterone synthase. This finding can be explained by the fact that the homology between the human and the rat enzyme is only 70%.

Further pursuing our POC, we describe in the present study the establishment of a new assay to identify a suitable candidate to investigate aldosterone-lowering effects in rats. A new cell line was constituted, that expresses the rat CYP11B2 enzyme to generate an *in vitro* tool for the identification of "rat-active" compounds. Various compounds from diverse substance classes were tested for inhibitory potency toward the rat aldosterone synthase recombinantly expressed in V79MZ cells. Moreover, an additional assay with rat adrenals was carried out to examine the results obtained from the *in vitro* assay. An active compound, which was identified in these assays, was evaluated for its ability to decrease plasma aldosterone levels in rats.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Carl Roth (Karlsruhe, Germany) or Sigma–Aldrich (Munich, Germany), unless otherwise stated. Cell culture reagents were obtained from c.c. pro (Oberdorla, Germany). [4-^{14}C]-Deoxycorticosterone (45–60 mCi/mmol) was purchased from NEN Life Science (Boston, USA).

2.2. Cell culture

The untransfected V79MZ Chinese hamster cells (provided by Prof. Bernhardt, Saarland University) and the rat CYP11B2 expressing V79MZ cell line were grown as monolayer culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% of fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and sodium pyruvate (1 mM) at 37 °C in 5% CO_2 in air. Transfected cells were maintained in the presence of 750 μg/ml G418 sulfate.

2.3. Cloning of rat CYP11B2 cDNA and plasmid construction

The cDNA of rat CYP11B2 was amplified from total RNA extracted from surgically removed rat adrenals after ACTH stimulation (1 mg/kg) of the animals. Total RNA was isolated with the GenElute™ Total Mammalian Miniprep Kit following the manufacturer's instructions. For cDNA synthesis 1 μg total RNA was used with 0.5 μg oligo-dT_{12-18}, 0.5 mM dNTP-mix and 1 μl Impromm-II™ Reverse Transcriptase (Promega, Madison, USA) in a synthesis buffer with 3 mM $MgCl_2$ according to Promega's standard reverse transcription protocol. This procedure was followed by a polymerase chain reaction (PCR) to amplify the rat CYP11B2 gene with two specific oligonucleotide primers that contained a cleavage site for EcoRI and corresponded to nucleotides 1–29 and 1475–1503 of the rat CYP11B2 cDNA published by Imai et al. [21]. The PCR reaction was performed with 2 μM of each primer, 0.2 mM dNTP-mix, 2.5 U of Hifi Taq polymerase (Roche, Mannheim, Germany) and 10 μl cDNA template of RT reaction described above in a reaction buffer containing 7.5 mM $MgCl_2$. The purified PCR-product (1512 bp) was cloned into MCS of pcDNA3.1/V5-His© TOPO® TA expression vector from invitrogen (Carlsbad, USA). The analysis of the nucleotide sequence of the cloned rat CYP11B2 gene was performed by GATC-Biotech (Konstanz, Germany).

2.4. Transfection procedure

One day before transfection was performed 2×10^5 untransfected V79MZ cells were seeded into 35 mm culture dishes (Nunc, Wiesbaden, Germany) and incubated overnight in complete growth medium. On the following day the approximately 70% confluent culture was transfected with the generated plasmid using the liposomal transfecting reagent Roti®-Fect following the manufacturer's recommendations.

2.5. Selection of stably transfected clones

Stably transfected clones carrying the Neomycin resistance marker were selected with G418 sulfate. First the killing concentration for the untransfected V79MZ cells was determined by using various concentrations of G418 sulfate (200–1000 μg/ml). After 6 days of incubation at a dose of 500 μg/ml G418 sulfate 50% of the cells were killed. Transfected cells were grown for 16 h in transfection medium without antibiotics before medium was replaced by complete growth medium. Selection of transfected clones started 2 days later by addition of 750 μg/ml G418 sulfate and was performed for 2 weeks while untransfected cells died. The isolated transfected cells were subsequently separated to create monoclonal cell lines. Therefore single cell clones were seeded into 96-well cell culture plates and grown in selective medium for further analysis.

2.6. Determination of rat aldosterone synthase activity

The rat aldosterone synthase activity of several monoclonal cell lines was determined by measuring the conversion of radiolabeled substrate ([4-^{14}C]-deoxycorticosterone) to the three products [4-^{14}C]-corticosterone, [4-^{14}C]-18-OH-corticosterone and [4-^{14}C]-aldosterone. For this purpose, transfected cell lines were seeded 1 day before into wells of a 24-well culture plate and incubated overnight to allow the attachment of the cells. Before testing growth medium was removed by aspiration and replaced by 500 μl fresh medium containing the substrate deoxycorticosterone (100 nM, 6 nCi of [4-^{14}C]-deoxycorticosterone in ethanol). After 6 h of incubation enzyme reaction was terminated by extraction of the supernatant with 500 μl ethyl acetate for steroid isolation. The organic solvent was pipetted into fresh cups and evaporated to dryness. The steroids were redissolved in 10 μl chloroform and analyzed by HPTLC (see Section 2.8). As a control the untransfected V79MZ cell line was also incubated with [4-^{14}C]-deoxycorticosterone to demonstrate the absence of enzymatic activity concerning this substrate.

2.7. Screening assay procedure in rat CYP11B2 expressing V79MZ cell line

For determination of inhibitory effects on the rat CYP11B2, transfected cells were grown in 24-well culture plates (1×10^6 cells/well) until confluence. Culture medium was removed by aspiration and replaced by 450 μl fresh medium containing 1% of the ethanolic dilution of the inhibitor resulting in a final concentration of 2 μM. Controls were treated in the same way without inhibitor but 1% ethanol. After a preincubation step of 60 min the enzyme reaction was started by addition of 50 μl DMEM in which the radiolabeled substrate (containing 6 nCi of [4-^{14}C]-deoxycorticosterone in ethanol, final concentration 500 nM) was dissolved. After 5 h incubation the steroids were extracted from supernatant with 500 μl ethyl acetate. The organic layer was separated and evaporated to dryness. Subsequently the steroids were redissolved in 10 μl chloroform and subjected to HPTLC analysis as described in Section 2.8.

2.8. HPTLC analysis and phosphoimaging of radiolabeled steroids

For analysis of the substrate conversion by rat CYP11B2 expressing V79MZ cell lines the redissolved steroids were transferred on a HPTLC plate (20 cm × 10 cm, Silicagel 60F_{254} with concentrating zone) from Merck (Darmstadt, Germany) and developed two times in chloroform:methanol:water (300:20:1) as solvent. Afterwards imaging plates BAS-MS2340 (Raytest, Straubenhardt, Germany) were exposed to the HPTLC plates for 48 h before they were scanned using a FLA3000 scanner (Raytest, Straubenhardt, Germany), substrate and products were quantified by AIDA® software (Raytest, Straubenhardt, Germany). The inhibitory potency of the compounds concerning rat aldosterone synthase activity was calculated from the reduced substrate conversion rate caused by the inhibitor.

2.9. In vitro assay for rat aldosterone synthase inhibitors on rat adrenals

Adrenals from male Wistar rats (Charles River, Germany) were surgically removed and transferred into wells of a 48-well plate containing 700 μl DMEM per well. After addition of inhibitors in final test concentrations of 50 and 250 μM (ethanol 1%, v/v) or 1% ethanol for control organs, preincubation was carried out for 60 min at 37 °C in 5% CO_2 in air. Further assay procedure was performed with 100 nM [4-^{14}C]-deoxycorticosterone for 6 h as described in Section 2.7. Every determination was performed with five organs.

2.10. In vivo aldosterone lowering

Animal care and experimental procedures were performed in accordance with the guidelines for the care and use of laboratory animals published by the National Institutes of Health and the German Animal Protection Law as approved by the local government. Male Wistar rats (Charles River, Germany) weighing 251–276 g (day: ACTH stimulation) and 263–281 g (day: test item application) were used in the present study. Animals were housed in a separate temperature-controlled room (20–24 °C) and maintained in a 12 h light/12 h dark cycle. Food and water were available ad libitum. At least 2 days before application, rats were anaesthetized with a ketamine (90 mg/kg)/xylazine (5 mg/kg) mixture, and cannulated with silicone tubing via the right jugular vein. Prior to the first blood sampling, the rats were connected to a counterbalanced system and tubing to perform blood sampling in the freely moving animal. Predose blood samples (400 μl) were taken before application of ACTH, before application of **7**/vehicle followed by sampling 0.25, 0.5, 1, 2, 3, and 6 h post-dose. Blood was collected in tubes, stored at room temperature until coagulation and subsequently centrifuged at 645 × *g* for 10 min at 4 °C. The harvested serum was kept at −20 °C until being assayed. After each blood sample an equal volume of saline (NaCl 0.9%, ~37 °C, 400 μl) was reinjected in order to keep the blood volume stabilized. Subcutaneous application of ACTH: the animals were stimulated by subcutaneous application of ACTH (1 mg/kg; 1 ml/kg) 16 h before test item application. Intravenous application of test item or vehicle: after sampling of 500 μl blood in a heparinized (15 μl, 500 IE/ml) syringe via the catheter, the formulation of **7** (10 mg/ml in 20% ethanol/50% PEG400/30% water) or the vehicle (20% ethanol/50% PEG400/30% water) was applied within 60 s on the same route to rats with an injection volume of 2 ml/kg. Time zero was registered when the compound was entering the circulation. After that, the 500 μl blood sample taken before was returned via the catheter in order to assure that the entire test compound was administered into the animal. Plasma aldosterone levels were measured using an aldosterone RIA kit from DRG (Marburg, Germany) according to the manufacturer's instructions.

3. Results

3.1. Selection of stably transfected cell clones containing rat CYP11B2 expression plasmid

About 60 h after transfection of V79MZ cells with the generated plasmid selection of clones carrying the Neomycin marker was started by addition of G418 sulfate. After 2 weeks of cultivation untransfected cells had died and resistant clones were separated to achieve monoclonal cell lines. For determination of rat aldosterone synthase activity the conversion rate of [4-^{14}C]-deoxycorticosterone to the three radiolabeled products corticosterone, 18-OH-corticosterone and aldosterone was measured. The untransfected V79MZ cell line was also incubated with the radiolabeled substrate to demonstrate that no substrate was converted. Out of all analyzed monoclonal cell lines the clone with the highest conversion rate was chosen for the development of a new screening assay to identify inhibitors of rat CYP11B2. This monoclonal cell line was named V79/CYP11B2-rat and exhibited a conversion rate of 25.3% within 6 h, whereas the substrate conversion of untransfected cells was less than 2%.

3.2. Establishment of a cellular screening assay for rat aldosterone synthase inhibitors

For determination of optimal test conditions different parameters were evaluated with the cell line V79/CYP11B2-rat. For this purpose, conversion rates of different numbers of cells were investigated in diverse multi-well cell culture plates at different time points. To reach an ideal substrate conversion of 12–20% with a confluent cell layer the following test conditions were elaborated: incubation of 1 × 10^6 cells/well in a 24-well plate for 5 h with a substrate concentration of 500 nM.

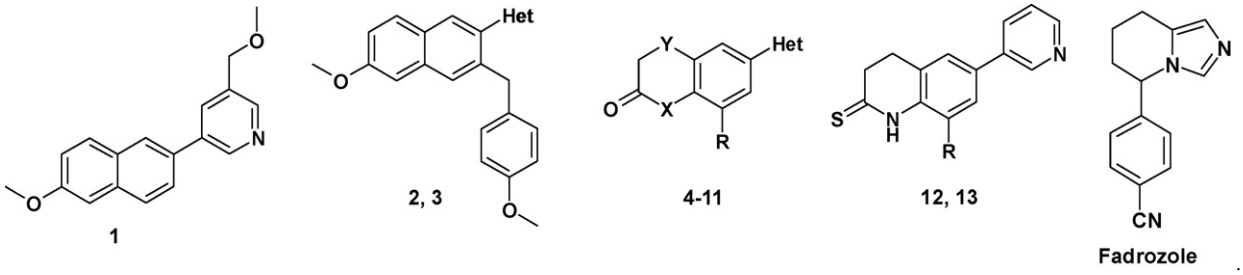
3.3. Screening of selected compounds

For the evaluation of the inhibitory potency toward rat aldosterone synthase, selected compounds were tested at a final concentration of 2 μM as described in Section 2.7. In order to determine a suitable candidate to investigate aldosterone-lowering effects in rats, we evaluated potent and selective inhibitors of human aldosterone synthase [18–20] for their ability to block aldosterone biosynthesis in V79MZ cells expressing rat CYP11B2 prior to *in vivo* experiments. In this screening assay the reference inhibitor *Fadrozole* showed a high inhibition of 73% at 0.5 μM. As shown in Table 1 only compound **7** (and to a minor degree also the *N*-methyl analogue **9**) showed a moderate inhibitory action (65% at 2 μM) on rat CYP11B2 *in vitro* within the class of heteroaryl substituted 3,4-dihydro-1*H*-quinolin-2-ones and structurally related substances. Compound **7** is a highly potent inhibitor of human CYP11B2 *in vitro* (IC_{50} = 0.2 nM) and also exhibits a pronounced selectivity versus other CYP enzymes [20]. All other tested compounds, which were in the human enzyme highly active, showed slight or no inhibition (0–16% at 2 μM) of the rat CYP11B2.

3.4. Inhibition of rat CYP11B2 evaluated in rat adrenals

For examination of the results we achieved by the *in vitro* screening assay compound **7** was also tested for its inhibitory potency against rat aldosterone synthase using rat adrenals, that were surgically removed from male Wistar rats. The CYP11B2 inhibitor SL272 (unpublished data), highly active and selective in human enzyme, but not active in the rat enzyme (0% inhibition), was taken as a reference (Fig. 1a). After preincubation of the organs with the inhibitors at two different concentrations (50 and 250 μM), the reduction of the substrate conversion caused by the substances compared to

Table 1
Percent inhibition at 0.5 μM and IC_{50} values for test compounds in V79MZ cells expressing the human CYP11B2 gene compared to percent inhibition in V79/CYP11B2-rat at 2 μM.



Compd	R	X	Y	Het	% inhibition[a,b] human CYP11B2[c] (V79MZ cells)	IC_{50} [nM][d,b] human CYP11B2[c] (V79MZ cells)	% inhibition[e] rat CYP11B2[f] (V79MZ cells)
1					96	0.2	2
2				3-Pyridine	95	11	16
3				4-Isoquinoline	94	5.0	11
4	H	NH	CH_2	3-Pyridine	88	28	4
5	H	NMe	CH_2	3-Pyridine	92	2.6	0
6	H	NH	CH_2	5-Methoxy-3-pyridine	91	2.7	16
7	H	NH	CH_2	4-Isoquinoline	94	0.2	65
8	H	NMe	CH_2	5-Methoxy-3-pyridine	94	0.2	0
9	H	NMe	CH_2	4-Isoquinoline	99	0.1	49
10	Cl	NH	CH_2	3-Pyridine	97	3.8	0
11	H	NH	S	3-Pyridine	92	12	0
12	H				97	3.1	4
13	Cl				97	4.2	3
Fadrozole						1.0	73 [0.5 μM]

[a] Mean value of at least four experiments, standard deviation less than 10%.
[b] Former published data [18–20].
[c] Hamster fibroblasts expressing human CYP11B2, substrate deoxycorticosterone, 100 nM.
[d] Mean value of at least four experiments, standard deviation usually less than 25%.
[e] Mean value of at least three experiments, standard deviation less than 15%.
[f] Hamster fibroblasts expressing rat CYP11B2, substrate deoxycorticosterone, 500 nM.

control organs was measured. In accordance with the results from the cellular inhibition assay the human CYP11B2 inhibitor SL272 did not decrease aldosterone synthase activity in rat adrenals at a concentration of 50 μM and led only to a 30% reduction of aldosterone formation at 250 μM (Fig. 1a). This effect was not significant. For compound **7**, which had shown a moderate inhibition at the rat enzyme (Table 1), the *in vitro* results could also be confirmed. The aldosterone biosynthesis in rat adrenals was significantly reduced by 76% at 50 μM ($P < 0.05$) and 89% at 250 μM ($P < 0.003$) (Fig. 1b).

3.5. Inhibition of aldosterone formation in vivo

Two days before application, adult male rats were cannulated with silicone tubing via the right jugular vein. Prior to the first blood sampling, the rats were connected to a counterbalanced system and tubing to perform blood sampling in the freely moving animal. Pre-dose blood samples were taken before application of ACTH and before application of **7** (animals 1–6) or vehicle (animals 7–10), followed by sampling 0.25, 0.5, 1, 2, 3, and 6 h post-dose. The animals received a subcutaneous injection of ACTH (1 mg/kg) 16 h before test item application to stimulate the corticoid biosynthesis. The aldosterone levels were determined using RIA. It becomes apparent that ACTH treatment induced a significant increase of the aldosterone levels. Within the vehicle-treated group (Fig. 2b), the concentrations found in the plasma were rather constant over the duration of the experiment (6 h). To six animals, herein after referred to as compound **7** group, isoquinoline derivative **7** was intravenously applied in a 20 mg/kg dose after 16 h upon ACTH treatment (Fig. 2a). As can be seen from the individual curves there are differences in the t_{max} values, course of the curves and maximal reduction values, however, there is no doubt that compound **7** is highly active in reducing aldosterone plasma concentrations.

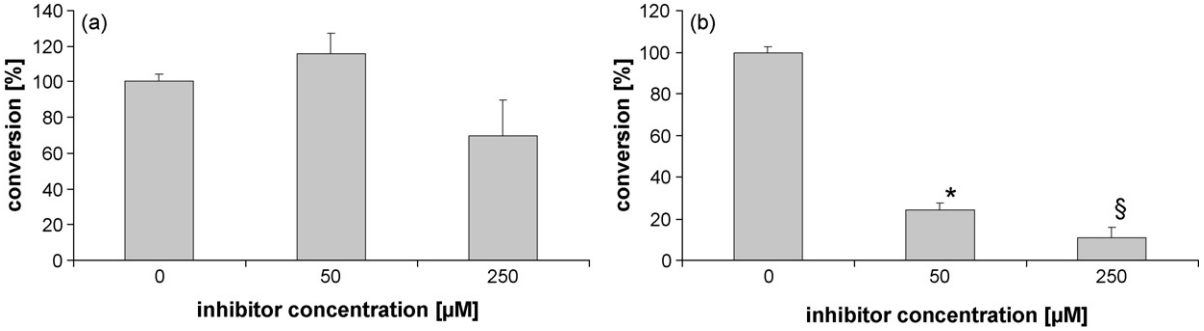
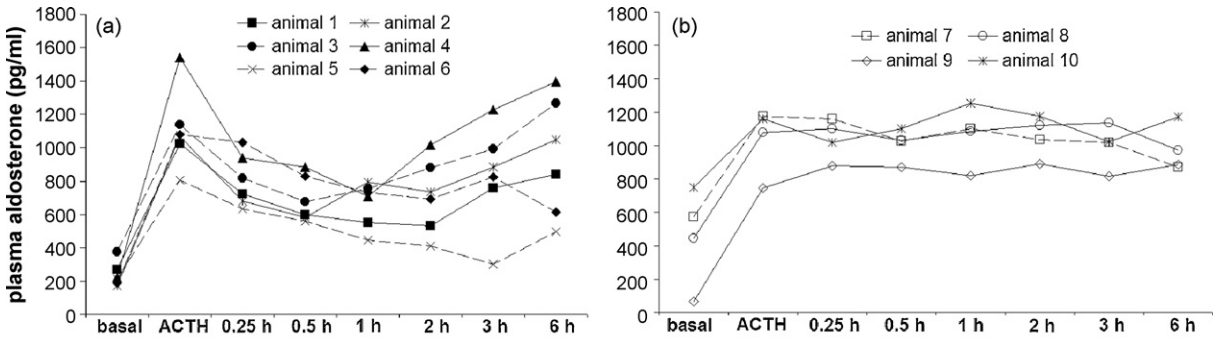


Fig. 1. Inhibition of aldosterone biosynthesis in rat adrenals by reference compound SL272 (a) and **7** (b) at 50 and 250 μM. Bars represent mean values ($n = 5$, ±SD) of relative formation compared to control. *$P < 0.05$ versus control; §$P < 0.003$ versus control.



animal no.	aldosterone plasma level (pg/ml) basal[a]	ACTH stimulated[b]	maximally reduced[c] [% reduction[d]]	$t_{max\ red}$ (h)[e]
1	270	1026	531 [48]	2
2	173	1068	585 [45]	0.5
3	375	1139	676 [41]	0.5
4	222	1543	708 [54]	1
5	217	805	302 [63]	3
6	188	1081	694 [36]	2

[a]Pre-dose blood sample taken 0.5 h before application of ACTH. [b]The animals were stimulated by subcutaneous application of ACTH (1 mg/kg) 16 h before test item application; blood sample taken before application of **7**. [c]Lowest aldosterone concentration measured for the individual animal. [d]Maximal reduction of aldosterone level compared to ACTH stimulated level. [e]Time of lowest aldosterone plasma concentration.

Fig. 2. Lowering of aldosterone plasma levels *in vivo*. (a) Compound **7** group (animals 1–6), (b) vehicle group (animals 7–10).

This effect could already be observed after 15 min. Within a timeframe of 0.5–3 h, the aldosterone concentrations were maximally reduced to 36–63% of the prior ACTH level for the individual animals of the compound **7** group. The aldosterone-lowering effect was highly significant ($P < 0.003$) 1 h after the compound **7** administration, and significant ($P < 0.05$) at all other time points between 0.25 and 2 h relating to ACTH stimulated plasma aldosterone levels. Furthermore, no obvious sign of toxicity was noted in any animal during the course of treatment. The rats were curious, showed normal behaviour and general conditions (e.g. breathing rate, mobility, normothermia).

4. Discussion and conclusion

The involvement of the major mineralocorticoid aldosterone in the development and progression of severe cardiovascular diseases is indisputable. The levels of plasma aldosterone in patients with untreated heart failure are significantly elevated and are strongly associated with mortality [22]. Moreover, high aldosterone levels cause excessive sodium retention with expansion of extracellular volume and loss of potassium. These instances lead to an electrolyte imbalance and increase the sensitivity of cardiac tissue to arrhythmias as well as the risk of sudden death [23]. In this context, it was reported, that aldosterone antagonists reduce the risk of sudden cardiac death in patients with heart failure [24]. Another important complication of chronically elevated plasma aldosterone levels is the development of myocardial fibrosis and as a consequence of this an increasing stiffness of the heart which further impairs the heart in a *circulus vitiosus*. It could be shown, that the activation of the mineralocorticoid receptor in the fibroblasts of the human heart promotes proliferation of these cells and increases production of collagen [25].

Since the nineties we propose aldosterone synthase (CYP11B2) as a new promising target for the treatment of these cardiovascular diseases and certain forms of hyperaldosteronism. Recently, we improved our lead structures to highly active and selective CYP11B2 inhibitors with ameliorated pharmacological characteristics [18–20]. For the further development of a drug candidate it is necessary to demonstrate its *in vivo* activity. After the evaluation of the *in vitro* profile of the compounds their *in vivo* efficacy had to be shown. Thus, with regard to problems due to species differences we established a further *in vitro* test to examine the activity of various compounds on the rat enzyme. For this purpose, we cloned the rat CYP11B2 encoding cDNA from rat adrenals in an appropriate expression vector and transfected the generated plasmid into V79MZ cells. After selection of stably transfected clones and isolation of monoclonal cell lines, the clone with the highest rat aldosterone synthase activity was chosen. Using the rat CYP11B2 expressing V79MZ cell line a new screening assay was established and our highly selective compounds were tested regarding the reduction of aldosterone formation.

Thereby, it turned out, that only compound **7** showed sufficient activity. All other tested compounds decreased the aldosterone synthesis only to a minor extent. The fact that our highly active compounds (IC_{50} values between 0.1 and 74 nM), show no or just slight inhibition on the rat enzyme is not really surprising as the identity between human and rat enzyme is only 70%. To verify the results obtained from our new screening assay, an additional *in vitro* assay using whole rat adrenals was performed with compound **7**. Herein, we also found a strong inhibition of aldosterone formation by 76 and 89% at 50 and 250 μM, respectively. After we had identified **7** to be a suitable candidate for investigation of aldosterone-lowering effects in rats, the *in vivo* assay was performed. A significant lowering of the plasma aldosterone levels in the range of 36–63% was observed within 0.5–3 h. This result not only supplies POC that inhibition of CYP11B2 leads to a reduction of plasma aldosterone concentration, but also identifies **7** as a scientific tool for general pharmacological studies at the RAAS.

Currently, further experiments in disease-oriented models are underway to determine the potential of our aldosterone synthase inhibitors to prevent or reverse myocardial fibrosis and reduce heart failure induced mortality. Our strategy of aldosterone synthase inhibition is a promising approach for the treatment of

certain forms of hyperaldosteronism and might improve the pharmacotherapy of heart failure and myocardial fibrosis. Aldosterone escape as described for ACE inhibitors should not be expected and severe side effects as observed for steroidal MR antagonists are not to be anticipated because of the non-steroidal structures of our compounds. The use of aldosterone synthase inhibitors should not lead to hyperkalemia, which is a common drawback of MR antagonists, as there is still stimulation of the mineralocorticoid receptor by the mineralocorticoids deoxycorticosterone and corticosterone as well as by cortisol.

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References

[1] T. Kawamoto, Y. Mitsuuchi, K. Toda, Y. Yokoyama, K. Miyahara, S. Miura, T. Ohnishi, Y. Ichikawa, K. Nakao, H. Imura, S. Ulick, Y. Shizuta, Role of steroid 11β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 1458–1462.
[2] D.F. Davila, T.J. Nunez, R. Odreman, C.A. de Davila, Mechanisms of neurohormonal activation in chronic congestive heart failure: pathophysiology and therapeutic implications, Int. J. Cardiol. 101 (2005) 343–346.
[3] C.G. Brilla, Renin–angiotensin–aldosterone system and myocardial fibrosis, Cardiovasc. Res. 47 (2000) 1–3.
[4] P. Lijnen, V. Petrov, Induction of cardiac fibrosis by aldosterone, J. Mol. Cell. Cardiol. 32 (2000) 865–879.
[5] A. Sato, T. Saruta, Aldosterone escape during angiotensin-converting enzyme inhibitor therapy in essential hypertensive patients with left ventricular hypertrophy, J. Int. Med. Res. 29 (2001) 13–21.
[6] B. Pitt, F. Zannad, W.J. Remme, R. Cody, A. Castaigne, A. Perez, J. Palensky, J. Wittes, The effect of spironolactone on morbidity and mortality in patients with severe heart failure, N. Engl. J. Med. 341 (1999) 709–717.
[7] B. Pitt, W. Remme, F. Zannad, J. Neaton, F. Martinez, B. Roniker, R. Bittman, S. Hurley, J. Kleiman, M. Gatlin, Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction, N. Engl. J. Med. 348 (2003) 1309–1321.
[8] D.N. Juurlink, M.M. Mamdani, D.S. Lee, A. Kopp, P.C. Austin, A. Laupacis, D.A. Redelmeier, Rates of hyperkalemia after publication of the Randomized Aldactone Evaluation Study, N. Engl. J. Med. 351 (2004) 543–551.
[9] J. Ménard, M.-F. Gonzalez, T.-T. Guyene, A. Bissery, Investigation of aldosterone-synthase inhibition in rats, J. Hypertens. 24 (2006) 1147–1155.
[10] R.W. Hartmann, Selective inhibition of steroidogenic P450 enzymes: current status and future perspectives, Eur. J. Pharm. Sci. 2 (1994) 15–16.
[11] S. Ulmschneider, U. Müller-Vieira, C.D. Klein, I. Antes, T. Lengauer, R.W. Hartmann, Synthesis and evaluation of (pyridylmethylene)tetrahydronaphthalenes/-indanes and structurally modified derivatives: potent and selective inhibitors of aldosterone synthase, J. Med. Chem. 48 (2005) 1563–1575.
[12] S. Ulmschneider, U. Müller-Vieira, M. Mitrenga, R.W. Hartmann, S. Oberwinkler-Marchais, C.D. Klein, M. Bureik, R. Bernhardt, I. Antes, T. Lengauer, Synthesis and evaluation of imidazolylmethylenetetrahydronaphthalenes and imidazolylmethyleneindanes: potent inhibitors of aldosterone synthase, J. Med. Chem. 48 (2005) 1796–1805.
[13] M. Voets, I. Antes, C. Scherer, U. Müller-Vieira, K. Biemel, C. Barrasin, S. Oberwinkler-Marchais, R.W. Hartmann, Heteroaryl substituted naphthalenes and structurally modified derivatives: selective inhibitors of CYP11B2 for the treatment of congestive heart failure and myocardial fibrosis, J. Med. Chem. 48 (2005) 6632–6642.
[14] M. Voets, I. Antes, C. Scherer, U. Müller-Vieira, K. Biemel, S. Oberwinkler-Marchais, R.W. Hartmann, Synthesis and evaluation of heteroaryl-substituted dihydronaphthalenes and indenes: potent and selective inhibitors of aldosterone synthase (CYP11B2) for the treatment of congestive heart failure and myocardial fibrosis, J. Med. Chem. 49 (2006) 2222–2231.
[15] P.B. Ehmer, M. Bureik, R. Bernhardt, U. Müller, R.W. Hartmann, Development of a test system for inhibitors of human aldosterone synthase (CYP11B2): screening in fission yeast and evaluation of selectivity in V79 cells, J. Steroid. Biochem. Mol. Biol. 81 (2002) 173–179.
[16] R.W. Hartmann, U. Müller, P.B. Ehmer, Discovery of selective CYP11B2 (aldosterone synthase) inhibitors for the therapy of congestive heart failure and myocardial fibrosis, Eur. J. Med. Chem. 38 (2003) 363–366.
[17] U. Müller-Vieira, M. Angotti, R.W. Hartmann, The adrenocortical tumor cell line NCI-H295R as an *in vitro* screening system for the evaluation of CYP11B2 (aldosterone synthase) and CYP11B1 (steroid-11β-hydroxylase) inhibitors, J. Steroid. Biochem. Mol. Biol. 96 (2005) 259–270.
[18] R. Heim, S. Lucas, C.M. Grombein, C. Ries, K.E. Schewe, M. Negri, U. Müller-Vieira, B. Birk, R.W. Hartmann, Overcoming undesirable CYP1A2 inhibition of pyridylnaphthalene-type aldosterone synthase inhibitors: influence of heteroaryl derivatization on potency and selectivity, J. Med. Chem. 51 (2008) 5064–5074.
[19] S. Lucas, R. Heim, M. Negri, I. Antes, C. Ries, K.E. Schewe, A. Bisi, S. Gobbi, R.W. Hartmann, Novel aldosterone synthase inhibitors with extended carbocyclic skeleton by a combined ligand-based and structure-based drug design approach, J. Med. Chem. 51 (2008) 6138–6149.
[20] S. Lucas, R. Heim, C. Ries, K.E. Schewe, B. Birk, R.W. Hartmann, In vivo active aldosterone synthase inhibitors with improved selectivity: lead optimization providing a series of pyridine substituted 3,4-dihydro-1H-quinolin-2-one derivatives, J. Med. Chem. 51 (2008) 8077–8087.
[21] M. Imai, H. Shimada, Y. Okada, Y. Matsushima-Hibiya, T. Ogishima, Y. Ishimura, Molecular cloning of a cDNA encoding aldosterone synthase cytochrome P-450 in rat adrenal cortex, FEBS Lett. 263 (1990) 299–302.
[22] K. Swedberg, Q. Eneroth, J. Kjekshus, L. Wilhelmsen, CONSENSUS Trial Study Group, Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality, Circulation 82 (1990) 1730–1736.
[23] H. Tsuji, F.J. Venditti Jr., J.C. Evans, M.G. Larson, D. Levy, The association of levels of serum potassium and magnesium with ventricular premature complexes (the Framingham Heart Study), Am. J. Cardiol. 74 (1994) 232–235.
[24] K. Anand, A.N. Mooss, S.M. Mohiuddin, Aldosterone inhibition reduces the risk of sudden cardiac death in patients with heart failure, J. Renin Angiotensin Aldosterone Syst. 7 (2006) 15–19.
[25] V. Robert, J.-S. Silvestre, D. Charlemagne, A. Sabri, P. Trouvé, M. Wassef, B. Swynghedauw, C. Delcayre, Biological determinants of aldosterone-induced cardiac fibrosis in rats, Hypertension 26 (1995) 971–978.