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The development of a whole-cell based medium throughput screening system for the discovery of human aldosterone synthase (CYP11B2) inhibitors: Old drugs disclose new applications for the therapy of congestive heart failure, myocardial fibrosis and hypertension

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ABSTRACT

Cytochrome P450 enzymes play an important role in steroid hormone biosynthesis of the human adrenal gland, e.g., the production of cortisol and aldosterone. Aldosterone, the most important human mineralocorticoid, is involved in the regulation of the salt and water homeostasis of the body and thus in the regulation of blood pressure, whereas cortisol is the most important glucocorticoid of the human body. CYP11B-dependent steroid hydroxylases are drug development targets, and since they are very closely related enzymes, the discovery of selective inhibitors has been subject to intense investigations for several years. Here we report the development of a whole-cell medium throughput screening technology for the discovery of CYP11B2 inhibitors. The new screening system displayed high reproducibility and was applied to investigate a library of pharmacologically active compounds. 1268 compounds were investigated during this study which revealed 5 selective inhibitors of CYP11B2 (after validation against CYP11B1). The new inhibitors of CYP11B2 are already existing drugs that could be used either in the treatment of hyperaldosteronism-related diseases or as lead compounds that could further be optimised to achieve safer and selective inhibitors of aldosterone synthase.

Article from the Special issue on 'Targeted Inhibitors'

1. Introduction

Aldosterone, the most important human mineralocorticoid, is involved in the regulation of the salt and water homeostasis of the body and thus in the regulation of blood pressure. It is unsurprising, therefore, that excessive aldosterone secretion has been reported in several cases of hypertension and has been correlated with higher mortality in congestive heart failure and fibrosis of the heart [1–4]. In addition to this, chronic elevation of aldosterone has also been found in the presence of adenoma, idiopathic hyperaldosteronism and insufficient renal flow [5]. Although the use of mineralocorticoid antagonists shows clinical benefit in the treatment of these diseases, it also leads to severe side effects like gynaecomastia and endocrinal dysregulation. Therefore, trials to inhibit the synthesis of aldosterone directly have been published [4,6–11]. Aldosterone synthesis in human is mediated through several cytochrome P450 enzymes, among which CYP11B2 catalyses the terminal three steps, the 11β-hydroxylation of 11-deoxycorticosterone (DOC) that leads to corticosterone (B), which is then 18-hydroxylated to yield 18-hydroxycorticosterone (18-OH-B) and finally oxidized to aldosterone (Fig. 1).

Thus, CYP11B2 comprises a target for drug treatment and selective inhibitors of the aldosterone-producing CYP11B2 enzyme are of high pharmacological interest [4,11,12]. However, it turned, out that CYP11B2 is not an easy drug target, since it shares 93% sequence identity on the protein level with the cortisol-producing cytochrome CYP11B1, which hydroxylates 11-deoxycortisol (RSS) into cortisol (F). This makes the development of selective inhibitors rather difficult. Both CYP11B1 and CYP11B2 11β-hydroxylate RSS and DOC *in vitro* [13–15]; however, the human CYP11B1 enzyme is a pure 11β-hydroxylase without 18-hydroxylase or 18-oxidase activity and is even unable to 11β-hydroxylate 18-OH-DOC [16]. Therefore, the inhibition of the CYP11B2-mediated bioconversion of DOC into aldosterone has been reported to be an useful tool in the management of hyperaldosteronism related diseases [4,12].

In our laboratory, two test systems have been developed for the evaluation of compounds with respect to their inhibitory effect on human CYP11B1 and CYP11B2, a mammalian cell culture system using recombinant V79 cells [6,15], and a fission yeast based system using recombinant *Schizosaccharomyces pombe* [7,17]. Although

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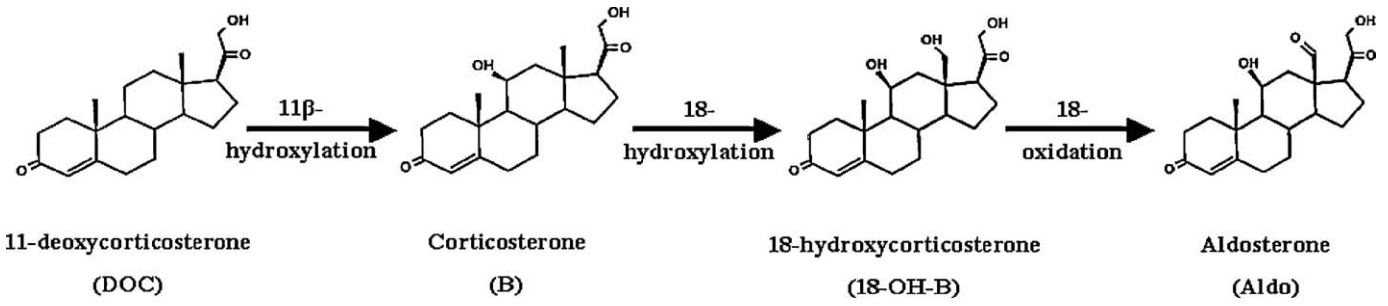

Fig. 1. CYP11B2 converts 11-deoxycorticosterone via corticosterone and 18-OH corticosterone to aldosterone.

every test system shows advantages and disadvantages [4], selective inhibitors of CYP11B2 could be identified [6–10,18]. Since these two systems are dependent on radioactive substrates and products, neither of them can be considered as high or medium throughput screening system for the discovery of aldosterone synthase inhibitors. Here, we report the development of an automated medium throughput screening system for the discovery of CYP11B2 inhibitors using a CYP11B2-expressing *Schizosaccharomyces pombe* strain in a 96-well microtiter plate format coupled to high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals

Radioactive steroids were obtained from Amersham Pharmacia Biotech (Freiburg, Germany) or NEN (Boston, MA, USA), ketoconazole, miconazole and clotrimazole were from FAGRON (Barsbüttel, Germany), non-radioactive steroids and a library of 1268 compounds from the LOPAC^{1280} library (Library of Pharmacologically Active Compounds) were obtained from SIGMA (Deisenhofen, Germany). The library is a collection of high quality innovative molecules that span a broad range of cell signalling and neuroscience areas. For more information about the LOPAC^{1280} library see http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/LO1280

2.2. Fission yeast strains and general techniques

Recombinant fission yeast strain MB164 that expresses human CYP11B2 [17] was used as whole-cell system to develop a screening system to evaluate compounds with respect to their inhibitory effect on CYP11B2. Furthermore, this strain as well as the recombinant fission yeast SZ1 that expresses human CYP11B1 [19] were used to validate the hit compounds concerning their selectivity. Yeast cells were cultivated at 30 °C in Edinburgh minimal media (EMM) as described in detail before [17,19–22].

2.3. CYP11B2-dependent steroid hydroxylation assays in 96-well plate format and the detection of conversion/inhibition using the HPLC

For the development of a new screening technology for the discovery of CYP11B2 inhibitors using the 96-well plate format and the HPLC as a detection method, the steroid bioconversion assay in the 96-well plate format established before for cytochrome CYP11B1 [22] was adapted and optimised for cytochrome CYP11B2 to achieve this aim. Plate preparation and steroid extraction were carried out automatically using a pipetting robot (Tecan Aquarius, Switzerland) [22]. After the incubation of the CYP11B2-expressing fission yeast strain MB164 with the substrate (DOC) and inhibitors, steroids were extracted automatically using the pipetting robot, which was programmed to perform several aspirating and dispensing steps (shaking-like process) in order to extract the steroids. The steroids-containing organic phase (chloroform) was then transferred to a new 96-well plate. Dried steroids were then resuspended in acetonitril and separated on a Jasco reversed phase HPLC system (Tokyo, Japan) composed of an auto-sampler AS-2050 plus, pump PU-2080, gradient mixer LG-2080-02 and an UV-detector UV-2075 plus equipped with a reversed phase Nova-Pak® C18 60Ao 4 μm column from Waters (Milford, MA, USA). The column temperature was kept constant at 25 °C with a peltier oven. The mobile phase used for steroid separation was a mixture of ACN:H_2O (60:40) with flow velocity of 1.2 ml/min. Steroids were detected at 240 nm, and peak identification was done using the ChromPass software (V.1.7.403.1, Jasco), pure steroids (>99%) were used as standards to identify the peaks on HPLC.

Since, the steroids present in sample are chemically and physically very similar molecules, it was assumed that the relative loss of steroids during the extraction procedure is equal for all steroids. Therefore, the ratio of product formation can be calculated depending on the intensity signal (I) of steroid of interest as shown below.

$$R\,\text{product}(\%) = \frac{I(\text{product})}{I(\text{product}) + I(\text{substrate})} \times 100$$

The intensity signal of a steroid is the peak area (A) of a certain steroid in the chromatogram with dimension mV.min in the case of HPLC or the radioactive signal (I_{radio}) in the case of HPTLC as shown below.

2.4. Validation of selectivity against CYP11B1/2 and the determination of the IC_{50} values

The compounds defined during the screening system, developed in this work, as potential inhibitors of CYP11B2 were further validated and investigated for selectivity against CYP11B2 as well as against CYP11B1. For this, fission yeast strains SZ1 and MB164 expressing human CYP11B1 and CYP11B2, respectively, were used to perform the validation assays as described before [7]. 500 μl cell suspensions with cell density of 5×10^7 cells/ml were prepared and pipetted in 1.5 ml Eppendorf tubes. Inhibitors were dissolved in DMSO at different concentrations, and equal volumes were used to achieve final concentrations of inhibitors ranged from 100 nM to 25 μM. Cells were pre-incubated at 30 °C and 1400 rpm using a tube shaker with the respective inhibitor solutions for 15 min prior to the addition of 100 nM steroid substrate dissolved in EtOH (11-deoxycortisol or 11-deoxycorticosterone in the case of CYP11B1 or CYP11B2, respectively). For the detection of CYP11Bs-dependent steroid bioconversion, 0.15 μCi [^{3}H] 11-deoxycortisol or 2.5 nCi [^{14}C] 11-deoxycorticosterone were added to each vial, respectively. After 3 h incubation at 30 °C and 1400 rpm, steroids were extracted with chloroform. Extracted, dried, radioactive samples were dissolved in 10 μl chloroform and applied on the concentrating zone

of an HPTLC silica gel 60 F254 plate (Merck, Darmstadt, Germany). The mobile phase for chromatography was $CHCl_3$:MeOH:H_2O (300:20:1). Radioactive decay signals were exposed to BAS-TR2040 (^{3}H) or BAS-IIIS (^{14}C) imaging plates (IP) from Fuji (Tokyo, Japan), and scanned with the BAS-2500 phosphoimager (BAS-2500, Fuji; Stamford, CT). Pure 10 mM steroid solutions dissolved in EtOH were used as reference substances for the identification of bands on the scanned IP.

Quantification data analysis procedures were performed using the open-source analysis software TINA v2.10g. The intensity of a region of interest on the imaging plate was reported in PSL (phosphostimulated luminescence) units, whereby the background exposure signal was subtracted from the raw PSL values prior to conversion calculations by the internal background quantification function. The intensity of the radioactive signal (I_{radio}) caused by a certain steroid is proportional to the amount of radioactively labelled steroid. The ratio of a certain steroid product P can be regarded as a function of the inhibitor concentration c_{inh}. The inhibition of the production of P (*INH*(P)) from the substrate is therefore defined as:

$$INH(P) = 1 - \frac{R(\mathrm{P}, c_{\mathrm{inh}})}{R(\mathrm{P}, c_{\mathrm{inh}} = 0)}$$

where $R(\mathrm{P},c_{inh} = 0)$ is the ratio of product in the control reaction without inhibitor. From the above formula one can clearly see that when $c_{inh} = 0$ or when the inhibitor shows no effect at all then $R(\mathrm{P},c_{inh} = 0) = R(\mathrm{P},c_{inh})$ for all c_{inh} and the inhibition is 0. Multiplying *INH*(P) by 100 displays the result in percent inhibition of the control reaction. The presentation of the data requires the following substitutions:

y:=*INH*(P),
x:=log(c_{inh}).

After inserting the data in a two dimensional scatter plot, the function of x and y was fitted and used to calculate the IC_{50} value. The IC_{50} value is the inhibitor concentration $c_{inh,50}$ where *INH* = 0.5 for the production of P.

2.5. Statistical analysis

To evaluate the results descriptive statistics were applied using the "Statistica" computer program. Moreover, *t*-test for independent samples was applied to evaluate the differences in means between two groups (with or without inhibitor), whereas the correlation coefficient Pearson *r*, was applied to investigate the correlation between the inhibition and concentration of inhibitor in different test media. The results of these statistical tests were considered significant when $p < 0.05$. Furthermore, the Z'-factor [23] was used for the evaluation and validation of the screening system developed in this work. The Z'-factor was determined from the inhibition assays of ketoconazole, clotrimazole and miconazole against CYP11B2 in recombinant fission yeast. The calculation of Z'-factor was carried out using the following formula:

$$Z' = 1 - \frac{3 \times \sigma_{\mathrm{PC}} + 3 \times \sigma_{\mathrm{NC}}}{|\bar{t}_{\mathrm{PC}} - \bar{t}_{\mathrm{NC}}|}$$

where $\bar{t}$ and σ are the mean and standard deviation of mean, respectively. PC refers to the positive control and NC to negative control.

2.6. Structure–activity relationship (SAR) study

The structure–activity analyses were performed using the Benchware HTS DataMiner (Tripos). Only the new selective CYP11B2 inhibitors defined in this work were included in this SAR study.

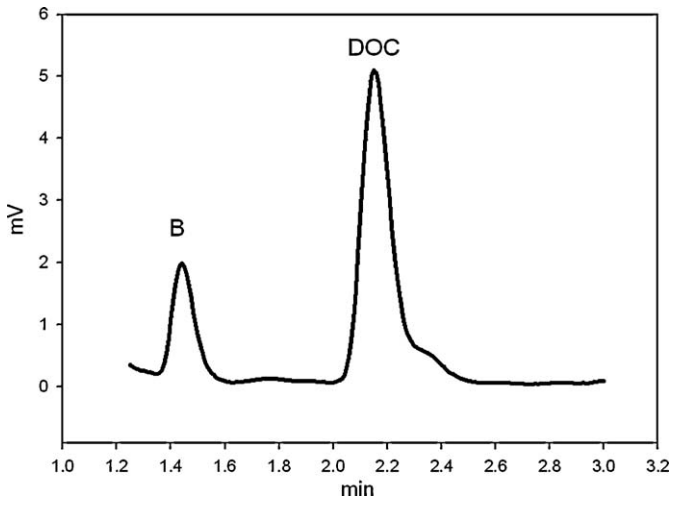

Fig. 2. HPLC trace of the CYP11B2-dependent bioconversion using the 96-well plate format. The bioconversion was carried out in a 96-well plate using the CYP11B2-expressing fission yeast strain MB164 incubated with 5 μM DOC for three hours. Steroid extraction was carried out with chloroform using the pipetting robot as described before.

3. Results

3.1. Development of an automated screening technology for the discovery of aldosterone synthase inhibitors using the 96-well plate format

Although CYP11Bs-expressing fission yeast strains have been reported for the investigation of the inhibitory effect of compounds against CYP11Bs, the described method (summarised in Section 2.4) is a multiple-point method that investigates the compounds at different concentrations using a radioactive substrate and Eppendorf vials in order to generate the IC_{50} value in the case of inhibition. In contrast to this manual method, which cannot be applied on a large scale to screen big libraries of compounds, the main purpose of this work was the development of an automated one-point screening technology that enables the parallel investigation of thousands of compounds for the discovery of new potential inhibitors of CYP11B2 in a medium throughput screening system.

For the establishment of the optimal conditions where a robust screening system can be developed, several parameters involving the cell density of the CYP11B2-expressing fission yeast strain MB164 and the concentrations of substrate and compounds were investigated. The investigations showed that a 600 μl cell suspension of MB14 with cell density of 10^8 cells/ml displays a detectable bioconversion of DOC into B (Fig. 2) after 3 h incubation with 5 μM DOC (10 μl from a 0.33 mM stock solution in EtOH) using a 96-well plate. Furthermore, different test media were investigated to find out the optimal test medium where a reproducible detection of conversion and inhibition can be carried out. For this purpose, the inhibition profile of the CYP11B2 inhibitor ketoconazole was investigated in different test media with various final concentrations from 5 to 60 μM. The ketoconazole inhibition profile in the fission yeast medium EMM displayed bad correlation between the inhibitor concentration and inhibition ($r = 0.548$) (Fig. 3A), whereas it is clearly shown that the inhibition profile of ketoconazole displays correlation contents between 0.71 and 0.81 when the test is performed in phosphate buffer (Fig. 3B–D). This buffer and the physiological pH (7.4) were finally chosen to perform the screening assay in this work.

Since the use of multiple-concentration assays at early screening stages of inhibitor discovery is very time- and resource-intensive, we aimed to develop a one-point method for the fast identifica-

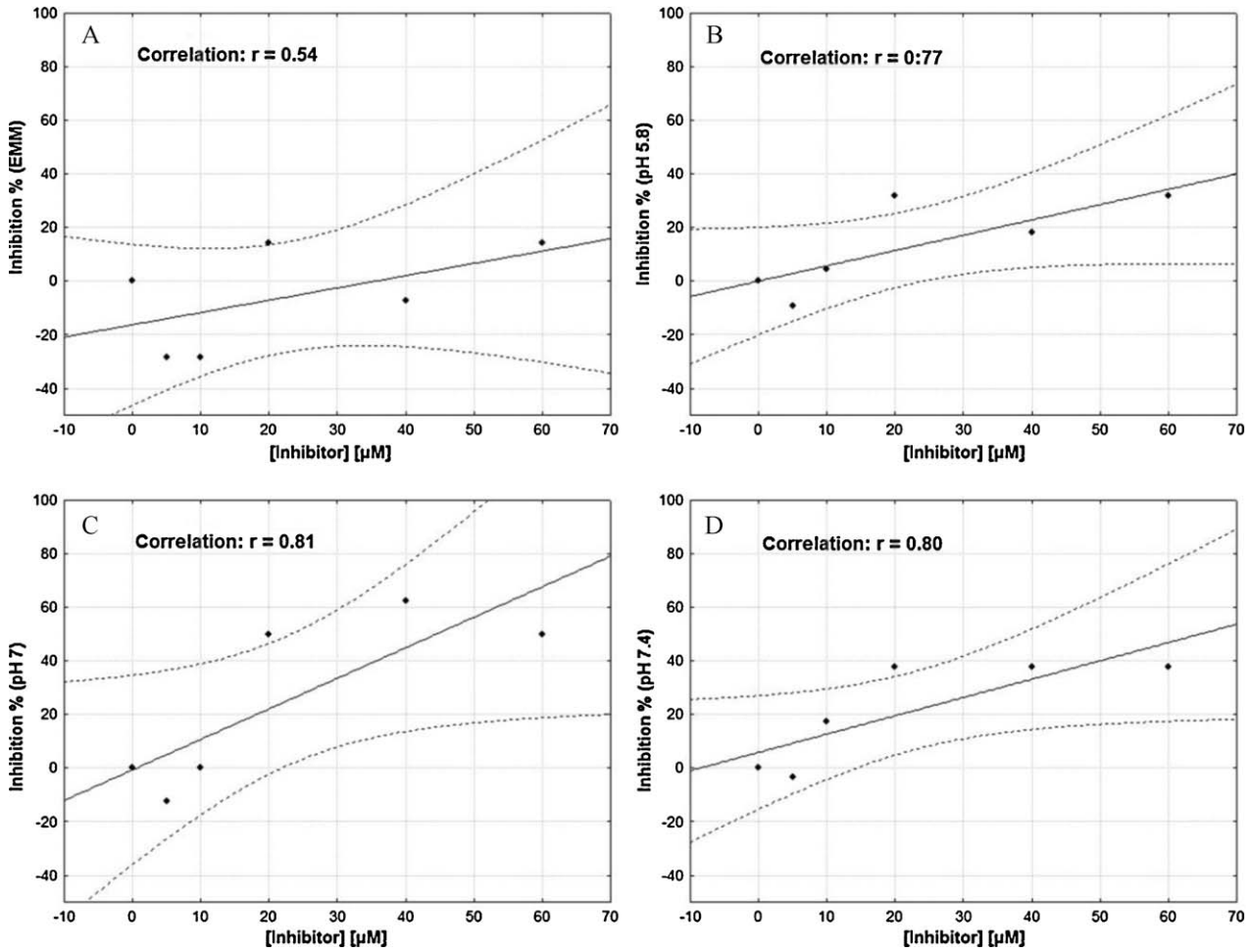

Fig. 3. Correlation between the concentration of ketoconazole and inhibition in different test media. (A; EMM) (potassium phosphate buffer 50 mM [B; pH 5.8], [C; pH 7.0], [D; pH 7.4]).

tion of CYP11B2 inhibitors. It has been found that a concentration of 41.6 μM (10 μl from a 2.5 mM stock solution in DMSO) of each inhibitor was optimal for the differentiation between strong (clotrimazole/ketoconazole) and moderate (miconazole) inhibitors of CYP11B2. The well known inhibitors of CYP11B2 displayed different inhibition levels during this test (Fig. 4). The mock-treated samples (DMSO) displayed a detectable conversion of DOC into B, whereas the presence of miconazole displayed moderate inhibition of CYP11B2. Clotrimazole or ketoconazole displayed strong inhibition of CYP11B2 under these conditions. These results confirm clotrimazole/ketoconazole and miconazole as potent and moderate inhibitors of CYP11B2, respectively. Moreover, these observations demonstrate a significant and logical correlation between the multiple-point assay reported before [7] and the one-point assay developed in this work. Hence, and since clotrimazole/ketoconazole display total inhibition of CYP11B2 under these test conditions, each compound with similar inhibition profile will be defined in our screening assay as clotrimazole-like inhibitor of CYP11B2, whereas compounds with moderate inhibition effect resembling the miconazole effect will be defined as miconazole-like inhibitors.

3.2. Validation of the new screening system and discovery of new potential inhibitors of CYP11B2

For the validation of the new screening system developed in this work, 32 independent experiments were carried out. The new screening system displayed highly reproducible results, which were statistically analysed using *t*-test ($p < 0.05$). The mock-treated samples (DMSO) displayed a CYP11B2 activity with a B/(B + DOC) ratio of 11.5%, whereas the presence of miconazole significantly ($p < 0.05$) decreased the activity of CYP11B2 to 6.5% showing 44%

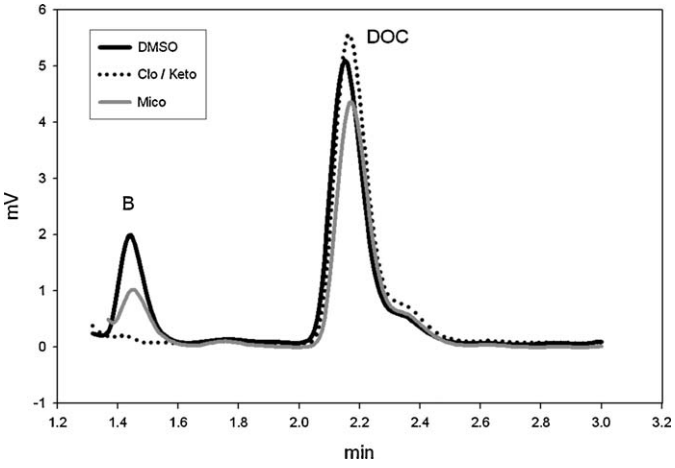

Fig. 4. HPLC traces of the CYP11B2-dependent conversion of DOC into B using MB164 in the presence of inhibitors. The presence of clotrimazole (Clo) or ketoconazole (Keto) causes total inhibition of CYP11B2, whereas the less potent inhibitor miconazole (Mico) partially inhibits CYP11B2 under these test conditions.

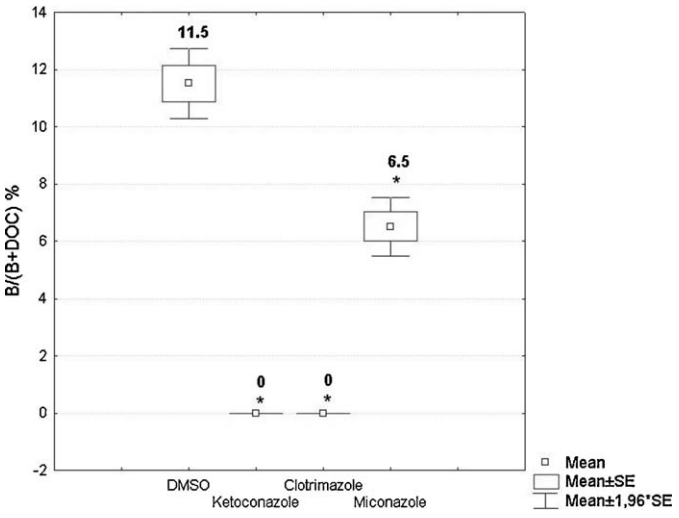

Fig. 5. Direct comparisons of the CYP11B2-dependent conversion rates of DOC into B during the screening assay. Values were calculated from 32 independent experiments and are presented as mean ± standard error of mean. Asterisks above boxes indicate a significant difference to the mock treated sample (DMSO) (*t*-test, $p < 0.05$).

inhibition under our test conditions. Furthermore, the presence of either clotrimazole or ketoconazole displayed total inhibition (100%) of CYP11B2 (Fig. 5). According to Zhang [23] the Z′-factor of the screening system was determined from the inhibition assays for clotrimazole, ketoconazole and miconazole as positive controls and DMSO as negative control. The screening assay gave a Z′-factor of 1.0 for clotrimazole, 1.0 for ketoconazole and 0.85 for miconazole, showing that the screening system is robust.

In a next step, the final screening protocol developed in this work (summarised in Fig. 6) was applied for the screening of a library of 1268 compounds from the LOPAC^{1280} library, which is a collection of pharmacologically active compounds.

The library was tested using triplicate assays. In addition to ketoconazole and clotrimazole which were also supplied in the library, the screening assay reported two novel clotrimazole-like inhibitors (Co_TH1, Co_TH11), whereas nine compounds were defined regarding to our definition as novel miconazole-like inhibitors (Table 1, Fig. 7).

3.3. Validation of the selectivity of the new potential CYP11B2 inhibitors identified during the screening assay

The potential inhibitors of CYP11B2 identified using the newly developed screening system (Table 1, Fig. 7) were defined as active compounds ("hits") and selected for further validation. At first, a cell viability assay was carried out to investigate the effect of the compounds on yeast growth. After incubation of fission yeast cultures with the different "hits" under the same conditions like in the screening assay, the yeast cells were plated on agar plates and incubated for 3 days, no significant changes ($p < 0.05$) regarding the colony numbers were observed in comparison with mock-treated samples. Moreover, no morphological changes were observed (color and shape). In a second step, the IC_{50} values of the "hits" with recombinant human CYP11B2 and CYP11B1 expressed in fission yeast (strains MB164 and SZ1, respectively) were estimated using the six-point method as described in Section 2.4. This validation assays are necessary to identify selective inhibitors of CYP11B2, which do not inhibit CYP11B1, which is >90% identical in its protein sequence to CYP11B2. Repeated steroid hydroxylation measurements with both systems were carried out and only highly correlative data sets ($R^2 > 0.90$) were used for the determination of the IC_{50} values. The validation assays reported five selective inhibitors of CYP11B2 and two inhibitors of both, CYP11B2 and CYP11B1 with compound Co_TH5 displaying unspecific inhibition and Co_TH11 binding stronger to CYP11B1 compared to CYP11B2 (Table 2). Four compounds showed no inhibitory effect against CYP11Bs under the validation assay conditions. Interestingly, out of the nine compounds defined during the screening assay as miconazole-like inhibitors, four compounds showed selective inhibition of CYP11B2. For this reason, it is important to include all miconazole-like inhibitors defined during the screening assay into the validation assay when the screening is carried out to discover selective inhibitors of CYP11B2.

The novel CYP11B2 inhibitors reported in this paper are pharmacologically active compounds. Co_TH1 is known as formestane (sold as Lentaron®) and described as an injectable steroidal aromatase inhibitor with significant activity against metastatic breast cancer [24]. Co_TH1 selectively inhibits CYP11B2 with an IC_{50} value of 2.5 μM (Table 2, Figs. 8 and 9), whereas no significant inhibition was detected against CYP11B1.

The closely related compound Co_TH2, which is androstenedion a testosterone precursor and metabolite with androgenic activity, is also a highly selective inhibitor. Interestingly, Co_TH2 was identified during the screening assay as miconazole-like inhibitor of CYP11B2, whereas Co_TH1 was identified as clotrimazole-like inhibitor. The validation assay reported an IC_{50} value of 3.11 μM for Co_TH2 and of 2.5 μM for Co_TH1. Co_TH3, Co_TH4 and Co_TH9 which were defined as miconazole-like inhibitors in the screening assay showed selective inhibition of CYP11B2 with IC_{50} values of 18 μM, 8.9 μM and 16 μM, respectively in the validation assay (Table 2).

4. Discussion and conclusion

CYP11B2 is an interesting new target for the development of drugs against hypertension and congestive heart failure. Here, we demonstrate the establishment of an efficient automated one-point screening system to identify inhibitors of human aldosterone synthase CYP11B2 (Fig. 6). The system uses recombinant fission yeast expressing CYP11B2 and is able to screen about 1200 compounds within 2 weeks.

The novel CYP11B2 inhibitors identified in this work are pharmacologically active compounds, which suggests that they could further be optimised to achieve more selective and safe inhibitors of CYP11B2. Furthermore, some of these compounds are already commercial drugs thus opening up the possibility to use them for a different therapeutic purpose. Other compounds are applied clinically with unexplained side effects and severe complications. These so far unexplained complications could be explained to some extent by their inhibitory effect on CYP11B2 as will be discussed below.

As mentioned above, Co_TH1 is marketed as formestane and described as an injectable steroidal aromatase inhibitor. Previous studies showed that systemic adverse effects occurred in about 12% of patients following intramuscular drug administration [25]. Many of these such as hot flushes, vaginal spotting and emotional liability were related to the mechanism of action of formestane, i.e., estrogen suppression. Lethargy, rash, nausea, dizziness, indigestion, ataxia, cramps and facial swelling have also been reported with an incidence of <7% [26]. Our results display that formestane (Co_TH1) selectively inhibits CYP11B2 (IC_{50}: 2.5 μM) leading to hypoaldosteronism, which could explain some of the side effects associated with formestane, i.e., nausea and dizziness. The closely related compound Co_TH2 is androstenedione, which is a testosterone precursor and metabolite with androgenic activity. Comparing with androstenedione, the steroidal scaffold of 4-hydroxy-androst-4-ene-3,17-dione the structure activity anal-

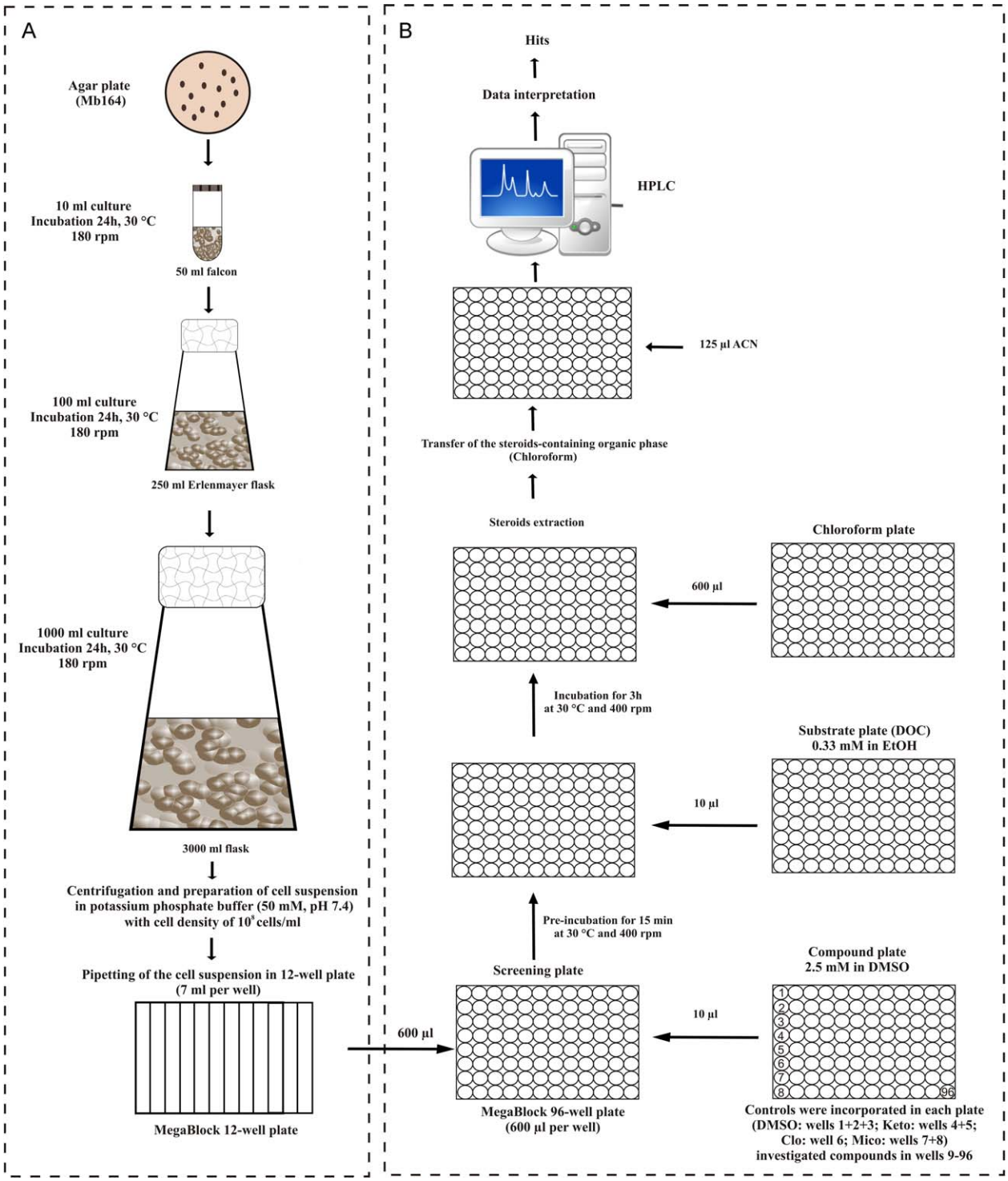

Fig. 6. Schematic overview of the new screening system developed in this work. Preparation of fission yeast cultures and the final cell suspension used in the screening assay were carried out manually (A), whereas all following steps including the addition of compounds/substrate, extraction of steroids and the preparation for the HPLC measurement were carried out automatically using the pipetting robot (B).

ysis (SAR) revealed that the ketone in position 17 (D-ring) is beneficial for activity, and an OH- residue at position 4 (A-ring) is necessary for potent inhibition of CYP11B2. Since androstenedione (Co_TH2) is a dehyhdroepiandrosteron (DHEA) metabolite, it might be of special interest that hormonal replacement therapy using DHEA will increase androstenedione. Therefore, DHEA replacement performed as anti-aging therapy [27] should take into consideration the possibility to develop salt depletion and unexplained hypotension.

The anti cancer drug Ellipticine (Co_TH4) identified during the screening assay as miconazole-like inhibitor of CYP11B2 showed selective inhibition of CYP11B2 with an IC_{50} of 8.9 µM. Recently, it has been demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with Cytochromes P450 [28]. Moreover, this compound was previously reported to be a strong inhibitor of CYP1A1/2 [29]. We found an inhibitory effect of ellipticine on the human CYP11B2. For this reason, it should be taken into consideration that the clinical application of ellipticine

Table 1
The new potential inhibitors of CYP11B2 identified during the screening assay in this work.

Compound code in this work	Compound code by SIGMA®	Name	Screening assay result	Description
Co_TH1	A5791	4-Androsten-4-ol-3,17-dione	Clotrimazole-like inhibitor	Aromatase inhibitor
Co_TH2	A9630	4-Androstene-3,17-dione	Miconazole-like inhibitor	Testosterone precursor and metabolite with androgenic activity
Co_TH3	C3635	DL-p-Chlorophenylalanine methyl ester hydrochloride	Miconazole-like inhibitor	Tryptophan hydroxylase inhibitor
Co_TH4	E3380	Ellipticine	Miconazole-like inhibitor	Cytochrome P450 (CYP1A1) and DNA topoisomerase II inhibitor
Co_TH5	I0782	Imazodan	Miconazole-like inhibitor	Selective phosphodiesterase II (PDEII) inhibitor
Co_TH6	L3791	Lamotrigine	Miconazole-like inhibitor	Anticonvulsant
Co_TH7	V1889	VER-3323 hemifumarate salt	Miconazole-like inhibitor	5-HT2C/5-HT2B serotonin receptor agonist
Co_TH8	L131	L-745,870 hydrochloride	Miconazole-like inhibitor	Selective D4 dopamine receptor antagonist
Co_TH9	P6777	Phenelzine sulfate salt	Miconazole-like inhibitor	Non-selective MAO-A/B inhibitor
Co_TH10	P8765	Ammonium pyrrolidinedithiocarbamate	Miconazole-like inhibitor	Prevents induction of nitric oxide synthase (NOS) by inhibiting translation of NOS mRNA
Co_TH11	T7313	1-[2-(Trifluoromethyl)phenyl] imidazole	Clotrimazole-like inhibitor	Potent nitric oxide synthase (NOS) inhibitor

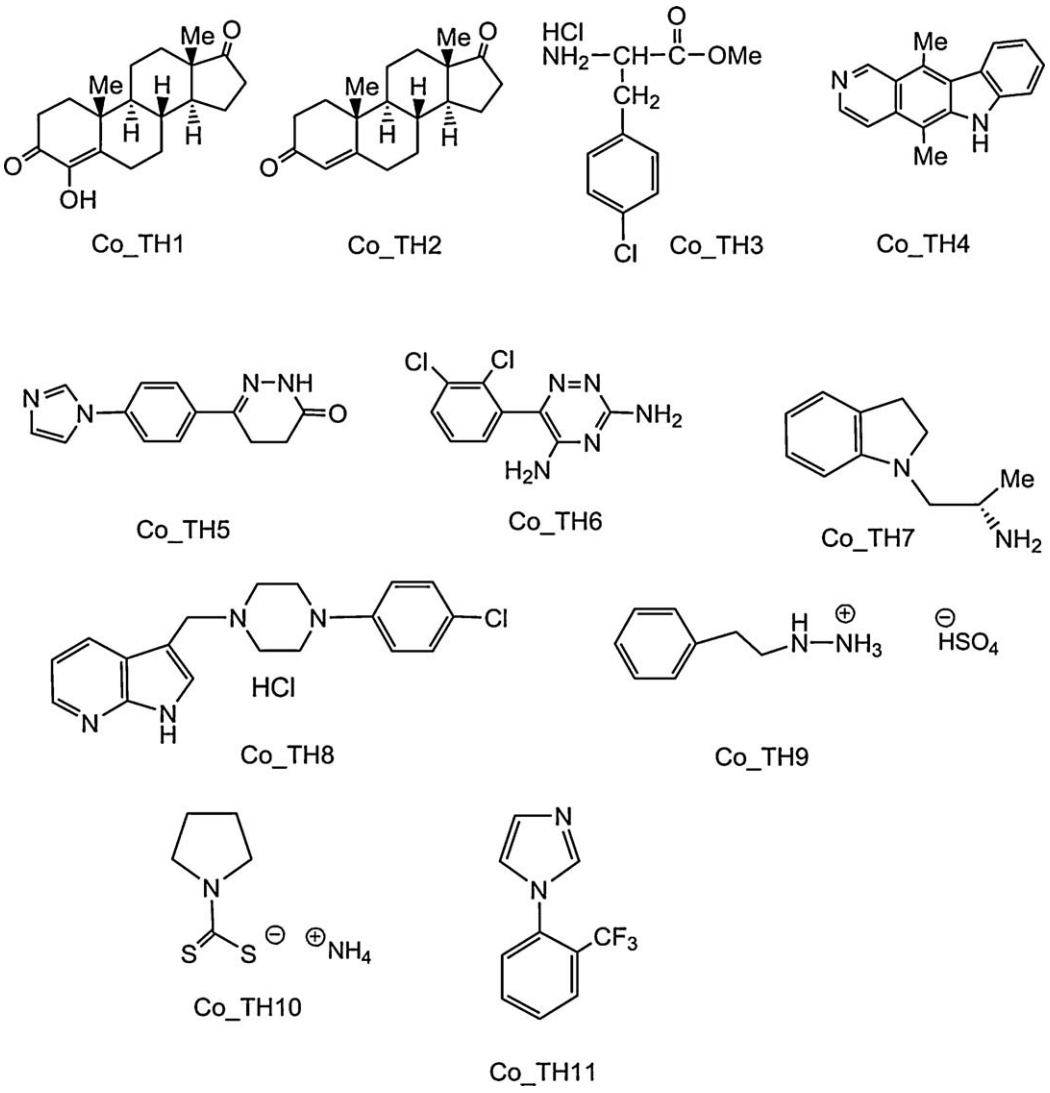

Fig. 7. Structures of the novel CYP11B2 inhibitors identified during the screening assay in this work.

Table 2
The inhibition profiles of the active compounds against CYP11B1 and CYP11B2 during the validation assay.

Compound code[a]	Name	Validation assay	
		CYP11B2 IC_{50} (R^2)	CYP11B1 IC_{50} (R^2)
Co_TH1	4-Androsten-4-ol-3,17-dione	2.5 μM (0.92)	–
Co_TH2	4-Androstene-3,17-dione	3.11 μM (0.95)	–
Co_TH3	DL-p-Chlorophenylalanine methyl ester hydrochloride	18 μM (0.99)	–
Co_TH4	Ellipticine	8.9 μM (0.93)	–
Co_TH5	Imazodan	#	#
Co_TH6	Lamotrigine	–	–
Co_TH7	VER-3323 hemifumarate salt	–	–
Co_TH8	L-745,870 hydrochloride	–	–
Co_TH9	Phenelzine sulfate salt	16 μM (0.99)	–
Co_TH10	Ammonium pyrrolidinedithiocarbamate	–	–
Co_TH11	1-[2-(Trifluoromethyl)phenyl]imidazole	1.37 μM (0.97)	0.7 μM (0.85)

(–) No significant inhibitory action was detectable under the conditions described. (#) Nonspecific inhibitory action was detected, and no IC_{50} value was calculated.
[a] In this work.

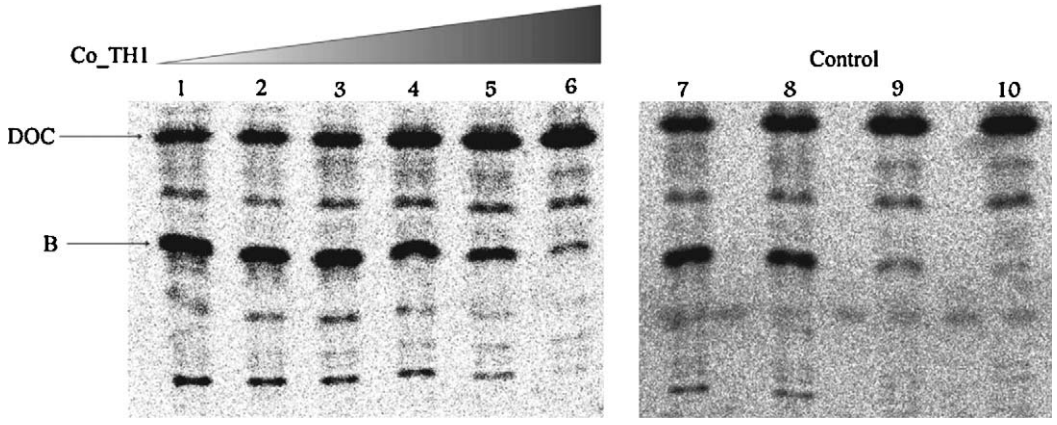

Fig. 8. Autoradiographic detection of steroid hydroxylation activity. Steroid hydroxylation assay using strain MB164 and different concentrations of Co_TH1 was carried out as described previously [7]. Co_TH1 concentrations were as follows: (line 1) 100 nM; (line 2) 200 nM; (line 3) 500 nM; (line 4) 2 μM; (line 5) 5 μM; (line 6) 25 μM; (lines 7, 8) mock-treated cells (DMSO); (lines 9, 10) 25 μM clotrimazole (positive control).

in the treatment of cancer could be associated with hypotension.

Compound Co_TH9 known as phenelzine (Sold as Nardil®) is a potent, irreversible inhibitor of monoamine oxidase (MAO)-A and -B that has been used to treat depression since the late 1950s [30]. Phenelzine toxicity is normally characterised by agitation, seizures, sweating, tachycardia and hypertension [31–33], although hypotension has also been described [34,35]. Interestingly, the unexplained phenelzine-induced hypotension could be treated successfully with salt tablets [36]. Several studies reported that a phenelzine-overdose induced complications which include severe and unexplained hypotension, impaired left ventricular function and acute myocarditis, death was also reported and should be considered in patients who develop unexplained hypotension after phenelzine overdose [35,37]. Since this paper reports that phenelzine inhibits the aldosterone synthase (CYP11B2) with an IC_{50} value of 16 μM, the side effect of this drug concerning salt loss and hypotension can be rationally explained with this result.

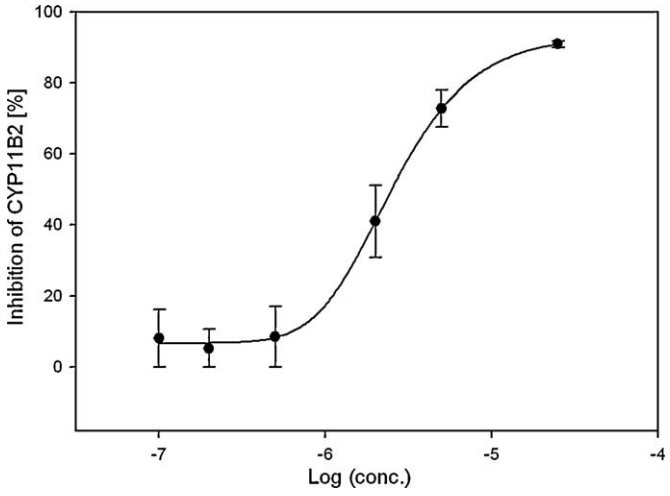

Fig. 9. The inhibitory effect of Co_TH1 on the activity of CYP11B2. Co_TH1 selectively inhibits CYP11B2 with an IC_{50} value of 2.5 μM (R^2 = 0.9984).

These findings could play an important role in the management of depressed patients receiving phenelzine and suggest to monitor the aldosterone concentration and blood pressure in these patients to avoid side effects.

In conclusion, these results indicate that the new screening system developed in this work is robust and that it can be applied to investigate libraries of existing drugs as well as new compounds to find novel CYP11B2 inhibitors. This screening enables the reinvestigation and reuse of existing drugs, which can save costs in the development of new CYP11B2 inhibitors. Although the test was developed and validated at the laboratory level, it could be used to screen up to 600 compounds per week. The throughput of the system could further increased by testing 10 compounds per well, which will increase the throughput of the system up to 6000 compounds per week.

Novel CYP11B2 inhibitors have been identified using this newly developed screening system. These inhibitors are drug-like compounds or new drugs that may be repositioned for possible use in

the treatment of hyperaldosteronism-related diseases or as lead compounds for further development of safer and more selective inhibitors of CYP11B2. Finally, some of the side-effects of these drugs can be explained by the inhibition of CYP11B2.

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