Hits Identified in Library Screening Demonstrate Selective CYP17A1 Lyase Inhibition
Rapid Communication

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[#] Dedicated to Prof. Dr. Dr. h.c. Theophil Eicher on the occasion of his 80th birthday.

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Abstract
A screening of structurally different steroid hormone synthesis inhibitors was performed in order to find a starting point for the development of a new inhibitor of the bifunctional steroidogenic enzyme CYP17A1. Emphasis was placed on determination of selectivity between the two catalytic steps, namely 17α-hydroxylase and $C_{17,20}$-lyase. For that purpose a new inhibition assay has been developed. Hits identified within this novel assay demonstrated selective inhibition of CYP17A1 lyase activity, and thus mark the basis for the development of selective $C_{17,20}$-lyase inhibitors for the treatment of prostate cancer.

Keywords: CYP17A1; Selectivity screening; Prostate cancer; Androgens; Hydroxylase assay; Lyase assay

Abbreviations:

17OH-P_4, 17α-hydroxyprogesterone; 17OH-P_5, 17α-hydroxypregnenolone; CRPC, castration resistant prostate cancer; CYP17A1, 17α-hydroxylase/$C_{17,20}$-lyase; DHEA, dehydroepiandrosterone; GnRH, gonadotropin-releasing hormone; hydroxylase, 17α-hydroxylase; lyase, $C_{17,20}$-lyase; P_4, progesterone; P_5, pregnenolone; PCa, prostate cancer

1. Introduction

Seventy years after the elucidation of the dependence of prostate cancer (PCa) on androgens [1], this disease still is amongst the five leading causes of cancer death in men in the U.S. and Western countries. Although established treatment methods, namely surgical or medical castration (with GnRH analogs) often in combination with antiandrogens, exist, they failed in advanced disease states, the so called castration resistant prostate cancer (CRPC). However, patients suffering from CRPC can clearly benefit from the newly approved drug abiraterone acetate [2]. This pregnenolone derivative was designed as an inhibitor of the enzyme 17α-hydroxylase/$C_{17,20}$-lyase (CYP17A1) [3] which catalyzes two key reactions in steroid hormone biosynthesis. In a first step the substrates progesterone (P_4) and pregnenolone (P_5) are hydroxylated in 17α position of the steroid to yield 17α-hydroxyprogesterone (17OH-P_4) and 17α-hydroxypregnenolone (17OH-P_5), respectively. At this node of glucocorticoid and androgen biosynthesis the $C_{17,20}$-lyase (lyase) activity of CYP17A1 cleaves the C_{17}-C_{20} bond of 17OH-P_5 to produce the C_{19}-steroid dehydroepiandrosterone (DHEA) (Fig. 1). In healthy men androgens are only produced in the testes (ca. 90%) and the adrenals, whereas in PCa patients there is notable production of androgens also in the tumor tissue. This is considered as one of the possible mechanisms of disease progression under conventional therapy, which only affects testicular androgen production. Although abiraterone acetate is successful in prolonging patients' lives, its steroidal structure in conjunction with the high daily dose could possibly result in unwanted activation of steroid hormone signaling via wild type or mutant steroid hormone receptor stimulation, although this has not yet been reported in clinical studies. Therefore contemporary research focus lies on non-steroidal inhibitors [4-11]. Furthermore abiraterone evidently blocks glucocorticoid biosynthesis by inhibition of both, hydroxylase and lyase, activities of CYP17A1 leading to a secondary mineralocorticoid excess that makes substitution with a synthetic glucocorticoid (e.g. prednisone) or co-medication with a mineralocorticoid receptor antagonist (e.g. eplerenone) necessary [12]. The missing selectivity between hydroxylase and lyase is often considered as inevitable because both CYP17A1 reactions occur in the same catalytic site. We [13, 14] and other researchers [15, 16] in the field of CYP17A1 inhibitor development consequently established inhibition assays which do not differentiate between the two reactions. Others considered this issue only late in their drug development process [8]. Aiming at the discovery of non-steroidal, selective lyase inhibitors as potential clinical candidates, a screening system capable of identifying suitable compounds in an early stage of development is required. In the present work we describe a new assay procedure which allows quick and easy to perform determination of lyase inhibition and demonstrate its usefulness by investigating selected structurally diverse compounds of our in-house steroidogenic CYP enzyme inhibitor library [17-20].

2. Materials and Methods

2.1. Chemicals

[7-^{3}H(N)]-pregnenolone (1 mCi/mL, 21.1 Ci/mmol) was obtained from PerkinElmer LAS (Rodgau, Germany).

2.2 CYP17A1 enzyme preparation

The coexpression of human CYP17A1 and rat NADPH-P450-reductase in *E. coli* and the isolation of the membrane fractions were performed as described previously [14]. Membrane fractions were diluted to a concentration that gives a 15-25 % conversion in the controls for the different assays.

2.3 $C_{17,20}$-lyase assay

The lyase activity of CYP17A1 was determined by measurement of the conversion of 17OH-P_5 to DHEA. In a first incubation step 17OH-P_5 was generated from P_5 by CYP17A1 itself. The assay procedure was as follows: An assay mixture consisting of 140 µL phosphate buffer (0.05 M, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM dithiothreitol), 50 µL NADPH generating system (in phosphate buffer with 50 mM glucose-6-phosphate, 5.75 mM $NADP^+$ and 27.5 U/mL glucose-6-phosphate dehydrogenase) and 5 µL substrate solution (25 µM [^{3}H]-P_5) was preincubated at 37 °C for 5 min. The reaction was started by addition of 50 µL enzyme preparation. After a 10 min incubation 98 µL of the reaction mixture were mixed with 25 µL 1 N HCl to stop the enzymatic reaction. Another 98 µL of the reaction mixture were mixed with 2 µL of a DMSO solution of the inhibitor (controls contained only DMSO). This mixture was incubated for additional 30 min at 37 °C. Subsequently the reaction was stopped by addition of 25 µL 1 N HCl. Extraction of the steroids was performed by addition of 500 µL ethylacetate and vigorous shaking for 10 min. After a centrifugation step (5 min, 15,000 g), 470 µL of the organic phase were removed and transferred into a fresh tube containing 125 µL phosphate buffer and 25 µL 1 N HCl. Shaking and centrifugation was repeated as described above. 400 µL ethylacetate solution was evaporated to dryness in a fresh tube and redissolved in 40 µL acetonitrile/water (3/2) for HPLC analysis.

2.4 17α-hydroxylase assay

Determination of the hydroxylase activity of CYP17A1 was performed by measurement of the conversion of P_5 to 17OH-P_5. An assay mixture consisting of 140 µL phosphate buffer (0.05 M, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM dithiothreitol), 50 µL NADPH generating system (in phosphate buffer with 50 mM glucose-6-phosphate, 5.75 mM $NADP^+$ and 27.5 U/mL

glucose-6-phosphate dehydrogenase) and 5 µL substrate solution (25 µM [^{3}H]-P_5) was preincubated at 37 °C for 5 min. The reaction was started by addition of 50 µL enzyme preparation. Compared to the lyase assay, however, enzyme concentration had to be reduced to keep control conversion in the favorable range of 15-25 % and to prevent DHEA formation. After a 30 minute incubation at 37 °C the enzyme reaction was stopped by addition of 50 µL 1 N HCl. Extraction of the steroids was performed by addition of 1000 µL ethylacetate and vigorous shaking for 10 min. After a centrifugation step (5 min, 15,000 g), 900 µL of the organic phase were removed and transferred into a fresh tube containing 250 µL phosphate buffer and 50 µL 1 N HCl. Shaking and centrifugation was repeated as described above. 800 µL ethylacetate solution was evaporated to dryness in a fresh tube and redissolved in 40 µL acetonitrile/water (1/1) for HPLC analysis.

2.5 HPLC Methods

HPLC separation of the steroids was performed using an Agilent 1100 HPLC system with PDA detector (Böblingen, Germany), a CC 125/3 Nucleodur 100-3 C-18 ec column (Macherey-Nagel, Düren, Germany) and a Berthold Radioflow Detector LB509 with Scintillator Pump (Bad Wildbad, Germany). Quickszint Flow 302 LSC Cocktail (Zinsser Analytic, Frankfurt/Main, Germany) was used as scintillator fluid.

HPLC conditions for the analysis of the lyase reaction were as follows: Eluent A was 0.1% trifluoroacetic acid in water, eluent B was 0.1% trifluoroacetic acid in acetonitrile. An eluent gradient of 60-75% eluent B in the first 30 sec was followed by a 3.5 min isocratic phase with 75% eluent B. From minute 4.0-4.5 the eluent was changed to 100% eluent B. After a 2.5 min phase with 100% eluent B the eluent system was set back to the original composition of 60% eluent B till the end of the run at 10.0 min. Solvent and scintillator flow was set to 0.5 mL/min.

For the analysis of the hydroxylase assay an isocratic method was used. A 1+1 mixture of water and acetonitrile with 0.1% trifluoroacetic acid was used as eluent. Solvent and scintillator flow was set to 0.9 mL/min.

3. Results and Discussion

For the discovery of selective lyase inhibitors a new assay was developed. Like other CYP17A1 assays used in literature the CYP17A1 assay performed and described by our group [14] is a hydroxylase assay that uses a high concentration of unlabeled P_4 as substrate. Due to the preference of human lyase for Δ_5-unsaturated steroids the reaction with the Δ_4-unsaturated steroid P_4 is at a dead end at the 17α-hydroxysteroid level and only 17OH-P_4 and the side product 16α-hydroxyprogesterone are yielded (data not shown). While others use 17OH-P_5 as a substrate for lyase inhibition assays we decided to establish a new assay closer to the natural conditions. As two substrates usually are available for the enzyme, 17α-unhydroxylated steroid and 17OH-P_5, this situation should be simulated by the use of tritium labeled P_5. The assay principle is illustrated in Fig. 2. The lyase substrate 17OH-P_5 is formed *in situ* by the CYP17A1 enzyme during a 10 min preincubation. At this timepoint almost all P_5 substrate has been transformed into 17OH-P_5 (>90%) and DHEA (<10%). The incubation of an aliquot is stopped using HCl and the concentration of DHEA is determined to be used as baseline value. At this timepoint the lyase assay is initiated by addition of test compound in different concentrations dissolved in DMSO (final concentration usually between 0 and 10 µM). After another 30 min of incubation and subsequent termination, lyase inhibition is calculated using the difference between the final DHEA concentration and the baseline value. These preincubation and incubation times were the results of a time course experiment aiming at the optimization of this process (see supporting information).
Although the lyase assay with labeled P_5 is associated with a slightly more laborious procedure, it has clear advantages. The substrate is less expensive than [^{3}H]-17OH-P_5 and readily available. As the assay is based on bacterially expressed human CYP17A1 in combination with a NADPH reductase [13] availability of enzyme is not a problem in contrast to protocols that use human testes homogenate [3]. Also side reactions catalyzed by other steroidogenic enzymes can be excluded using human CYP17A1 from bacterial source. Although bacterial and eukaryotic protein expression are known to differ with regard to posttranslational modifications, an impact of the latter on the assay result is unlikely. Phosphorylation, the only described posttranslational modification for human CYP17A1 know to the authors, increases lyase activity [21], presumably by enhancing the interaction between CYP17A1 and the NADPH reductase. It is probable that this does not affect ligand accommodation and inhibitor potency. Influence on the results due to other possible, but yet undescribed posttranslational modifications remains to be elucidated. Addition of cytochrome b_5, a cofactor reported to facilitate the lyase reaction [8, 22], was found to be not required.

With the new tool in hands twenty selected steroidogenic enzyme inhibitors were tested for hydroxylase and lyase inhibition. All compounds were non-steroidal and taken from our in-house

library, which was build over the past decade as a result of our group's research on CYP enzyme inhibitors and thus represents a wide-ranged collection of compounds. A feature they all have in common is a nitrogen atom able to interact with the heme iron located in the active centre of CYP enzymes. The compounds were carefully selected with regard to diversity of the scaffold, different nitrogen containing heterocycles, varying nitrogen positions in the heterocyle and a different hydroxylase inhibition profile. The steroidal CYP17A1 inhibitor abiraterone was used as reference compound. The results of the most interesting compounds regarding chemical diversity and lyase selectivity are shown in Table 1. Abiraterone is a more potent inhibitor of the hydroxylase reaction ($IC_{50;\ hydroxylase}$ = 1.7 nM) which is in consistence with the observation of secondary mineralocorticoid excess reported in clinical trials (e.g. [23, 24]). By comparison compounds **1, 3, 4** and **5** are potent lyase inhibitors and show IC_{50} values less than 40 nM, while compound **2** is less potent (IC_{50} = 2936 nM). Although none of the test compounds is more active than abiraterone, compounds **1**, **4** and **5** show a better selectivity profile. Especially **4**, which had been developed in our group as a CYP11B1 inhibitor, is an interesting candidate for further investigation having a 15-fold greater potency for lyase than for hydroxylase inhibition. A compound with these properties should be able to prevent androgen formation while retaining glucocorticoid synthesis. In consequence, the secondary mineralocorticoid excess resulting from low glucocorticoid levels should be prevented and thus co-medication be avoided, which is needed to manage the side effects of unselective CYP17A1 inhibition.

Even though a selective lyase inhibitor would be a safer drug for PCa patients, it should only be administered to patients with clearly defined state of the androgen receptor. Described mutations like L701H and H877A result in a broadened ligand spectrum activating the receptor [25]. In consequence, the receptor is no longer exclusively activated by androgens, but also by some synthetic and natural corticosteroids and presently available antiandrogens. Combination of a selective lyase inhibitor with a next generation androgen receptor antagonist like Enzalutamide (previously known as MDV3100), a drug which has proven to be safe and efficient in the AFFIRM phase III clinical trial [26, 27], or administration of a dual inhibitor of CYP17A1 and CYP11B1 [5] would in these cases be the treatment of first choice.

4. Conclusion

As selective lyase inhibitors should be advantageous for the treatment of PCa as they do not decrease glucocorticoid levels a new lyase selectivity assay was developed. With this assay procedure selective lyase inhibition of recently described steroidogenic CYP inhibitors was discovered and led to the identification of compound **4** as a new lead structure for the development of an improved drug against PCa. Further optimization of compound **4** is necessary as it also inhibits CYP11B1. The application of different types of CYP17A1 inhibitors (selective lyase inhibitors and dual inhibitors of CYP17A1 and CYP11B1) according to different types of androgen receptors will maximize the benefits for the PCa patients with diverse disease states.

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Conflict of Interest

The authors have declared no conflict of interest.

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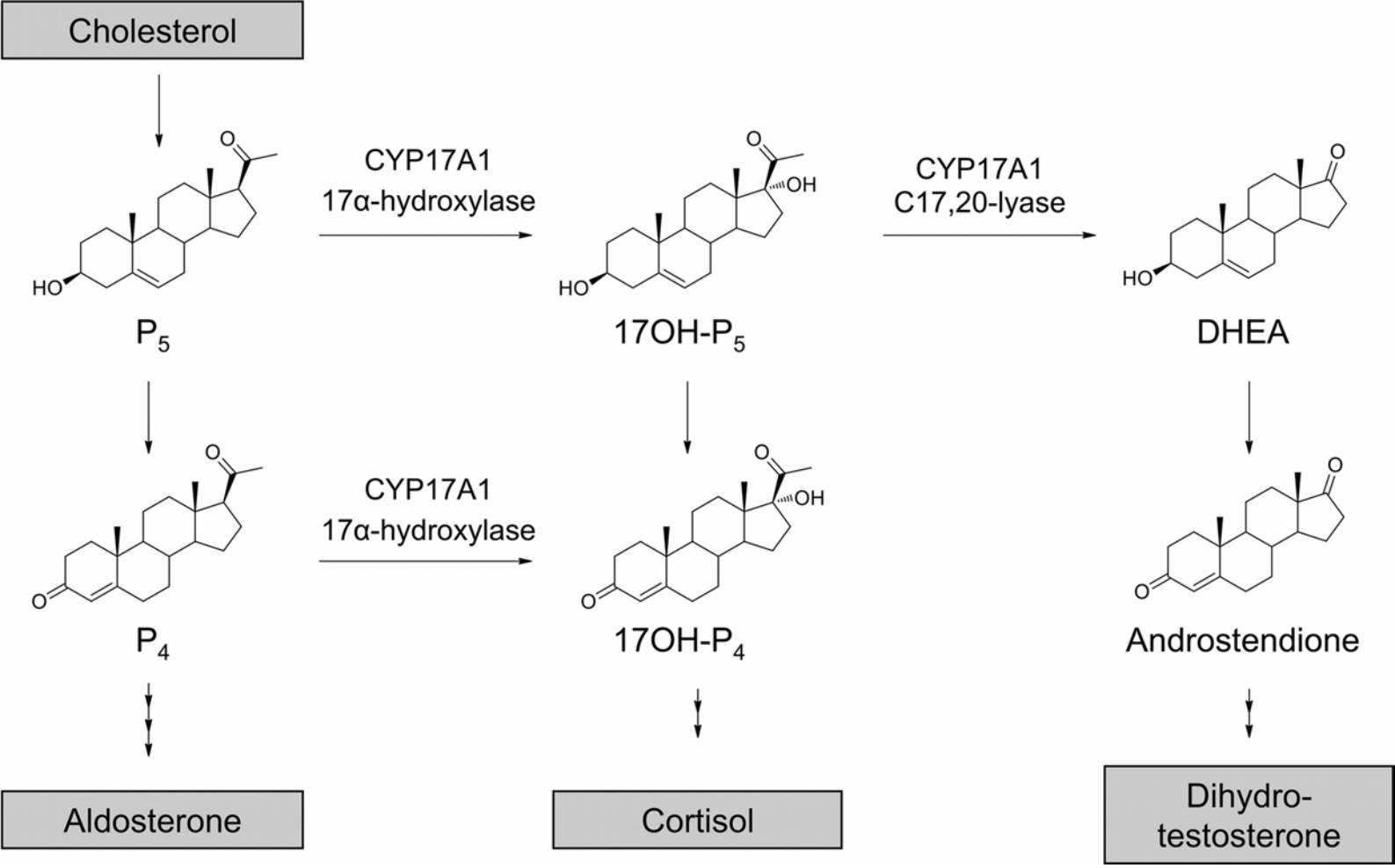
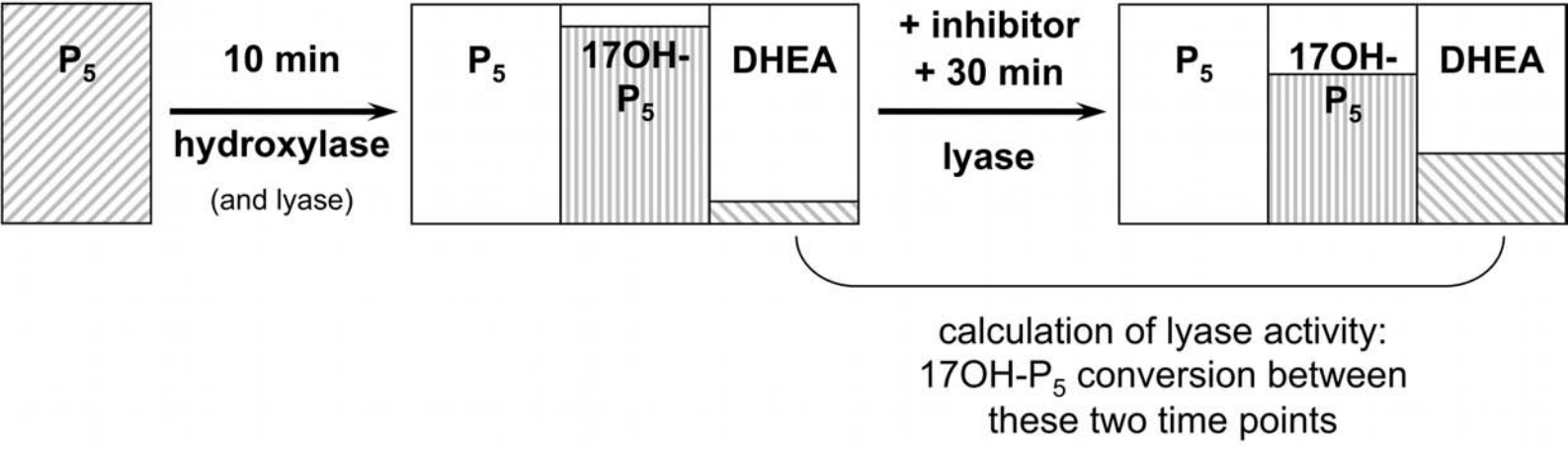
Figure Captions

Figure 1. The role of CYP17A1 in mineralocorticoid, glucocorticoid and androgen biosynthesis

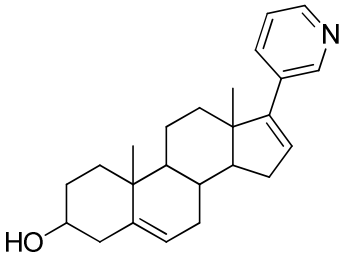
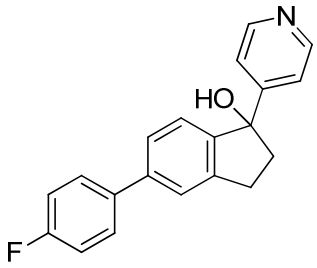
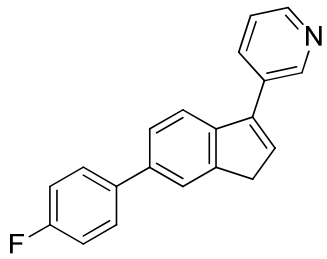
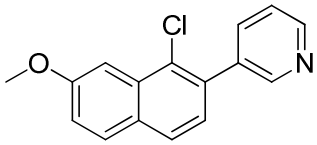
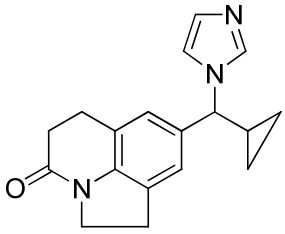
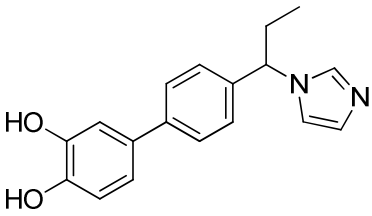
Figure 2. Schematic representation of the lyase assay procedure


Cholesterol
CYP17A1
17α-hydroxylase
CYP17A1
C17,20-lyase
HO
O
OH
P5
17OH-P5
DHEA
P4
17OH-P4
Androstendione
Aldosterone
Cortisol
Dihydro-
testosterone


P5
10 min
hydroxylase
(and lyase)
P5
17OH-P5
DHEA
+ inhibitor
+ 30 min
lyase
P5
17OH-P5
DHEA
calculation of lyase activity:
17OH-P5 conversion between
these two time points

- CYP17A1 lyase activity can be selectively inhibited over 17α-hydroxylase activity.
- Selective lyase inhibitors are identified with new assays.
- The novel lead structure may lay the basis for a safer prostate cancer therapy.
- A new and rapid screening procedure is reported.

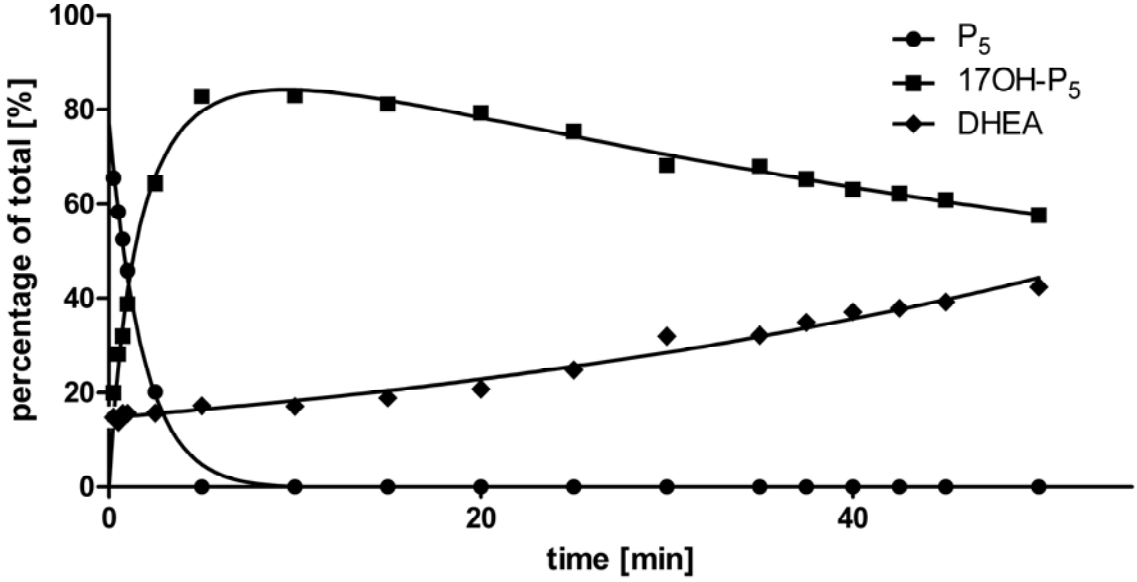
Table 1. In vitro CYP17A1 hydroxylase and lyase inhibition assay data

Compound		IC_{50} [nM] [a]	
		Hydroxylase[b]	Lyase
ABT		1.7	15.3
1		73.2	36.6
2		23,595	2936
3		3.0	21.5
4		351	23.5
5		46.1	25.8

[a] Concentration of inhibitors required to give 50% inhibition. Assays were performed as described in Experimental. 500 nM P_5 was used as substrate. Data represent the mean values of at least three experiments. The deviations were within ± 25%

[b] Note that IC_{50} values for hydroxylase previously reported by our group differ from the given values due to different assay protocols.

Supplemental Figure 1. Incubation with P_5 and time dependant production of the hydroxylase product 17OH-P_5 and the lyase product DHEA by CYP17A1. The curve represents the average of an experiment performed in triplicate.



The assay procedure of the new lyase assay includes a splitting of the test samples after complete conversion of P_5 to 17OH-P_5, a further incubation of one part of the sample whereas the other part is stopped and comparison of the DHEA amount formed in both sample parts. In order to find a suitable splitting and stopping time, the P_5 conversion was monitored over time. The resulting curve is depicted in Supplemental Figure 1. Five minutes after starting the enzyme reaction the original substrate P_5 is completely converted. Until then DHEA production is at a minimum level (note that the baseline DHEA level until minute 10' is due to an impurity of the substrate with the same retention time as DHEA). This indicates that the hydroxylase product 17OH-P_5 is released from the enzyme before further conversion by the lyase reaction and is consistent with the observation of Soucy and Luu-The [1]. Based on these results the time for splitting the samples was set to ten minutes after starting the incubation and the time for stopping all samples was set to forty minutes after the start. The DHEA production is linear with time in this frame.

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