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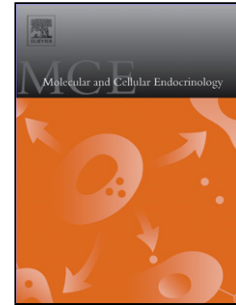
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**Development of a biological screening system for the evaluation of highly active and selective 17 $\beta$ -HSD1-inhibitors as potential therapeutic agents**

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Abbreviations: 17 $\beta$ -HSD1, 17 $\beta$ -hydroxysteroid dehydrogenase type 1; 17 $\beta$ -HSD2, 17 $\beta$ -hydroxysteroid dehydrogenase type 2; E1, estrone; E2, 17 $\beta$ -estradiol; ER, estrogen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ; PAMPA, parallel artificial membrane permeability assay; SAR, structure-activity relationship; TEER, transepithelial electrical resistance

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Keywords: 17 $\beta$ -HSD1-inhibitors, drug development, estrogen dependent disease, metabolic stability, screening system, selectivity (17 $\beta$ -HSD2, hepatic CYPs, estrogen receptor).

**Abstract**

17 $\beta$ -Hydroxysteroid dehydrogenase type 1 (17 $\beta$ -HSD1) catalyses the intracellular conversion of oestrone (E1) to oestradiol (E2). E2 is known to be involved in the development and progression of breast cancer and endometriosis. Since 17 $\beta$ -HSD1 is overexpressed in these oestrogen-dependent diseases, inhibition of this enzyme may be a more target-directed therapeutical approach compared to established medical treatments. For the identification of highly active and selective 17 $\beta$ -HSD1-inhibitors that are suitable for application as potential therapeutics, there is a need for an appropriate, efficient and reliable screening system. Here, we report the development and application of our screening system using our in house library of potential 17 $\beta$ -HSD1-inhibitors. Four potent and selective compounds with a good first pharmacokinetic profile were identified.

## 1. Introduction

17 $\beta$ -HSD1 catalyses the NADPH dependent reduction of the weak oestrogen E1 to E2 (final step of oestrogen biosynthesis). E2 is the principal and most potent oestrogen in humans. In addition to non-genomic effects, it performs crucial functions in the endocrine system by binding to the oestrogen receptors (ERs). However, it has also been shown to stimulate the proliferation of diseases such as endometriosis (Zeitoun et al., 1998) and breast cancer (Thomas, 1984; Vihko et al., 2003). 17 $\beta$ -HSD1 is overexpressed in endometrial tissue (Šmuc et al., 2007) and in many breast tumours. This leads to a high intracellular concentration of E2 which activates cell proliferation (Miyoshi et al., 2001). Inhibition of this enzyme is therefore an attractive novel concept for the treatment of oestrogen-dependent diseases. Compared to established endocrine therapies that systemically reduce E2 action, fewer side effects are expected.

To be suitable as potential therapeutics 17 $\beta$ -HSD1-inhibitors must meet several requirements. High 17 $\beta$ -HSD1 inhibitory activity and low inhibition of its physiological counterpart 17 $\beta$ -HSD2 (Wu et al., 1993) are essential. Besides, the inhibitors must not activate the ERs  $\alpha$  and  $\beta$  to avoid stimulation of tumour growth. Moreover, good intestinal absorption, sufficient metabolic stability and low interaction with hepatic CYP-enzymes are very important. Consequently, it is necessary to have a biological screening system on hand for the evaluation of active compounds. Here, we describe our biological test system that enabled us to identify two series of highly active non-steroidal 17 $\beta$ -HSD1-inhibitors with good selectivities towards 17 $\beta$ -HSD2 and both ER subtypes (Bey et al., 2008; Frotscher et al., 2008; Marchais-Oberwinkler et al., 2008).

## 2. Materials and methods

### 2.1 Cell-free competitive inhibition assay on human 17 $\beta$ -HSD1

The assay was performed similar as described by Sam et al. (Sam et al., 1998). Briefly, human enzyme was partially purified from placental tissue and incubated with [2, 4, 6, 7-<sup>3</sup>H]-E1 (500 nM) for 10 min at 37 °C in the presence of NADH. After ether extraction, substrate and product were separated by HPLC and detected with a radioflow detector (Berthold Technologies, Bad Wildbad) (Frotscher et al., 2008).

### 2.2 Cell-free competitive inhibition assay on human 17 $\beta$ -HSD2

Human enzyme is partially purified from placental tissue and incubated with [2, 4, 6, 7-<sup>3</sup>H]-E2 (500 nM) for 20 min at 37 °C in the presence of NAD<sup>+</sup>. After ether extraction, substrate and product were separated by HPLC and detected with a radioflow detector (Frotscher et al., 2008).

### 2.3 Competitive ER binding assay

ER-binding affinity was determined similarly to a previously described method (Zimmermann et al., 2005). ER $\alpha$  or ER $\beta$  was incubated with [2, 4, 6, 7-<sup>3</sup>H]-E2 and inhibitor for 1 h at room temperature. Receptor-bound and free E2 were separated by means of hydroxyapatite. The amount of bound radioactivity was measured in a liquid scintillation counter. Non-specific binding was determined with diethylstilbestrol (Bey et al., 2008).

### 2.4 Cytotoxicity assay

For evaluation of cytotoxicity, conversion of MTT was determined as described by Denizot and Lang with minor modifications (Denizot and Lang, 1986). T47-D cells were incubated with the inhibitor for 3 h at 37 °C in a 5 % CO<sub>2</sub> humidified atmosphere. After an MTT-incubation of 3 h the cleavage of MTT to a blue formazane by mitochondrial succinate-dehydrogenase was quantified spectrophotometrically at 590 nm.

### 2.5 Cellular activity

T47-D cells were pre-incubated with the inhibitor for 30 min. After 30 min incubation with [2, 4, 6, 7-<sup>3</sup>H]-E1 at 37 °C in a 5 % CO<sub>2</sub> humidified atmosphere the supernatant was extracted with ether. Probe analysis was performed according to the cell-free inhibition assays.

### 2.6 Oestrogenicity assay

T47-D cells were grown in phenolred-free medium supplemented with stripped fetal calf serum. T47-D cells were incubated with the compounds for 10 days at 37 °C in a 5 % CO<sub>2</sub> humidified atmosphere. The medium containing the respective inhibitor was exchanged every 2 to 3 days. E2 was used as positive, ethanol as a negative control. Determination of cell number was performed by MTT-conversion as described for the cytotoxicity assay.

### 2.7 CaCo2-permeability assay

CaCo-2 cell culture and transport experiments are performed as described by Yee (Yee, 1997) with small modifications. Four reference compounds (atenolol, testosterone, ketoprofen, erythromycin) were used in each assay for validation. Samples were taken from the acceptor side after 0, 60, 120 and 180 min, and from the donor side after 0 and 180 min. The integrity of the monolayers is checked by measuring the transepithelial electrical resistance (TEER) before the transport experiments and by measuring lucifer yellow permeability after each assay. All samples of the CaCo-2 transport experiments are analysed by HPLC-MS/MS.

### 2.8 Metabolic stability assay

The assay is performed with liver microsomes from rats (Sprague Dawley, male pool; BD Gentest, USA). The incubation consists of a microsomal suspension of 0.33 mg/ml protein in phosphate buffer NADP<sup>+</sup>-regenerating system (NADP<sup>+</sup>, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, MgCl<sub>2</sub>). The reaction is initiated by the addition of test compound (final concentration 1 µM) to the pre-incubated microsomes/buffer mix at 37 °C. The samples are removed from the incubations after 0, 15, 30, and 60 min, and processed for acetonitrile precipitation. Analysis is performed by LC-MS/MS.

### 2.9 Inhibition of hepatic CYP-enzymes

The commercially available P450 inhibition kits from BD Gentest (Heidelberg, Germany) are used according to the manufacturer's instructions. Compounds are tested for inhibition of the following enzymes: CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4. Inhibitory potencies are determined as IC<sub>50</sub>-values.

### 2.10 *In vivo* pharmacokinetics

Male Wistar rats weighing 300-330 g (Janvier, France) are housed in a temperature-controlled room (20-22 °C) and maintained in a 12 h light/ 12 h dark cycle. Food and water are available *ad libitum*. Compounds are applied orally in a cassette dosing to four rats by using a feeding needle. The compounds are dissolved in a mixture labrasol/water (1:1) and given at a volume of 5 ml/kg. Blood samples (0.2 ml) are taken at 0, 1, 2, 3, 4, 6, 8, 10 and 24 h postdose and analysed by HPLC-MS/MS.

## 3. Results

### 3.1 Requirements of 17β-HSD1-inhibitors as potential therapeutic agents

For the design of a screening system that can assess relevant aspects of 17β-HSD1-inhibitors as potential therapeutics it is necessary to establish the corresponding single tests for the evaluation of different properties and combine them into an efficient screening system. Important properties of the inhibitors as well as appropriate tests are given in Table 1.

**Table 1**

## 3.2 Description of the developed screening system

The first and principle feature is a high inhibitory activity towards the target enzyme 17 $\beta$ -HSD1. It can be evaluated quickly by a competitive inhibition assay using the partially purified human enzyme. With this test it is possible to deduce reliable structure-activity relationships (Salhab et al.). Besides, it is also important that inhibitors are active intracellularly. This property can be investigated by a cellular assay. Moreover, this assay provides first insights into cell permeability, intracellular metabolism and protein adsorption. To avoid side effects the physiological counterpart of the target enzyme, 17 $\beta$ -HSD2, which catalyses the reverse reaction, should not be blocked (Fig. 1).

**Figure 1**

Therefore, a cell free competitive inhibition assay using the human 17 $\beta$ -HSD2 must be applied at an early stage to gain additional SAR regarding selectivity. For a comparison of the inhibitors, IC<sub>50</sub>-values are determined in both cell-free assays and selectivity factors are calculated. A second aspect of selectivity concerns the ERs. Neither an agonistic nor antagonistic effect is acceptable, as side effects could also be evoked via ER-antagonism. For this reason, binding affinity is evaluated for each subtype of human ER ( $\alpha$  and  $\beta$ ) in a competitive binding assay. Going beyond the step of ligand binding in a second functional test, the proliferation stimulating properties of the inhibitors are evaluated in a cellular system with the oestrogen-dependent breast cancer cell line T47-D. For the evaluation of toxic effects, a further cellular assay has been established. Acute cytotoxicity is assessed by determining the number of living cells after a short incubation time using the MTT-test. Chronic cytotoxic effects may also be seen in the oestrogenicity assay, in which the cells are incubated with inhibitor for ten days.

In addition to these pharmacodynamic considerations pharmacokinetic aspects play also an important role in preclinical drug design. A prerequisite for the application of a compound as therapeutic is good absorption in the gastrointestinal tract. A first evidence on cell permeability is given by the cellular inhibition test mentioned before. For the determination of intestinal absorption a CaCo2-permeability assay is performed. In contrast to artificial membrane models such as PAMPA (parallel artificial membrane permeability assay) this colon carcinoma derived cell line allows for the observation of relevant metabolic steps and active transport processes in the gastrointestinal tract. To ensure a high throughput a cassette dosing procedure has been developed. Interference with liver metabolism is of special interest in drug development. On the one hand the compounds must display sufficient metabolic stability in order to reach the target. Therefore, metabolic stability is assessed by the determination of the half life time in rat liver microsomes. On the other hand the compounds should not inhibit the degradation of other endogenous or exogenous substances. To evaluate this effect inhibition of the six most important hepatic CYP-enzymes is determined in a cell-free assay. The final step in the evaluation process is an *in vivo* test in rats that is performed to assess the pharmacokinetic profile of the inhibitors.

**Figure 2**

For the development of a powerful screening system that provides a maximum of information it is necessary to apply the single evaluation steps in a reasonable and efficient order (Fig. 2). In every single step inhibitors undergo selection. By application of this screening sequence hit structures can be identified, the development to lead structures is supported, input in the optimisation of these lead structures is provided, and candidate substances for further evaluation can be proposed. For the fast determination of SAR three cell-free activity and selectivity assays are performed. Before applying cellular assays, cytotoxic effects are determined to make sure that the measured inhibitions are not artifactual. Subsequently, intracellular inhibition, as well as oestrogenic effects, is determined. Intestinal absorption is only determined for compounds which are intracellularly active and non oestrogenic. After



that the interplay of inhibitors and hepatic enzymes is evaluated in two steps followed by pharmacokinetic studies in rats. For the determination of *in vivo* activity several models are described (Day et al., 2008; Einspanier et al., 2006; Grümmer et al., 2001; Husen et al., 2006; Laschke et al., 2005). Presently, the most appropriate for our screening procedure is being elaborated.

### Figure 3

#### 3.3 Application of the established screening system

For the evaluation of the effectiveness of the developed screening system 214 non-steroidal compounds of different structural classes were designed, synthesised and tested (Fig. 3). Eighty-two compounds were highly active 17 $\beta$ -HSD1 inhibitors with IC<sub>50</sub>-values below 1  $\mu$ M, and 23 of these were also selective against 17 $\beta$ -HSD2. The latter compounds were evaluated in the ER-affinity tests. Sixteen turned out to have a very low affinity to both ER subtypes and no cytotoxic effects on T47-D cells. Five of these showed a good intracellular activity. After performing the oestrogenicity test, four compounds turned out to be candidates for extended pharmacokinetic evaluation.

## 4. Discussion and Conclusion

As mentioned above, non-steroidal compounds are of major interest. Fewer side effects are expected from these compounds, since the non-steroidal scaffold should show less affinity to other steroid-binding proteins. Nevertheless, the affinity of our compounds for the ERs is of particular interest, because ER $\alpha$  agonism would lead to further disease progression and thus impairs the therapeutic benefit. An agonistic effect on ER $\beta$  might have a benefit in the treatment of oestrogen-dependent diseases due to the putative antiproliferative effect of this receptor (Matthews and Gustafsson, 2003; Ström et al., 2004). However, this may lead to ER $\beta$ -derived side effects. Conversely, ER $\alpha$  antagonists decrease proliferation, but they also show typical anti-oestrogenic side effects. Therefore, an ER $\alpha$  antagonism is also not tolerable for this approach. Since a deficiency of ER $\beta$  might lead to uterine hyperproliferation, ER $\beta$ -antagonism is also undesirable (Weihua et al., 2000). For these reasons, we performed two ER-binding assays that enabled us to deduce SAR for the development of compounds with no or low affinity to the ERs.

For a closer investigation of the compounds there is a need for a cellular model that fulfills the following requirements. First, the cells should be of mammalian origin and be related to one of the oestrogen-dependent diseases. Secondly, they must express 17 $\beta$ -HSD1 and 17 $\beta$ -HSD2. Stronger expression of the former is desirable, as this is the case in the diseased tissue. Moreover, the cells should express ER $\alpha$  and ER $\beta$ , which leads to oestrogen dependent proliferation. For this reason, we consider that T47-D cells are very appropriate for the *in vitro* studies.

After investigating the important aspects as mentioned above, the compounds must be evaluated in disease models. Using different animal models, 17 $\beta$ -HSD1 has been validated as an appropriate target for treating breast cancer and endometriosis (Einspanier et al., 2006; Grümmer et al., 2001). For the investigation of the *in vivo* activity of our compounds we are presently examining their PK-profile and their inhibitory potency on E2-formation in the corresponding species.

In the present study we have established several single tests and combined them into a powerful screening system. In this test sequence, relevant properties of potential therapeutics were investigated at an early stage of inhibitor development. A number of non-steroidal compounds were evaluated. Using the single assays SAR were deduced and applied for the optimisation of the synthesised compounds. As a result two series of novel 17 $\beta$ -HSD1-inhibitors with promising features were discovered. The most interesting compounds were highly active and selective inhibitors of 17 $\beta$ -HSD1 and showed good pharmacokinetic properties. They are candidates for a further preclinical development.

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Table 1 Requirements for inhibitors and corresponding tests

	Necessary properties for 17 $\beta$ -HSD1-inhibitors as potential therapeutic agents	Relevant assays for the evaluation of the inhibitors
Pharmacodynamic aspects	<p>High inhibitory activity at the target enzyme</p> <p>Selectivity towards 17<math>\beta</math>-HSD2</p> <p>No (anti-)oestrogenic effects</p> <p>No acute or chronic toxicity</p>	<ul style="list-style-type: none"> <li>▪ Cell-free 17<math>\beta</math>-HSD1 inhibition assay</li> <li>▪ Cellular 17<math>\beta</math>-HSD1 inhibition assay</li> <li>▪ Cell-free 17<math>\beta</math>-HSD2 inhibition assay</li> <li>▪ Competitive ER<math>\alpha</math> and ER<math>\beta</math> binding assay</li> <li>▪ T47-D proliferation test</li> <li>▪ T47-D MTT-cytotoxicity assay (acute)</li> <li>▪ T47-D proliferation test (chronic)</li> </ul>
Pharmacokinetic aspects	<p>Good cellular absorption</p> <p>Low interference with liver metabolism</p> <p>Pharmacokinetic properties</p>	<ul style="list-style-type: none"> <li>▪ Cellular inhibition assay</li> <li>▪ CaCo2-permeability test</li> <li>▪ Rat liver microsome stability assay</li> <li>▪ CYP-inhibition assays</li> <li>▪ <i>In vivo</i> test in rats</li> </ul>

Figure 1:

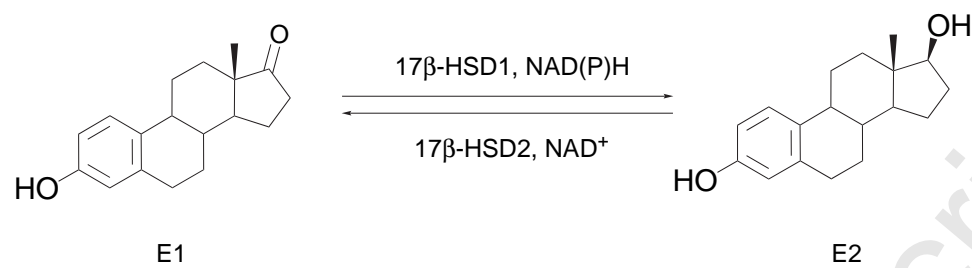
Interconversion of estrone (E1) and 17 $\beta$ -estradiol (E2) by 17 $\beta$ -HSDs

Figure 2: Combination of single assays to a powerful screening system

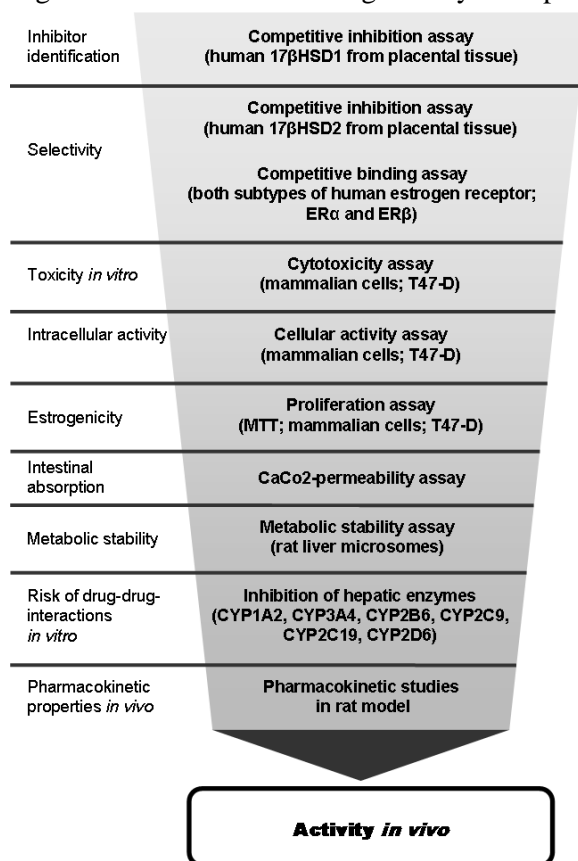




Figure 3: Efficiency of the screening system

