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Towards the evaluation in an animal disease model: Fluorinated 17 β -HSD1 inhibitors showing strong activity towards both the human and the rat enzyme

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Abstract

17 β -Estradiol (E2), the most potent human estrogen, is known to be involved in the etiology of estrogen-dependent diseases (EDD) like breast cancer and endometriosis. 17 β -Hydroxysteroid dehydrogenase type 1 (17 β -HSD1) catalyses the last step of E2 biosynthesis and is thus a promising target for the treatment of EDD. The previously described bicyclic substituted hydroxyphenyl methanones (BSHs) display high inhibitory potency towards human 17 β -HSD1, but marginal activity towards rodent 17 β -HSD1, precluding a proof of principle study in an animal endometriosis model. The aim of this work was to perform structural optimizations in the BSHs class to enhance inhibitory activity against rodent (mouse and rat) 17 β -HSD1 while maintaining activity against the human enzyme. The introduction of fluorine atoms on the benzoyl moiety resulted in compounds with the desired properties. Molecular docking and homology modelling were applied to elucidate the binding mode and interspecies differences in activity. Compound **33** is the most potent inhibitor of both *human* and *rat* 17 β -HSD1 up to date (IC₅₀ = 2 nM and 97 nM, respectively).

Keywords: 17 β -Hydroxysteroid dehydrogenase type 1, Interspecies differences, Estrogen-dependent diseases, Estrogen mimetics, Non-steroidal inhibitors

Abbreviations: 17 β -HSD1, 17 β -hydroxysteroid dehydrogenase type 1; 17 β -HSD2, 17 β -hydroxysteroid dehydrogenase type 2; E1, estrone; E2, 17 β -estradiol; EDD, estrogen-dependent disease; PoP, proof of principle; SERM, selective estrogen receptor modulator; GnRH, gonadotropin-releasing hormone; NADP(H), nicotinamide adenine dinucleotide phosphate; NAD(H), nicotinamide adenine dinucleotide; ADME, absorption, distribution, metabolism, and excretion; *h*17 β -HSD1, *human* 17 β -hydroxysteroid dehydrogenase type 1; *m*17 β -HSD1, *mouse* 17 β -hydroxysteroid dehydrogenase type 1; *r*17 β -HSD1, *rat* 17 β -hydroxysteroid dehydrogenase type 1; BSHs, bicyclic substituted hydroxyphenylmethanones; HPLC, high performance liquid chromatography; CC, column chromatography; Cpd., compound; TLC, thin layer chromatography; rt, room temperature; DCM, dichloromethane; BBr₃, boron tribromide; DME, dimethoxyethane; CCl₄, carbon tetrachloride; NaH, sodium hydride; MeI, methyl iodide; DMF, *N,N*-dimethyl formamide; Pd(dppf)Cl₂, [1,1'-Bis(diphenylphosphino)ferrocene]palladium(II) dichloride; Pd(PPh₃)₄, Tetrakis(triphenylphosphine)palladium(0); HEK293 cells, Human Embryonic Kidney 293 cells; PDB, Protein Data Bank.

1. Introduction

17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1) catalyses the NAD(P)H dependent transformation of estrone (E1) to 17 β -estradiol (E2) which represents the most potent estrogen in humans (fig. 1).[1] E2 is known to play a crucial role in the development and progression of several estrogen dependent diseases (EDD).[2-5] Increased E2/E1 ratios as well as elevated 17 β -HSD1 mRNA levels are indicators of the involvement of 17 β -HSD1 in breast cancer[6, 7], ovarian tumor[8], endometriosis[9], endometrial hyperplasia[10] and other EDD[11]. Consequently, the selective inhibition of this enzyme is considered as a valuable treatment option. This intracrine concept has recently been supported by the observation that an 17 β -HSD1 inhibitor tool compound can effectively block the increased E2 synthesis in endometriotic tissue specimens.[12] Due to the tissue-selective expression and the intracrine mode of action of 17 β -HSD1[13], its inhibition should be associated with less side effects compared to established treatments using GnRH-analogs, aromatase inhibitors, anti-estrogens or selective estrogen receptor modulators (SERMs).[14-16] 17 β -HSD2 catalyses the reverse reaction (inactivation of E2 to E1, fig. 1). Therefore, inhibitors of 17 β -HSD1 should be selective over the type 2 enzyme.

Figure 1

Therapeutics interfering with intracrine pathways of hormone biosynthesis are already in clinical use, e.g. 5 α -reductase inhibitors.[17-20] Although a number of steroidal and non-steroidal inhibitors of 17 β -HSD1[21-23] are described and there is experimental evidence for the effectiveness of 17 β -HSD1 inhibition against human tumor cell lines *in vitro* and in animal models, there is no inhibitor under clinical evaluation. Moreover, no proof of principle

study in an animal disease model has been conducted for the indication endometriosis, although this is a widespread disease for which no adequate medical treatment is available.

A reason for this are interspecies differences between human 17 β -HSD1 and animal orthologs which impede corresponding *in vivo* experiments, for instance in rodents.[24] Thus, human and mouse 17 β -HSD1 are 83 % similar in the first 287 amino acids and show significant differences concerning the topology of the substrate binding site.[25, 26] As a consequence, compounds highly active towards the human enzyme are often weakly active or inactive towards rodent 17 β -HSD1.

The aim of this study was the design of compounds inhibiting both human and rodent 17 β -HSD1. Such a compound may be used in a proof of principle study in an animal endometriosis model and may serve as potential candidate for clinical development afterwards. Starting point for the design was the class of bicyclic substituted hydroxyphenylmethanones (BSHs).[27-29] In contrast to most other classes of 17 β -HSD1 inhibitors described by us[30-38], it contains members which not only are strong inhibitors of the human enzyme but also show inhibition of the murine ortholog (previously unpublished results, fig.2). Examples are compounds **D** and **E** (fig. 2).[27, 28] Drug design was focused on compound **E** as a lead and included structural variations with the aim of enhancing inhibitory activity towards rodent 17 β -HSD1 while maintaining activity towards the human enzyme.

Figure 2

2. Design

In a screening across all compound classes of our in-house compound library, the BSH **E** (fig. 2) showed the highest inhibitory activity against murine 17 β -HSD1 (for data of other compound classes cf. Supplementary Table S3). Compounds from the same class lacking the aromatic sulfonamide moiety displayed slightly inferior activity (compare compounds **D** and **E**, fig. 2). Thus, the aromatic sulfonamide moiety in combination with the BSH scaffold not only leads to a favourable activity profile against human 17 β -HSD1 and 2 [28], but it also appears to be attractive in terms of activity against murine 17 β -HSD1.

The fact that **E** was the only BSH bearing an aromatic sulfonamide group prompted us to investigate this structural element in more detail (chart 1). Its optimal position on the phenyl ring C was determined (compounds **1-10**), and analogous amides were investigated as possible bioisosteres (compounds **11** and **12**). Moreover, compounds **13-29** with diverse substituents on ring D were prepared in a parallel synthesis approach in order to explore the chemical space more thoroughly.

Judging from previous data, fluorination of ring A could be beneficial. However, the introduction of a single fluorine atom was not expected to lead to a pronounced enhancement of activity.[27, 39-41] Therefore, a structural optimization was performed which focused on the introduction of two fluorine atoms into the benzoylic moiety (ring A; compounds **30-33**), considering the following structure activity relationships obtained in previous studies: [27, 28]

- the OH-group on the benzoylic moiety (ring A) is very important for activity

- only minor structural variations in this part are tolerated by the human target enzyme

Finally, ring D was replaced with heterocycles or cyclopropyl in order to decrease the lipophilicity of the fluorinated sulfonamide compounds (compounds **34-38**).

Chart 1

An overview on the synthesised compounds **1-38** is given in the supporting information (Chart S1)

3. Chemistry

The key intermediates **I** and **II** were synthesised in good yields from the appropriate benzoyl chlorides and 2-bromothiophene using Friedel-Crafts conditions (scheme 1). Subsequent ether cleavage[42] (method A) yielded **III** and **IV** in quantitative yields, respectively.

III was reacted with 4-aminophenylboronic acid pinacol ester in a Suzuki cross coupling reaction[43] to afford the final compound **1**. Compounds **2** and **30** were prepared from **I** and **II**, respectively, using the same methods as for **1** but in reversed order because the intermediates **2a** and **30a** were needed as starting materials in the syntheses according to scheme 2, *vide infra*.

The sulfonamides **3-28** and **34-36** were synthesised by reaction of **1**, **2** and **30**, respectively, with the corresponding sulfonic acid chlorides at room temperature. Cleavage of the ether function of **28** gave the dihydroxylated compound **29**. (Scheme 1)

Scheme 1

Reaction of **2a** with 3-methoxybenzenesulfonyl- or 3-methoxybenzoyl chloride yielded the corresponding intermediates **10a** and **11a**, which were submitted to ether cleavage to give the final compounds **10** and **11**, respectively. (Scheme 2)

N-Methylation of compound **11a** with methyl iodide afforded **12a** which was reacted with BBr₃ in dichloromethane (method A) to give **12**.

Interestingly, the synthesis of the fluorinated sulfonamides **31-33** starting from the phenol **30** (in analogy to the preparation of the fluorinated sulfonamides **34-36**) proved to be difficult due to purification issues. This problem could be solved by employing the ether intermediate

30a instead of the phenol **30** as starting material and conducting *O*-demethylation of the intermediates **31a-33a** as the final step.

Scheme 2

The synthesis of compounds **37** and **38** started with the reaction of 3-methyl-5-bromoaniline and cyclopropanesulfonyl chloride to afford **37b** (Scheme 3). Subsequent methylation yielded the *N*-methyl sulfonamide **38b**. Intermediates **37b** and **38b** were converted to the corresponding boronic acid esters **37a** and **38a** by a Pd-catalyzed reaction with bis(pinacolato)diboron in the presence of potassium acetate in DMSO. Suzuki cross coupling reaction with **IV** afforded the final compounds **37** and **38**.

Scheme 3

4. Biological results

Inhibition of human and rodent 17 β -HSD1 and 2

Human placenta was used as source for *h*17 β -HSD1, the enzyme was obtained according to described methods.[44-46] Recombinant *mouse* 17 β -HSD1 and *rat* 17 β -HSD1 were transiently expressed in HEK293 cells. Incubations were run with cytosolic fractions, tritiated E1, cofactor and inhibitor. The type 2 enzymes were obtained from the microsomal fractions of human placenta or mouse and rat liver, respectively. See experimental part for details. The separation of substrate and product was accomplished by HPLC (tables 1-3).

Inhibition of *h*17 β -HSD1 by compounds **3-8** was strongly dependent on the position of the sulfonamide nitrogen at the phenyl ring C: compounds bearing the sulfonamide moiety in *m*-position to the thiophene ring (compounds **6-8**, table 1) showed significantly higher inhibitory potency compared to their corresponding *p*-substituted isomers **3-5**. Interestingly, the parent anilines **1** and **2** displayed a reversed correlation between position of the nitrogen atom and inhibitory potency. Bioisosteric replacement of the benzenesulfonic moiety of compound **6** with a thiophenesulfonic group led to compound **9** with similar activity. The *m*-sulfonamide function may be bioisosterically replaced by the amide function as can be seen by comparison of the inhibitory potencies of compounds **10** and the only slightly less active **11** (table 1). Methylation of the amide group decreased activity (compound **12**). Among compounds **1-12**, inhibitor **7** showed the highest activity towards *h*17 β -HSD1 (IC₅₀ = 8 nM) and the highest selectivity towards the human type 2 enzyme (SF = 25). Representative compounds bearing

electron-donating or –withdrawing substituents were tested for inhibition of *m*17 β -HSD1 but only showed weak activities of 26 % and below at 1 μ M.

Table 1

The favourable *h*17 β -HSD1 inhibition and selectivity data of compound **7** prompted us to investigate the effect of different substituents on the benzenesulfonyl moiety on inhibitory potency in more detail. Thus, compounds **13-29**, bearing the sulfonamide moiety in the 3-position of the phenyl ring (ring C) were synthesised (table 2). The compounds basically showed comparable inhibition of *h*17 β -HSD1, independent of the nature of the substituent (electron-donating or –withdrawing). It seemed, however, that bulky and/or hydrophilic substituents somewhat decrease potency (e.g. compounds **17** and **25**). None of the compounds **13-29**, however, exceeded the activity of compound **7**. Also from this group of compounds, representative members bearing electron-donating or –withdrawing substituents were tested for inhibition of *m*17 β -HSD1 but did not show pronounced activities (table 2).

Table 2

Fluorination of the benzoyl[39-41] part of the inhibitors eventually led to a substantial increase of inhibitory activity (table 3). Compounds **30-38** showed very strong inhibition of *h*17 β -HSD1, with IC₅₀-values generally in the one-digit nanomolar range, at the expense, however, of a low selectivity over the human type 2 enzyme. In addition, most of the compounds **30-38** displayed inhibition of the murine type 1 enzyme. Compound **33** was the most active inhibitor of *m*17 β -HSD1. Intrigued by the potency of the fluorinated compound **33** and analogues towards *m*17 β -HSD1, we tested compounds **30-38** for activity towards the rat enzymes as well. **32** and **33** showed strong inhibition of *r*17 β -HSD1 (IC₅₀ = 80 and 97 nM, respectively). Moreover, the activity towards 17 β -HSD2 was less pronounced in case of the rat compared to mouse.

Table 3

5. Molecular modeling

5.1. Choice of Receptor Coordinates

A multitude of ligand-*h*17 β -HSD1 complex structures have been solved to date and are available from the protein data bank.[47] As the choice of receptor coordinates as a starting point for *in silico* studies may significantly influence computed results, we carefully surveyed selected ternary structures with cofactor and additional ligand coordinates for their applicability in our experiments. Interestingly, some important structural differences can be observed when comparing for example the co-crystal of estradiol bound to *h*17 β -HSD1 (PDB-ID: 1FDT) with that one of reported inhibitor **E₂B** (PDB-ID 3HB5).[48, 49] In the former complex, the side chain of Lys195 is oriented inwards and towards the active site, thereby occupying a notable volume of the inhibitor binding region (see Supporting Figure S1). On the other hand, in 3HB5 this side chain is oriented outside of the protein. This is enabled by a quite significant change in backbone conformation. As a consequence, the otherwise partly occluded carboxamide group of Asn152 is easily accessible for attractive interactions with the inhibitor. In a previous study, we proposed that such interactions might be important for *h*17 β -HSD1 inhibitors structurally related to the compounds reported herein.[28] Hence, we reasoned that 3HB5 could be the ideal starting point for further *in silico* studies.

5.2. Docking to *h*17 β -HSD1, Refinement and Analysis

Initial attempts to directly dock highly potent inhibitor **33** to *h*17 β -HSD1 yielded ambiguous results only. We accounted this observation to the high degree of conformational freedom within this structure due to seven rotatable bonds. Hence, we followed a more user-guided approach towards binding pose prediction. Initially, structure preparation was performed. Then, pharmacophore features were defined which were based on the co-crystallised inhibitor **E₂B** showing some structural similarity to our inhibitor scaffold (see Supplementary Figure S1). A pharmacophore-guided docking was performed using compound **30** which has only a few rotatable bonds and, thus, displays the lowest degree of conformational freedom among the highly potent *h*17 β -HSD1 inhibitors. The docking result was further refined by energy minimization. Finally, the additional bulky sulfonamide moiety of **33** was modelled into the ligand. The resulting predicted complex structure is given in Figure 3A and B. Due to the rather linear shape of the active site, the sulfonamide and the D ring are very likely to be oriented towards the exit of the binding pocket, providing the possibility to form a favourable hydrogen bond with His221.

As a control, we also docked **E₂B** using the pharmacophore-guided procedure and the resulting binding pose showed an RMSD value of 0.3758 Å compared to the co-crystal structure (Supplementary Figure S2). Finally, we also applied this docking protocol to compound **33**. Again, ambiguous results were obtained. However, among the suggested binding poses very similar *h*17 β -HSD1:**33** complex structures were identified showing the same key interactions (see Supplementary Figure S2).

Our proposed binding model provides plausible explanations for the key SAR observations described above. The fluorination of the A ring had a noticeable beneficial effect on binding affinity to *h*17 β -HSD1. This is nicely reflected by the strength of the hydrogen bond between Asn152 and the 3-hydroxy group computed for compound **33** (Figure 3C) and a non-

fluorinated hypothetical derivative (Figure 3D). Additional calculations of pK_a values for this hydroxyl group showed that the presence of fluorine substituents on ring A strongly influences the polarization of the O-H bond through modulation of electron density at the oxygen (Table S2 Supporting Information). This leads to a more acidic character and renders the hydroxyl group a better hydrogen bond donor. Hence, we conclude that possible additional direct interactions between the fluorine atoms and the protein or an ortho-effect on ligand conformation are at least of minor importance for the beneficial outcome of fluorination.

Figure 3

5.3. Homology Modeling of *m17 β -HSD1*

Finally, we generated a homology model for the homologous mouse enzyme in complex with inhibitor **33**. To this end, we employed a built-in protocol of MOE which allows to employ ligand atoms as environment for side chain refinement. The respective *m17 β -HSD1:33* complex was generated using this method. Interestingly, the Asparagine residue which we propose to be involved in the described affinity-determining hydrogen bond is replaced by Histidine (Figure 3E). Although the imidazole side chain functionality can in principle exert the same interactions as the carboxamide group of Asn152, it is obvious that this exchange has a huge spatial and electronic impact on the predicted interaction. In line with these considerations, we find that the strength of the possible hydrogen bond between Histidine and inhibitor within the *m17 β -HSD1* complex is dramatically decreased in comparison to its (human) Asparagine congener.

5.4 Difference in activity towards mouse and rat *17 β -HSD1*

The observed general preference of the *17 β -HSD1* inhibitors towards the rat over the mouse isoform might be caused by rather subtle changes within the protein structure, as these enzymes are very similar, sharing a sequence identity of 93 %. Comparing both sequences and taking our homology model of *m17 β -HSD1* into account, we identified four sites with amino acid exchanges that might have an influence on inhibitor binding: Tyr194His, Val198Glu, Leu220Gln, and Gln281Arg. Actually, these are the only residues in proximity to the ligand binding site which show a difference between mouse and rat. In the proposed binding mode no direct interactions between these residues and the ligand could be detected. However, Tyr194His is located in the helix/loop region which adopts different conformations within available X-ray structures for the human homolog (e.g. 1FDT vs 3HB5, *vide supra* 5.1 Choice of Receptor Coordinates). As this part of the protein is obviously quite flexible in case of *h17 β -HSD1*, we speculate that both rodent enzymes may also adopt conformations where Tyr194 or His194 are facing towards the ligand binding site, facilitating direct interactions with the ligand or indirectly influencing side chain geometries of neighboring residues,

affecting ligand affinity. Hence, we hypothesize that this amino acid exchange is the reason for the observed differences between *m*17 β -HSD1 and *r*17 β -HSD1 inhibition. The other mentioned residues (Val198Glu, Leu220Gln, and Gln281Arg) are located at more distant positions (see Supplementary Figure S5). However, an indirect effect on inhibitor binding through subtle influence on the backbone or the side chains which are in contact with the ligands cannot be excluded.

6. Discussion and Conclusions

The aim of the present study was the search for potent inhibitors of both human and rodent 17 β -HSD1, which can pave the way to a proof of principle study in an animal disease model for EDD. The class of sulfonamide-substituted BSHs was chosen as a starting point because this compound class, in contrast to others, is characterized not only by high inhibitory potency towards human 17 β -HSD1, but also by activity towards the murine enzyme.

Modifying the substitution pattern on the sulfonamide moiety did not result in compounds with the desired inhibitory profile. The introduction of fluorine atoms on the benzoyl moiety, however, led to the discovery of highly active inhibitors of *h*17 β -HSD1. In addition, strong inhibition of the rodent enzymes, especially rat 17 β -HSD1, was observed for some compounds. Computational analyses suggested that the increase of inhibitory activity was due to a beneficial influence of fluorination on the hydrogen bonding properties of the adjacent OH-group. Lower potencies towards rodent compared to human 17 β -HSD1 are in agreement with differences in the architectures of the ligand binding sites and nearby regions, as comparisons between a crystal structure of *h*17 β -HSD1 and homology models of the corresponding murine and rat enzymes indicated.

Compound **33** is one of the most active inhibitors of human 17 β -HSD1 ever described. Moreover, it displayed strong inhibition of the rat ortholog. Its inhibition of the type 2 enzyme is less pronounced for the rat than for the mouse. Thus, compound **33** is a lead for further optimisations in order to obtain a suitable candidate which can be used in a proof of principle study in a rat endometriosis model. The minimally required 17 β -HSD1 selectivity over 17 β -HSD2 is not known at the present time. In endometriotic tissue of patients with endometriosis, due the increased activity of 17 β -HSD1, higher than expected concentrations of inhibitor may be required for normalization of E2 synthesis. In healthy endometrial tissue, the intracellular E2 concentrations during the proliferative phase are 5-8 fold higher than in the serum [50]. In endometriotic tissue samples of patients with endometriosis, the intracellular E2 concentrations remain higher over E1 throughout the whole menstrual cycle. In the rat endometriosis model, endometrial explants, that are autologously transplanted to ectopic sites outside the uterus, also contain very high levels of E2, about two orders of magnitude higher than in the serum [51]. Yet, at the same time, because of significantly increased intracellular concentration of E2 in endometriotic tissue [50, 51], it may also be difficult for the compound to compete with E2, and thus a higher than expected selectivity towards 17 β -HSD1 inhibition is also possible. Recently, a more than 100-fold selective (and estrone-derived) 17 β -HSD1

inhibitor has been shown to effectively block the increased E2 synthesis in human endometriotic tissue specimens [12]. A 100-300-fold selectivity may thus be sufficient for a proof of principle study in a rat endometriosis model.

7. Experimental Section

7.1 Chemical Methods. Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Maybridge, Combi Blocks, Merk, or Fluka and were used without purification.

Column chromatography (CC) was performed on silica gel (70 – 200 μ m), reaction progress was monitored by thin layer chromatography (TLC) on Alugram SIL G UV₂₅₄ (Macherey-Nagel).

¹H NMR and ¹³C NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) or Bruker AM300 spectrometer (300 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard (CDCl₃: δ = 7.24 ppm (¹H NMR) and δ = 77 ppm (¹³C NMR), CD₃OD: δ = 3.35 ppm (¹H NMR) and δ = 49.3 ppm (¹³C NMR), CD₃COCD₃: δ = 2.05 ppm (¹H NMR) and δ = 29.9 ppm (¹³C NMR), CD₃SOCD₃: δ = 2.50 ppm (¹H NMR) and δ = 39.5 ppm (¹³C NMR)). Signals are described as s, d, t, dd, ddd, m, dt, q, sep, br. for singlet, doublet, triplet, doublet of doublets, doublet of doublets of doublets, multiplet, doublet of triplets, quadruplet, septet, and broad, respectively. All coupling constants (*J*) are given in hertz (Hz).

Mass spectra (ESI) were recorded on a TSQ Quantum (Thermo Finnigan) instrument.

Tested compounds are >95% chemical purity as measured by HPLC. The methods for HPLC analysis and a table of data for all tested compounds are provided in the supplementary data section.

The following compounds were prepared according to previously described procedures: (5-bromothiophene-2-yl)(3-methoxyphenyl)methanone (**I**),[27] (5-bromothiophene-2-yl)(3-hydroxyphenyl)methanone (**III**),[27] (5-(3-aminophenyl)thiophene-2-yl)(3-methoxyphenyl)methanone (**2a**),[52] (5-(3-aminophenyl)thiophene-2-yl)(3-hydroxyphenyl)methanone (**2**),[52] 4-bromo-*N*-(3-(5-(3-hydroxybenzoyl)thiophene-2-yl)phenyl)-2-trifluoromethoxybenzenesulfonamide (**7**),[52].

The Supplementary Data section reports the synthesis of compounds **4-28**, **10a**, **11a**, **30a**, **29-38** and **37b**. For each general synthetic procedure, one representative example is given below

7.1.1 (5-Bromothiophene-2-yl)-(3-ethoxy-2,6-difluorophenyl)methanone (**II**).

An ice-cooled mixture of 2-bromothiophene (2.217 g, 13.60 mmol), 2,6-difluoro-4-ethoxybenzoyl chloride (3.000 g, 13.60 mmol) and aluminum chloride (1.813 g, 13.60 mmol) in anhydrous dichloromethane (25 mL) was warmed to room temperature and stirred for 2-4

h. 1M HCl (50 mL) was used to quench the reaction. The aqueous layer was extracted with dichloromethane (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC (hexane/ethyl acetate 97:3); yield: 79% (3740 mg). ¹H NMR (300 MHz, acetone-d₆) δ 7.34 (dd, *J* = 4.1, 0.9 Hz, 1H), 7.22 (dd, *J* = 6.7, 4.7 Hz, 2H), 6.98 (td, *J* = 9.0, 2.0 Hz, 1H), 4.05 (q, *J* = 7.0 Hz, 2H), 1.27 (t, *J* = 7.0 Hz, 3H); ¹³C NMR (75 MHz, acetone-d₆) δ 179.03, 152.19 (dd, *J* = 242.2, 5.8 Hz), 148.69 (dd, *J* = 250.5, 7.4 Hz), 145.09, 143.94 (dd, *J* = 10.6, 3.3 Hz), 136.88, 132.66, 128.96, 124.50, 117.11 (dd, *J* = 9.3, 3.2 Hz), 111.26 (dd, *J* = 22.6, 4.1 Hz), 65.44, 14.08; MS (ESI): 448.64 (M+H)⁺.

7.1.2. General procedure for ether cleavage. Method A (IV, 10-12, 29-33)

To a solution of methoxybenzene derivative (8 mmol, 1 equiv) in anhydrous dichloromethane (20 mL) at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1M, 3 equiv per methoxy function) was added dropwise. The reaction mixture was stirred overnight at room temperature under nitrogen atmosphere. Water (50 mL) was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC.

7.1.3 (5-Bromothiophene-2-yl)-(2,6-difluoro-3-hydroxyphenyl)methanone (IV).

The title compound was prepared by reaction of (5-bromothiophene-2-yl)-(3-ethoxy-2,6-difluorophenyl)methanone (V) (3.00 g, 8.64 mmol) and boron tribromide (43.2 mmol, 5 equiv) according to Method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 84% (2.310 g). MS (ESI): 320.12 (M+H)⁺.

7.1.4. General procedure for Suzuki coupling. Method B (1, 30a, 37, 38)

A mixture of arylbromide (0.50 mmol, 1 equiv), boronic acid derivative (1.2 equiv), cesium carbonate (4 equiv) and tetrakis(triphenylphosphine) palladium (0.01 equiv) was suspended in an oxygen-free DME/water (1:1, 25 mL) solution and refluxed under nitrogen atmosphere. The reaction mixture was cooled to room temperature. The aqueous layer was extracted with ethyl acetate (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

7.1.5. (5-(4-Aminophenyl)thiophene-2-yl)(3-hydroxyphenyl)methanone (1)

The title compound was prepared by reaction of (5-bromothiophene-2-yl)(3-hydroxyphenyl)methanone (III) (140 mg, 0.50 mmol), 4-aminophenylboronic acid pinacol ester (155 mg, 0.71 mmol), cesium carbonate (658 mg, 2.02 mmol) and tetrakis(triphenylphosphine) palladium (6.00 mg, 5.00 μmol) according to Method B. The product was purified by CC (dichloromethane/methanol 99:1); yield: 82% (120 mg). ¹H NMR (500 MHz, CDCl₃) δ 8.75 (br. s, 1H), 7.65 (d, *J* = 4.0 Hz, 1H), 7.44 (d, *J* = 4.0 Hz, 1H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.33 (dt, *J* = 7.6, 1.3 Hz, 1H), 7.31 – 7.29 (m, 1H), 7.13 (t, *J* = 7.8 Hz, 1H), 7.10 (ddd, *J* = 7.9, 2.5, 1.2 Hz, 1H), 7.07 (t, *J* = 1.9 Hz, 1H), 6.99 (ddd, *J* = 7.6, 1.7, 0.9

Hz, 1H), 6.71 (ddd, $J = 8.0, 2.2, 0.8$ Hz, 1H), 4.85 (br., 2H, NH₂); ¹³C NMR (125 MHz, CDCl₃) δ 186.80, 157.50, 153.62, 149.30, 141.63, 139.55, 135.88, 129.86, 129.65, 129.65, 123.79, 120.14, 119.23, 115.36, 115.29, 114.53, 111.59; MS (ESI): 296.23 (M+H)⁺; HPLC purity $\geq 98\%$ ($R_t = 7.90$ min).

7.1.6. General procedure for sulfonamide/amide coupling. Method C (**3-9**, **10a**, **11a**, **13-28**, **34-36**, **37b**)

The amino phenyl derivative (0.4 mmol, 1 equiv) was dissolved in pyridine absolute (3 mL) and was spiked with sulfonyl chloride/acid chloride (1.5 equiv). The reaction mixture was stirred overnight at rt (refluxed in case of amide coupling). The reaction was quenched by adding 10 mL of 2N HCl and extracted with ethyl acetate (3 x 50 mL). The organic layers were washed with saturated NaHCO₃ (50 mL) and brine (50 mL), dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

7.1.7. *N*-(4-(5-(3-Hydroxybenzoyl)thiophene-2-yl)phenyl)benzenesulfonamide (**3**)

The title compound was prepared by reaction of (5-(4-aminophenyl)thiophene-2-yl)(3-hydroxyphenyl)methanone (**1**) (100 mg, 0.34 mmol) and benzenesulfonyl chloride (90 mg, 0.51 mmol) according to Method C. The product was purified by CC (dichloromethane/methanol 99.5:0.5); yield: 77% (114 mg). ¹H NMR (500 MHz, CD₃OD) δ 9.18 (br. s, 1H), 8.76 (br. s, 1H), 7.74 – 7.70 (m, 2H), 7.52 (d, $J = 4.0$ Hz, 1H), 7.51 – 7.48 (m, 2H), 7.46 (dt, $J = 2.3, 1.6$ Hz, 1H), 7.39 (dd, $J = 10.5, 4.7$ Hz, 2H), 7.28 (d, $J = 4.0$ Hz, 1H), 7.24 (t, $J = 7.8$ Hz, 1H), 7.19 – 7.16 (m, 1H), 7.13 – 7.10 (m, 1H), 7.10 – 7.06 (m, 2H), 6.95 (ddd, $J = 8.1, 2.5, 1.0$ Hz, 1H); ¹³C NMR (125 MHz, MeOD) δ 189.73, 158.89, 154.09, 142.82, 141.08, 140.52, 140.13, 137.95, 134.08, 130.75, 130.67, 130.18, 128.20, 128.16, 125.13, 122.05, 121.26, 120.62, 116.48; MS (ESI): 436.24 (M+H)⁺; HPLC purity $\geq 97\%$ ($R_t = 11.57$ min).

7.1.8. 3-Methoxy-*N*-(3-(5-(3-methoxybenzoyl)thiophene-2-yl)phenyl)-*N*-methylbenzamide (**12a**)

A mixture of 3-methoxy-*N*-(3-(5-(3-methoxybenzoyl)-thiophene-2-yl)phenyl)benzamide (**11a**) (666 mg, 1.50 mmol, 1 equiv) and NaH (60% suspension in mineral oil, 3.00 mmol, 2 equiv) in DMF (20 mL) was stirred for 30 min at room temperature, then iodomethane (213 mg, 1.50 mmol, 2 equiv) was added. After 30 min the reaction mixture was poured into water (50 mL). The resulting precipitate was collected, washed with water (50 mL), dried, and purified by CC (hexane/ethyl acetate 3:2); yield: 42% (290 mg). ¹H NMR (500 MHz, CDCl₃) δ 7.66 (d, $J = 4.0$ Hz, 1H), 7.52 – 7.48 (m, 2H), 7.46 (t, $J = 7.7$ Hz, 1H), 7.43 (dd, $J = 2.5, 1.4$ Hz, 1H), 7.41 (t, $J = 1.8$ Hz, 1H), 7.35 (t, $J = 7.9$ Hz, 1H), 7.24 (d, $J = 4.0$ Hz, 1H), 7.21 – 7.17 (m, 1H), 7.17 – 7.11 (m, 2H), 6.98 (dd, $J = 2.5, 1.5$ Hz, 1H), 6.96 – 6.92 (m, 1H), 6.88 – 6.83 (m, 1H), 3.93 (s, 3H), 3.73 (s, 3H), 3.59 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 187.55, 170.40, 159.64, 159.10, 151.55, 145.67, 142.74, 139.13, 136.88, 135.69, 134.33, 129.91, 129.42, 128.97, 126.94, 124.54, 124.26, 121.58, 121.03, 118.57, 116.22, 113.74, 113.63, 55.45, 55.23, 38.24; MS (ESI): 458.19 (M+H)⁺.

7.1.9. Cyclopropanesulfonic acid (3-methyl-5-(4,4,5,5-tetramethyl-(1,3,2)dioxaborolan-2-yl)-phenyl)-amide (37a) and cyclopropanesulfonic acid methyl-(3-methyl-5-(4,4,5,5-tetramethyl-(1,3,2)dioxaborolan-2-yl)-phenyl)-amide (38a)

Cyclopropanesulfonic acid (3-bromo-5-methylphenyl)-amide (**37b**) (800 mg, 2.76 mmol, 1 equiv) or cyclopropanesulfonic acid (3-bromo-5-methylphenyl)methyl-amide (**38b**) (260 mg, 0.86 mmol, 1equiv) was dissolved in 10 mL of dry dimethyl sulfoxide (3 mL for compound **38b**) under N₂ atmosphere to prepare the title compounds **37a** and **38a**, respectively. Bis(pinacolato)diborone (1.5 equiv), potassium acetate (3 equiv) and Pd(dppf)Cl₂ (0.05 equiv) were added. The reaction mixture was heated to 80°C over night. The reaction mixture was cooled down to room temperature, quenched with water (50 mL) and diethyl ether (50 mL), filtered over celite and extracted three times with diethyl ether (3 x 50 mL). The ethereal solutions were washed three times with water (50 mL) and brine (50 mL), dried over magnesium sulphate, filtered and evaporated under reduced pressure. Purification was accomplished by CC (dichloromethane pure to dichloromethane/methanol 8:2); yield: 16% (150 mg, compound **37a**) and 50% (150 mg, compound **38a**). The product was used in the next step without characterization.

7.1.10. Cyclopropanesulfonic acid (3-bromo-5-methyl-phenyl)-methyl-amide (38b)

Cyclopropanesulfonic acid (3-bromo-5-methylphenyl)amide (**37b**) (100mg, 0.345mmol, 1equiv) was dissolved in 1 mL anhydrous dimethyl formamide and cooled to 0°C under N₂ atmosphere. Sodium hydride (10.0 mg, 0.414 mmol, 1.2 equiv) was added and the solution was stirred for 10 min. Methyl iodide (54 mg, 0.024 mL, 0.38 mmol, 1.1equiv) was added slowly. The mixture was stirred at room temperature over night. The mixture was quenched with water (50 mL) and extracted three times with diethyl ether (3 x 50 mL). The ethereal solutions were combined, washed 5 times with water (50 mL) and brine (50 mL) and dried over sodium sulphate. The solvent was evaporated under reduced pressure. No further purification was required; yield: 81% (85 mg). ¹H NMR (500 MHz, acetone-d₆) δ 7.44 (s, 1H), 7.29 (s, 1H), 7.16 (s, 1H), 3.31 (s, 3H), 2.56-2.54 (m, 1H), 2.33 (s, 3H), 0.99-0.96 (m, 4H).

7.2. Biological methods. [2,4,6,7-³H]-E1 and [2,4,6,7-³H]-E2 were bought from Perkin-Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. For a brief description of the biological assays see Supporting Data.

7.2.1. Preparation of human 17β-HSD1 and 17β-HSD2.

Human 17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures.[44] Fresh human placenta was homogenized, and cytosolic fraction and microsomes were separated by fractional centrifugation. For the partial purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction.

7.2.2. Inhibition of human 17 β -HSD1.

Inhibitory activities were evaluated by an established method with minor modifications.[44] Briefly, the enzyme preparation was incubated with NADPH (500 μ M) in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20% of glycerol and EDTA (1 mM). Inhibitor stock solutions were prepared in DMSO. The final concentration of DMSO was adjusted to 1% in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2,4,6,7-³H]-E1 (final concentration: 500 nM, 0.15 μ Ci). After 10 min, the incubation was stopped with HgCl₂ and the mixture was extracted with diethylether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 reverse phase chromatography column (Nucleodur C18 125/3 100-5, Macherey-Nagel) connected to a HPLC-system (Agilent 1200 Series, Agilent Technologies). Detection and quantification of the steroids were performed using a radioflow detector (Ramona, raytest). The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{conversion} = [\% \text{E2} / (\% \text{E2} + \% \text{E1})] \times 100$$

Each value was calculated from at least three independent experiments.

7.2.3. Inhibition of human 17 β -HSD2.

The *h*17 β -HSD2 inhibition assay was performed similarly to the *h*17 β -HSD1 procedure. The microsomal fraction was incubated with NAD⁺ [1500 μ M], test compound and a mixture of unlabelled- and [2,4,6,7-³H]-E2 (final concentration: 500 nM, 0.11 μ Ci) for 20 min at 37 °C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{conversion} = [\% \text{E1} / (\% \text{E1} + \% \text{E2})] \times 100$$

7.2.4. Preparation of mouse and rat 17 β -HSD1 and 2.

Recombinant mouse and rat 17 β -HSD1 enzymes were produced by transfection of HEK293 cells with a mouse or rat 17 β -HSD1 expression plasmid purchased from OriGene Technologies, Inc. (Rockville, USA). 48 hours after transfection, cells were homogenised by ultrasonication (3x10s) in a buffer containing sucrose (40 mM Tris, 250 mM sucrose, 5 mM EDTA, 7 mM DTT, 1 mM PMSF, pH 7.5). Cell lysates were centrifuged (1,000g, 15 min, 4°C) and 20% glycerol was added to the supernatant before aliquots were frozen and stored at -70°C. Microsomal 17 β -HSD2 fractions were obtained from mouse or rat liver. Fresh liver tissues were processed as described for the human placental 17 β -HSD2 preparation. The pellet fractions containing the microsomal 17 β -HSD2 were used for the determination of E1 formation.

7.2.5. Inhibition of mouse and rat 17 β -HSD1.

Inhibitory activities of the compounds towards mouse or rat 17 β -HSD1 were evaluated by a method similar to the human 17 β -HSD1 assay. The recombinant enzyme preparations were incubated with NADPH (500 μ M), test compound and a mixture of unlabeled- and [2,4,6,7- 3 H]-E1 (final concentration: 10 nM, 0.15 μ Ci) for 10 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{conversion} = [\% \text{E2} / (\% \text{E2} + \% \text{E1})] \times 100$$

7.2.6. Inhibition of mouse and rat 17 β -HSD2.

The mouse and rat 17 β -HSD2 inhibition assays were performed similarly to the human 17 β -HSD2 procedure. The microsomal fractions were incubated with NAD $^+$ (1,500 μ M), test compound and a mixture of unlabelled- and [2,4,6,7- 3 H]-E2 (final concentration: 10 nM, 0.15 μ Ci) for 20 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation:

$$\% \text{ conversion} = [\% \text{E1} / (\% \text{E1} + \% \text{E2})] \times 100$$

7.3. *In silico* studies

Docking and Binding Pose Refinement.

The protein structure 3HB5 was prepared for docking by applying the LigX protocol of MOE (Chemical Computing Group) using the default parameters. Hence, structure preparation and protonate 3D (allowing side chain flips) algorithms were applied and receptor atoms were tethered with a strength of 10 and a buffer of 0.25. Additionally, all atoms farther than 8 Å from the ligand were automatically fixed and the refinement step used a gradient of 0.1 kcal/mol/Å.

After structure preparation, a constrained four-feature pharmacophore model was set up to guide the initial ligand placement in the docking procedure (see Supplementary Figure S1, Supporting Information). The native ligand was used to define the binding site. Then, compounds **30**, **E2B**, and compound **33** (see Supplementary Figure S2) were docked to the prepared protein-cofactor complex using the “induced fit” protocol with default parameters and the described pharmacophore features being active. The placement algorithm was “Triangle Matcher” with 30 retained poses and “London dG” as rescoring function. The refinement step was carried out using the “Forcefield” option and “GBVI/WSA dG” as the second rescoring function with 30 retained poses.

The ten highest scoring binding poses of compound **30** were evaluated and the most abundant ligand orientation (8/10) was further refined by optimizing hydrogen bond geometry followed by an additional energy minimization step. Then, the 2-trifluoromethylbenzenesulfonamide moiety of **33** was modelled into the generated binding pose of compound **30** by adding the respective atoms starting with the sulfonamide moiety expanding the molecule with minimal clashes. Finally, energy minimization yielded proposed binding pose of compound **33** (see also Supplementary Figure S2). All steps were performed with MOE using the AMBER10:EHT force field and the R-Field solvation model

Homology modelling

The models of the mouse and rat enzymes (*m17 β -HSD1* and *r17 β -HSD1*) were generated using the built-in “homology model” function of MOE. We used the structure of our ternary *h17 β -HSD1:33:cofactor* complex (see above) as template coordinates. The sequences of the mouse and rat isoforms were obtained from NCBI via accession codes NP_034605.1 (mouse) and NP_036983.1 (rat). First, the sequence of respective isoform was loaded into MOE and the sequence was aligned to the human isoform (see also Supplementary Figure S3). Then, ligand atoms (compound **33** and cofactor NADP) were selected as environmental constrain for induced fit optimisation. The default parameters were used (10 models with 1 side chain sample at 300K). The resulting 10 models were scored by the built-in GB/VI (default) algorithm. The highest scoring model was automatically refined with an RMS gradient of 0.5 (default). The yielded ligand binding poses for compound **33** bound to *m17 β -HSD1* and *r17 β -HSD1* were optimised using the LigX protocol with default parameters. All steps were performed using the AMBER10:EHT force field and the R-Field solvation model.

For evaluating model geometry we created Phi-Psi plots of the template coordinates (PDB-ID: 3HB5) and the homology models (see Supplementary Figure S4). In all three models only three out of 284 residues lay outside of the theoretically favoured regions indicating a good overall geometry.

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- [1] J.G. Liehr, Is estradiol a genotoxic mutagenic carcinogen?, *Endocr Rev*, 21 (2000) 40-54.
- [2] D.B. Thomas, Do hormones cause breast cancer?, *Cancer*, 53 (1984) 595-604.
- [3] J. Russo, S.V. Fernandez, P.A. Russo, R. Fernbaugh, F.S. Sheriff, H.M. Lareef, J. Garber, I.H. Russo, 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells, *FASEB J*, 20 (2006) 1622-1634.
- [4] G.S. Dizerega, D.L. Barber, G.D. Hodgen, Endometriosis: role of ovarian steroids in initiation, maintenance, and suppression, *Fertil Steril*, 33 (1980) 649-653.

- [5] K. Zeitoun, K. Takayama, H. Sasano, T. Suzuki, N. Moghrabi, S. Andersson, A. Johns, L. Meng, M. Putman, B. Carr, S.E. Bulun, Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol, *J Clin Endocrinol Metab*, 83 (1998) 4474-4480.
- [6] Y. Miyoshi, A. Ando, E. Shiba, T. Taguchi, Y. Tamaki, S. Noguchi, Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers, *Int J Cancer*, 94 (2001) 685-689.
- [7] A. Jansson, 17Beta-hydroxysteroid dehydrogenase enzymes and breast cancer, *J Steroid Biochem Mol Biol*, 114 (2009) 64-67.
- [8] C.H. Blomquist, M. Bonenfant, D.M. McGinley, Z. Posalaky, D.J. Lakatua, S. Tuli-Puri, D.G. Bealka, Y. Tremblay, Androgenic and estrogenic 17beta-hydroxysteroid dehydrogenase/17-ketosteroid reductase in human ovarian epithelial tumors: evidence for the type 1, 2 and 5 isoforms, *J Steroid Biochem Mol Biol*, 81 (2002) 343-351.
- [9] T. Smuc, M.R. Pucelj, J. Sinkovec, B. Husen, H. Thole, T. Lanisnik Rizner, Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis, *Gynecol Endocrinol*, 23 (2007) 105-111.
- [10] T. Saloniemi, P. Jarvensivu, P. Koskimies, H. Jokela, T. Lamminen, S. Ghaem-Maghami, R. Dina, P. Damdimopoulou, S. Makela, A. Perheentupa, H. Kujari, J. Brosens, M. Poutanen, Novel hydroxysteroid (17beta) dehydrogenase 1 inhibitors reverse estrogen-induced endometrial hyperplasia in transgenic mice, *Am J Pathol*, 176 (2010) 1443-1451.
- [11] T. Kasai, M. Shozu, K. Murakami, T. Segawa, K. Shinohara, K. Nomura, M. Inoue, Increased expression of type I 17beta-hydroxysteroid dehydrogenase enhances in situ production of estradiol in uterine leiomyoma, *J Clin Endocrinol Metab*, 89 (2004) 5661-5668.
- [12] B. Delvoux, T. D'Hooghe, C. Kyama, P. Koskimies, R.J. Hermans, G.A. Dunselman, A. Romano, Inhibition of type 1 17beta-hydroxysteroid dehydrogenase impairs the synthesis of 17beta-estradiol in endometriosis lesions, *J Clin Endocrinol Metab*, 99 (2014) 276-284.
- [13] T.M. Penning, Molecular endocrinology of hydroxysteroid dehydrogenases, *Endocr Rev*, 18 (1997) 281-305.
- [14] A. Cavalli, A. Bisi, C. Bertucci, C. Rosini, A. Paluszczak, S. Gobbi, E. Giorgio, A. Rampa, F. Belluti, L. Piazzzi, P. Valenti, R.W. Hartmann, M. Recanatini, Enantioselective nonsteroidal aromatase inhibitors identified through a multidisciplinary medicinal chemistry approach, *J Med Chem*, 48 (2005) 7282-7289.
- [15] M. Le Borgne, P. Marchand, M. Duflos, B. Delevoye-Seiller, S. Piessard-Robert, G. Le Baut, R.W. Hartmann, M. Palzer, Synthesis and in vitro evaluation of 3-(1-azolylmethyl)-1H-indoles and 3-(1-azolyl-1-phenylmethyl)-1H-indoles as inhibitors of P450 arom, *Arch Pharm (Weinheim)*, 330 (1997) 141-145.
- [16] C. Jacobs, M. Frotscher, G. Dannhardt, R.W. Hartmann, 1-imidazolyl(alkyl)-substituted di- and tetrahydroquinolines and analogues: syntheses and evaluation of dual inhibitors of thromboxane A(2) synthase and aromatase, *J Med Chem*, 43 (2000) 1841-1851.
- [17] E. Baston, R.W. Hartmann, N-substituted 4-(5-indolyl)benzoic acids. Synthesis and evaluation of steroid 5alpha-reductase type I and II inhibitory activity, *Bioorg Med Chem Lett*, 9 (1999) 1601-1606.
- [18] W. Reichert, J. Jose, R.W. Hartmann, 5 alpha-reductase in intact DU145 cells: evidence for isozyme I and evaluation of novel inhibitors, *Arch Pharm (Weinheim)*, 333 (2000) 201-204.
- [19] F. Picard, E. Baston, W. Reichert, R.W. Hartmann, Synthesis of N-substituted piperidine-4-(benzylidene-4-carboxylic acids) and evaluation as inhibitors of steroid-5alpha-reductase type 1 and 2, *Bioorg Med Chem*, 8 (2000) 1479-1487.
- [20] E. Baston, O.I. Salem, R.W. Hartmann, 6-Substituted 3,4-dihydro-naphthalene-2-carboxylic acids: synthesis and structure-activity studies in a novel class of human 5alpha reductase inhibitors, *J Enzyme Inhib Med Chem*, 17 (2002) 303-320.
- [21] J.M. Day, P.A. Foster, H.J. Tutill, M.F. Parsons, S.P. Newman, S.K. Chander, G.M. Allan, H.R. Lawrence, N. Vicker, B.V. Potter, M.J. Reed, A. Purohit, 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer, *Int J Cancer*, 122 (2008) 1931-1940.

- [22] S. Marchais-Oberwinkler, C. Henn, G. Moller, T. Klein, M. Negri, A. Oster, A. Spadaro, R. Werth, M. Wetzel, K. Xu, M. Frotscher, R.W. Hartmann, J. Adamski, 17beta-Hydroxysteroid dehydrogenases (17beta-HSDs) as therapeutic targets: protein structures, functions, and recent progress in inhibitor development, *J Steroid Biochem Mol Biol*, 125 (2011) 66-82.
- [23] S. Starčević, P. Brožič, S. Turk, J. Cesar, T.L. Rižner, S. Gobec, Synthesis and biological evaluation of (6- and 7-phenyl) coumarin derivatives as selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1, *J Med Chem*, 54 (2011) 248-261.
- [24] G. Möller, B. Husen, D. Kowalik, L. Hirvelä, D. Plewczynski, L. Rychlewski, J. Messinger, H. Thole, J. Adamski, Species used for drug testing reveal different inhibition susceptibility for 17beta-hydroxysteroid dehydrogenase type 1, *PLoS One*, 5 (2010) e10969.
- [25] A. Azzi, P.H. Rehse, D.W. Zhu, R.L. Campbell, F. Labrie, S.X. Lin, Crystal structure of human estrogenic 17 beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol, *Nat Struct Biol*, 3 (1996) 665-668.
- [26] T. Klein, C. Henn, M. Negri, M. Frotscher, Structural basis for species specific inhibition of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): computational study and biological validation, *PLoS One*, 6 (2011) e22990.
- [27] A. Oster, S. Hinsberger, R. Werth, S. Marchais-Oberwinkler, M. Frotscher, R.W. Hartmann, Bicyclic substituted hydroxyphenylmethanones as novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases, *J Med Chem*, 53 (2010) 8176-8186.
- [28] A. Oster, T. Klein, C. Henn, R. Werth, S. Marchais-Oberwinkler, M. Frotscher, R.W. Hartmann, Bicyclic substituted hydroxyphenylmethanone type inhibitors of 17 beta-hydroxysteroid dehydrogenase Type 1 (17 beta-HSD1): the role of the bicyclic moiety, *ChemMedChem*, 6 (2011) 476-487.
- [29] A.S. Abdelsamie, E. Bey, N. Hanke, M. Empting, R.W. Hartmann, M. Frotscher, Inhibition of 17beta-HSD1: SAR of bicyclic substituted hydroxyphenylmethanones and discovery of new potent inhibitors with thioether linker, *Eur J Med Chem*, 82 (2014) 394-406.
- [30] E. Bey, S. Marchais-Oberwinkler, M. Negri, P. Kruchten, A. Oster, T. Klein, A. Spadaro, R. Werth, M. Frotscher, B. Birk, R.W. Hartmann, New insights into the SAR and binding modes of bis(hydroxyphenyl)thiophenes and -benzenes: influence of additional substituents on 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) inhibitory activity and selectivity, *J Med Chem*, 52 (2009) 6724-6743.
- [31] E. Bey, S. Marchais-Oberwinkler, R. Werth, M. Negri, Y.A. Al-Soud, P. Kruchten, A. Oster, M. Frotscher, B. Birk, R.W. Hartmann, Design, synthesis, biological evaluation and pharmacokinetics of bis(hydroxyphenyl) substituted azoles, thiophenes, benzenes, and aza-benzenes as potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1), *J Med Chem*, 51 (2008) 6725-6739.
- [32] M. Frotscher, E. Ziegler, S. Marchais-Oberwinkler, P. Kruchten, A. Neugebauer, L. Fetzter, C. Scherer, U. Muller-Vieira, J. Messinger, H. Thole, R.W. Hartmann, Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases, *J Med Chem*, 51 (2008) 2158-2169.
- [33] S. Marchais-Oberwinkler, M. Frotscher, E. Ziegler, R. Werth, P. Kruchten, J. Messinger, H. Thole, R.W. Hartmann, Structure-activity study in the class of 6-(3'-hydroxyphenyl)naphthalenes leading to an optimization of a pharmacophore model for 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) inhibitors, *Mol Cell Endocrinol*, 301 (2009) 205-211.
- [34] S. Marchais-Oberwinkler, P. Kruchten, M. Frotscher, E. Ziegler, A. Neugebauer, U. Bhoga, E. Bey, U. Muller-Vieira, J. Messinger, H. Thole, R.W. Hartmann, Substituted 6-phenyl-2-naphthols. Potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation, and pharmacokinetics, *J Med Chem*, 51 (2008) 4685-4698.
- [35] S. Marchais-Oberwinkler, M. Wetzel, E. Ziegler, P. Kruchten, R. Werth, C. Henn, R.W. Hartmann, M. Frotscher, New drug-like hydroxyphenylnaphthol steroidomimetics as potent and selective

17 β -hydroxysteroid dehydrogenase type 1 inhibitors for the treatment of estrogen-dependent diseases, *J Med Chem*, 54 (2010) 534-547.

[36] C. Henn, A. Einspanier, S. Marchais-Oberwinkler, M. Frotscher, R.W. Hartmann, Lead optimization of 17 β -HSD1 inhibitors of the (hydroxyphenyl)naphthol sulfonamide type for the treatment of endometriosis, *J Med Chem*, 55 (2012) 3307-3318.

[37] A. Spadaro, M. Frotscher, R.W. Hartmann, Optimization of hydroxybenzothiazoles as novel potent and selective inhibitors of 17 β -HSD1, *J Med Chem*, 55 (2012) 2469-2473.

[38] P. Miralinaghi, C. Schmitt, R.W. Hartmann, M. Frotscher, M. Engel, 6-Hydroxybenzothiophene Ketones: Potent Inhibitors of 17 β -Hydroxysteroid Dehydrogenase Type 1 (17 β -HSD1) Owing to Favorable Molecule Geometry and Conformational Preorganization, *ChemMedChem*, (2014) *In Press*.

[39] A.V. Razgulin, S. Mecozzi, Binding properties of aromatic carbon-bound fluorine, *J Med Chem*, 49 (2006) 7902-7906.

[40] H.J. Bohm, D. Banner, S. Bendels, M. Kansy, B. Kuhn, K. Muller, U. Obst-Sander, M. Stahl, Fluorine in medicinal chemistry, *Chembiochem*, 5 (2004) 637-643.

[41] K. Muller, C. Faeh, F. Diederich, Fluorine in pharmaceuticals: looking beyond intuition, *Science*, 317 (2007) 1881-1886.

[42] B.E. Fink, D.S. Mortensen, S.R. Stauffer, Z.D. Aron, J.A. Katzenellenbogen, Novel structural templates for estrogen-receptor ligands and prospects for combinatorial synthesis of estrogens, *Chem Biol*, 6 (1999) 205-219.

[43] N. Miyaura, T. Yanagi, A. Suzuki, The Palladium-Catalyzed Cross-Coupling Reaction of Phenylboronic Acid with Haloarenes in the Presence of Bases, *Synthetic Communications*, 11 (1981) 513-519.

[44] P. Kruchten, R. Werth, S. Marchais-Oberwinkler, M. Frotscher, R.W. Hartmann, Development of a biological screening system for the evaluation of highly active and selective 17 β -HSD1-inhibitors as potential therapeutic agents, *Mol Cell Endocrinol*, 301 (2009) 154-157.

[45] W. Qiu, R.L. Campbell, A. Gangloff, P. Dupuis, R.P. Boivin, M.R. Tremblay, D. Poirier, S.X. Lin, A concerted, rational design of type 1 17 β -hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity, *FASEB J*, 16 (2002) 1829-1831.

[46] K.M. Sam, S. Auger, V. Luu-The, D. Poirier, Steroidal spiro-gamma-lactones that inhibit 17 β -hydroxysteroid dehydrogenase activity in human placental microsomes, *J Med Chem*, 38 (1995) 4518-4528.

[47] H.M. Berman, T.N. Bhat, P.E. Bourne, Z. Feng, G. Gilliland, H. Weissig, J. Westbrook, The Protein Data Bank and the challenge of structural genomics, *Nat Struct Biol*, 7 Suppl (2000) 957-959.

[48] R. Breton, D. Housset, C. Mazza, J.C. Fontecilla-Camps, The structure of a complex of human 17 β -hydroxysteroid dehydrogenase with estradiol and NADP⁺ identifies two principal targets for the design of inhibitors, *Structure*, 4 (1996) 905-915.

[49] M. Mazumdar, D. Fournier, D.W. Zhu, C. Cadot, D. Poirier, S.X. Lin, Binary and ternary crystal structure analyses of a novel inhibitor with 17 β -HSD type 1: a lead compound for breast cancer therapy, *Biochem J*, 424 (2009) 357-366.

[50] K. Huhtinen, R. Desai, M. Stahle, A. Salminen, D.J. Handelsman, A. Perheentupa, M. Poutanen, Endometrial and endometriotic concentrations of estrone and estradiol are determined by local metabolism rather than circulating levels, *J Clin Endocrinol Metab*, 97 (2012) 4228-4235.

[51] Y. Chen, C. Chen, S. Shi, J. Han, J. Wang, J. Hu, Y. Liu, Z. Cai, C. Yu, Endometriotic implants regress in rat models treated with puerarin by decreasing estradiol level, *Reprod Sci*, 18 (2011) 886-891.

[52] R. Hartmann, M. Frotscher, S. Marchais-Oberwinkler, A. Oster, A. Spadaro, Preparation of heteroaryl phenyl ketones and amides as selective 17 β -hydroxysteroid dehydrogenase type 1 inhibitors, in, 2012, pp. 132pp.

Figure 1. Interconversion of Estrone (E1) and 17 β -Estradiol (E2).

Compound A

*h*17 β -HSD1: IC₅₀ = 13 nM
*m*17 β -HSD1: no inhibn @ 1 μ M

Compound B

*h*17 β -HSD1: IC₅₀ = 20 nM
*m*17 β -HSD1: no inhibn. @ 1 μ M

Compound C

*h*17 β -HSD1: IC₅₀ = 8 nM
*m*17 β -HSD1: 10 % inhibn. @ 1 μ M

Compound D

*h*17 β -HSD1: IC₅₀ = 21 nM
*m*17 β -HSD1: 16 % inhibn. @ 1 μ M

Compound E

*h*17 β -HSD1: IC₅₀ = 12 nM
*m*17 β -HSD1: 25 % inhibn. @ 1 μ M

Figure 2. Different in-house classes of highly potent 17 β -HSD1 inhibitors: Representative members and their activities against the human and the murine enzyme

Figure 3. Computational investigation of inhibitor-enzyme interactions. A) Overlay of the proposed binding mode of compound **33** (blue carbon; sticks) and the previously reported co-crystallised ligand E₂B (PDB-ID 3HB5; dark grey carbon; sticks) in complex with *h*17β-HSD1 (grey ribbon and transparent pocket surface) and NADP (yellow carbon; sticks). B) 2D depiction of ligand interactions for compound **33** with *h*17β-HSD1. Calculated distances (in Å) and strengths (in kcal·mol⁻¹) of main interactions are given. C) Close-up showing ring A of compound **33** and its main interactions (kcal·mol⁻¹) with *h*17β-HSD1 (grey). D) Close-up showing ring A of a hypothetical non-fluorinated derivative of compound **33** and its main interactions (kcal·mol⁻¹) with *h*17β-HSD1 (grey). E) Close-up showing ring A of compound **33** and its main interactions (kcal·mol⁻¹) with the homology model for *m*17β-HSD1 (cyan).

Chart 1. Design of Synthesised Compounds **1 – 38**.

Cpd	R ₄	Cpd	R ₄	Cpd	R ₄
3	H	16	4-NO ₂	25	3-NHCOMe
4	4-Br-2-OCF ₃	17	2,3-di-NO ₂	26	2-Me
5	3-CN	18	2-F	27	3-Me

6	H	19	3-F	28	2-F-4-OMe
7	4-Br-2-OCF ₃	20	4-F	29	2-F-4-OH
8	3-CN	21	4-Br	34
13	4-CN	22	4-Br- 2,5-di-F	35
14	2-NO ₂	23	3,5-di-Cl-4-OH	36
15	3-NO ₂	24	3-OMe		

Scheme 1: synthesis of compounds **1-9**, **13-30** and **34-36**. a) AlCl₃, anhydrous CH₂Cl₂, 0 °C, 0.5h and then rt, 3h. b) method A, BBr₃, CH₂Cl₂, -78°C to rt, overnight. c) Method B, 3-aminophenylboronic acid or 4-aminophenylboronic acid pinacol ester, Cs₂CO₃, Pd(PPh₃)₄, DME/water (1:1), reflux, 4h. d) Method C, pyridine, corresponding sulfonyl chloride, rt, overnight.

Scheme 2: synthesis of compounds **10-12** and **31-33**. a) Method C, pyridine, ArXCl, rt (reflux in case of $X = \text{CO}$), overnight. b) method A, BBr_3 , CH_2Cl_2 , -78°C to rt, overnight. c) NaH, DMF, MeI, 0°C to rt, 0.5h.

Scheme 3: synthesis of compounds **37** and **38**. a) Method C, pyridine, cyclopropanesulfonyl chloride, rt, overnight. b) bis(pinacolato)diboron, Pd(dppf)Cl₂, KOAc/DMSO, 2h. c) NaH, DMF, MeI, 0°C to rt, 0.5h. d) Method B, **IV**, Cs₂CO₃, Pd(PPh₃)₄, DME/water (1:1), reflux, 3 days.

Table 1. Inhibition of *human* 17 β -HSD1, *human* 17 β -HSD2 and *murine* 17 β -HSD1 by compounds **1-12**.

1, 2		3 - 8, 10		9		11-12
Cpd.	Ring C	R	<i>h</i> 17 β -HSD1 ^a	<i>h</i> 17 β -HSD2 ^b	SF ^d	<i>m</i> 17 β -HSD1 ^e
			IC ₅₀ [nM] ^c	IC ₅₀ [nM] ^c		% inh. @ 1 μ M ^c
1	-	4-NH ₂	145	120	0.8	n.d. ^f
2	-	3-NH ₂	77 % ^g	68 % ^g	-	n.i. ^h
3	4	H	195	459	2.3	n.d. ^f
4	4	4-Br-2-OCF ₃	169	716	4.2	n.d. ^f
5	4	3-CN	151	763	5.0	n.d. ^f
6	3	H	21	112	5.2	n.d. ^f
7	3	4-Br-2-OCF ₃	8	199	25	21
8	3	3-CN	23	171	7.5	n.d. ^f
9	-	-	39	114	3.0	n.d. ^f
10	3	3-OH	16	88	5.4	26
11	-	H	33	159	4.8	26
12	-	CH ₃	71	218	3.0	17

^a Human placenta, cytosolic fraction, substrate [³H]-E1, 500 nM, cofactor NADH, 500 μ M;

^b Human placenta, microsomal fraction, substrate [³H]-E2, 500 nM, cofactor NAD⁺, 1500 μ M; ^c Mean value of three determinations, standard deviation less than 15%; ^d SF = selectivity factor: *h*IC₅₀(17 β -HSD2) / *h*IC₅₀(17 β -HSD1); ^e Mouse recombinant enzyme, expressed in HEK 293, substrate [³H]-E1, 10 nM, cofactor NADH, 500 μ M; ^f n.d.: not determined; ^g Inhibition @ 1 μ M (inhibitor concentration); ^h n.i.: no inhibition.

Table 2 Inhibition of *human* 17 β -HSD1, *human* 17 β -HSD2 and *murine* 17 β -HSD1 by compounds **13-29**.

13-29

Cpd.	R	IC ₅₀ [nM] ^c		SF ^d	<i>m</i> 17 β -HSD1 ^e
		<i>h</i> 17 β -HSD1 ^a	<i>h</i> 17 β -HSD2 ^b		% inh.@1 μ M ^c
13	4-CN	56	392	7.0	8
14	2-NO ₂	32	148	4.6	n.d. ^f
15	3-NO ₂	47	130	2.7	n.d. ^f
16	4-NO ₂	42	320	7.7	n.d. ^f
17	2,3-di-NO ₂	182	322	1.8	n.d. ^f
18	2-F	50	116	2.3	n.d. ^f
19	3-F	38	185	4.9	n.d. ^f
20	4-F	30	83	2.7	25
21	4-Br	50	162	3.2	n.d. ^f
22	4-Br- 2,5-di-F	109	144	1.3	n.d. ^f
23	3,5-di-Cl-4-OH	135	126	0.9	16
24	3-OMe	35	130	3.7	n.d. ^f
25	3-NHCOMe	311	282	0.9	13
26	2-Me	17	104	6.1	51
27	3-Me	18	99	5.5	n.d. ^f
28	2-F-4-OMe	51	112	2.2	n.d. ^f
29	2-F-4-OH	112	215	1.9	n.d. ^f

^a Human placenta, cytosolic fraction, substrate [³H]-E1, 500 nM, cofactor

NADH, 500 μ M. ^b Human placenta, microsomal fraction, substrate [³H]-E2, 500 nM, cofactor NAD⁺, 1500 μ M. ^c Mean value of three determinations, standard deviation less than 15%. ^d SF = selectivity factor: $hIC_{50}(17\beta\text{-HSD2}) / hIC_{50}(17\beta\text{-HSD1})$. ^e Mouse recombinant enzyme, expressed in HEK 293, substrate [³H]-E1, 10 nM, cofactor NADH, 500 μ M. ^f n.d.: not determined.

Table 3. Inhibition of human, mouse, and rat 17 β -HSD1 and 17 β -HSD2 by compounds **30-38**.

<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>30</p> </div> <div style="text-align: center;"> <p>31-33</p> </div> <div style="text-align: center;"> <p>34-38</p> </div> </div>											
Cpd.	R ₁	R ₂	R ₃	IC ₅₀ [nM] ^c		SF ^d	inhibn.@1 μ M ^c		inhibn.@250nM ^c		cLogD pH 7.4
				<i>h</i> 17 β - HSD1 ^a	<i>h</i> 17 β - HSD2 ^b		<i>m</i> 17 β - HSD1 ^e	<i>m</i> 17 β - HSD2 ^f	<i>r</i> 17 β - HSD1 ^g	<i>r</i> 17 β - HSD2 ^h	
30	-	-	-	10	25	2.5	30%	n.d. ⁱ	51%	n.d. ⁱ	
31	4-Br-2- OCF ₃	-	-	2	17	8.5	71%	100 %	56%	86%	5.1
32	2-OCF ₃	-	-	3	13	4.3	62%	100%	80 nM ^j	38 nM ^j	4.7
33	2-CF ₃	-	-	2	6	3.0	250 nM ^j	20 nM ^j	97 nM ^j	49 nM ^j	4.8
34		H	H	4	3.7	0.9	47%	92%	32%	28%	2.6
35		H	H	6	2.7	0.4	10%	47%	29%	31%	2.5
36		H	H	2	6.5	3.2	76%	100%	51%	49%	3.3
37		CH ₃	H	5	8	1.6	600 nM ^j	55 nM ^j	61%	78%	3.8
38		CH ₃	CH ₃	5	20	4.0	510 nM ^j	129 nM ^j	48%	56%	4.0

^a Human placenta, cytosolic fraction, substrate [³H]-E1, 500 nM, cofactor NADH, 500 μM; ^b Human placenta, microsomal fraction, substrate [³H]-E2, 500 nM, cofactor NAD⁺, 1500 μM; ^c Mean value of three determinations, standard deviation less than 15%; ^d SF = selectivity factor: *hIC*₅₀(17β-HSD2) / *hIC*₅₀(17β-HSD1). ^e Mouse recombinant enzyme, expressed in HEK 293, substrate [³H]-E1, 10 nM, cofactor NADH, 500 μM; ^f Mouse liver, microsomal fraction, substrate [³H]-E2, 10 nM, cofactor NAD⁺, 1500 μM; ^g Rat recombinant enzyme, expressed in HEK 293, substrate [³H]-E1, 10 nM, cofactor NADH, 500 μM; ^h Rat liver, microsomal fraction, substrate [³H]-E2, 10 nM, cofactor NAD⁺, 1500 μM; ⁱ n.d. not determined; ^j IC₅₀-value for compounds showing ≥ 80 % inhibition of 17β-HSD1 of the respective species.

Graphical Abstract