Structure-Activity Study in the Class of 6-(3'-Hydroxyphenyl)naphthalenes Leading to an Optimization of a Pharmacophore Model for 17 β -Hydroxysteroid Dehydrogenase Type 1 (17 β -HSD1) Inhibitors

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Structure-Activity Study in the Class of 6-(3'-Hydroxyphenyl)naphthalenes Leading to an Optimization of a Pharmacophore Model for 17 β -Hydroxysteroid Dehydrogenase Type 1 (17 β -HSD1) Inhibitors.

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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; ER, estrogen receptor; PDB-ID: protein data bank identification code; RBA, relative binding affinity; SAR, structure activity relationship.

Keywords: 17 β -HSD1; 17 β -HSD2; non-steroidal inhibitor; ER; estrogen-dependent diseases; pharmacophore model

Abstract

17 β -Hydroxysteroid dehydrogenase type 1 (17 β -HSD1) catalyzes the transformation of estrone (E1) into the most potent estrogen, estradiol (E2), which stimulates cell proliferation and decreases apoptosis. 17 β -HSD1 is often strongly overexpressed in estrogen-dependent diseases (like breast cancer and endometriosis). Thus, this enzyme is a promising novel target for the development of selective inhibitors, which could be used as drugs for the treatment of these diseases.

Using a structure- and ligand-based approach, a pharmacophore model was proposed and a new class of non-steroidal inhibitors of 17 β -HSD1 was designed. Enzyme inhibition was evaluated *in vitro* using the human enzyme. After identification of the 6-(3'-hydroxyphenyl)-2-naphthol scaffold **1**, the potency of this class of inhibitors was further improved by substitution of the 1 position of the naphthalene ring by a phenyl group (compound **18**, IC₅₀= 20 nM). Compound **18** also showed a good selectivity toward 17 β -HSD2 and the estrogen receptors α and β .

1. Introduction

Estrogens, especially estradiol (E2), the most potent member of this family, have many important physiological activities being for example involved in the development and function of the female reproduction system and maintenance of bone mineral density. However, they are also known to have a major impact on the development of hormone-dependent diseases like breast cancer and endometriosis (Dizerega et al., 1980; Travis et al., 2003).

The highly active E2 is formed in the target tissues after reduction of the less potent estrone (E1) by the enzyme 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1). It has been suggested that 17 β -HSD1 plays an important role in the *in situ* regulation of estrogens in hormone-dependent diseases (Sasano et al., 1996; Suzuki et al. 2000, Vihko et al., 2004). In addition, in primates this enzyme is often over expressed in breast cancer (Suzuki et al., 2000; Gunnarsson et al., 2001; Miyoshi et al., 2001; Gunnarsson et al., 2005) and endometriotic tissues (Šmuc et al., 2007), resulting in high intracellular E2 concentrations. 17 β -HSD1 is therefore an attractive target for the development of inhibitors for the treatment of estrogen-dependent diseases.

17 β -HSD1 is a soluble cytosolic enzyme, which belongs to the SDR superfamily. It is predominantly expressed in human ovaries, breast tissue and placenta. The main function of 17 β -HSD1 is the NADPH-dependent reduction of estrogens (E1 to E2, last step of E2 biosynthesis) and to a minor extent the reduction of androgens (dehydroepiandrosterone to 5-androstene-3 β ,17 β -diol).

Recently, several groups have reported about 17 β -HSD1 inhibitors, most of them having a steroidal structure (Poirier, 2003; Brožič et al., 2007). Recently, a special interest appeared for non-steroidal inhibitors (Messinger et al., 2006; Allan et al., 2008; Bey et al., 2008; Frotscher et al., 2008).

The work described in the following focuses on 6-(3'-hydroxyphenyl)-2-naphthols as a new class of potent and selective non-steroidal inhibitors of 17 β -HSD1. A pharmacophore model, the search for the most appropriate scaffold as well as the investigation of the best position for introduction of substituents will be presented.

2. Investigation of the core structure

The active site of 17 β -HSD1 has been studied by crystallisation (16 crystal structures of 17 β -HSD1 with different ligands are available) and mutagenesis. It is a narrow hydrophobic tunnel with two polar amino acids at each end (His221/Glu282 on one side

and Ser142/Tyr155 on the other side). In the X-ray structure of the ternary complex (enzyme, steroid and cofactor, PDB-ID (Berman et al., 2000): 1FDT), there are surprisingly two polar amino acids, Tyr218 and Ser222, located close to the hydrophobic B/C ring region of the steroid.

E1 is the favourite substrate of 17 β -HSD1. Important structural features are the hydroxyphenyl moiety (A ring) as well as the 17-ketone group at the D ring. These two polar moieties are separated by an hydrophobic central core (B/C rings).

Based on the X-ray structure analysis of the enzyme active site (PDB-ID: 1FDT), on the structure study of its natural ligand E1 and on SAR studies of known inhibitors (Deluca et al., 2006; Fischer et al., 2005), we propose a pharmacophore model (Chart 1) for non-steroidal inhibitors of 17 β -HSD1. It contains two polar regions A and B, which can be hydrogen bond donors or acceptors, mimicking the steroid-O3 and O-17, respectively. A distance of about 11 Å between A and B, as observed in the steroid, should be optimum. Another polar group, C, could also be considered for interaction with the two polar amino acids Tyr218 and Ser222 identified in the X-ray structure. These three regions A, B and C should be connected to a non polar core. A substituent R could be introduced on the hydrophobic scaffold to increase the activity by additional interactions.

Chart 1

Following the pharmacophore model, we designed hydroxyphenylnaphthol derivatives (Chart 2) as they fulfilled the requirements. They were synthesized (Frotscher et al., 2008; Marchais-Oberwinkler et al., 2008) varying the number and the position of the hydroxy substituents and tested. The inhibition assay of 17 β -HSD1 was performed according to previously described procedures (Lin et al., 1992; Sam et al., 1995, Sam et al., 1998). The biological activities of the synthesized compounds are shown in Table 1.

Table 1

The dihydroxylated compound **1**, bearing one hydroxy group in position 1 of the naphthalene ring and a second hydroxy group in position 4' of the phenyl moiety is a weak inhibitor of 17 β -HSD1 (55 % inhibition at 1 μ M, no inhibition at 100 nM). Moving the 1-hydroxy group to the 2 position on the naphthalene (compound **2**) resulted in a complete loss of activity. The distance between the two hydroxy groups (about 13 Å) is certainly too large compared to the steroid. Shifting the 4'-hydroxy group to the 3' position on the phenyl (compound **3**), resulted in a highly potent inhibitor of 17 β -HSD1 (91 % inhibition at 100 nM). This is remarkable as the distance between the two hydroxy

groups is identical to the one in compound **1**. These results show an exceptionally sharp SAR, which might be due to a low flexibility of the enzyme active site.

In order to find out about the importance of each hydroxy function present in compound **3**, mono hydroxylated derivatives of **3** were synthesized. Taking away the hydroxy moiety at the naphthalene core decreased the activity dramatically (compound **4**, 26% inhibition at 100 nM versus 91% for the dihydroxylated **3**). Removing the hydroxy moiety at the phenyl group (compound **5**) led to a complete loss of activity. From this observation, it is obvious that both hydroxy functions are necessary for high activity, the phenolic OH-group being more important than the OH on the naphthalene moiety. The two hydroxy groups are most likely involved in the stabilization of the molecule in the active site, establishing H-bond interactions with His221/Glu282 on the one side and with Ser142/Tyr155 on the other side.

Starting from the optimized substitution pattern identified in compound **3**, a nitrogen atom was introduced in the naphthalene ring to establish further H-bond interactions with the two polar amino acids (Tyr218 and Ser222) present in the active site next to the B/C ring of the steroid, leading to quinolines **6** and **7**. In both cases, a dramatic decrease of activity was observed compared to the naphthalene **3** (24 and 14% inhibition at 100 nM, for **6** and **7**, respectively, versus 91% for **3**), indicating that no H-bond interactions may be formed as expected or that the basicity inherent to the nitrogen may not be well tolerated in this area. In terms of enzyme inhibition, the quinoline is suitable to only a limited extent to replace the naphthalene. The quinoline **6** was docked into the active site of the enzyme to obtain a better insight in these results: it was evident that the OH of Tyr218 was too far away to establish H-bond interactions (4.6 Å). Due to the flexibility of the side chain of Ser222, its OH group very likely can adopt different conformations, the oxygen being located at a distance varying between 3.5 and 5.6 Å to the nitrogen of quinoline **6** (data not shown). A molecular dynamic study was performed to find out the preferred position of this side chain. The minimum distance between OH of Ser222 and the N-quinoline was chosen as starting geometry. After a short time, the CH₂-OH rotates away from the ligand, making it unavailable for H-bond interactions for the rest of the simulation time of 500 ps.

The polar area C introduced in the pharmacophore model leads to a decrease in activity and should not be further considered. The hydrophobic naphthalene ring is therefore the best central scaffold identified in this study. The most potent compound **3**

can be look upon as a core structure for introduction of substituents in order to increase activity.

3. Substitution of the core structure

Monosubstitutions of compound **3** were performed in the phenyl ring, investigating positions 4' (compound **8**) and 5' (compounds **9-12**). A selection of several groups with different properties concerning size, H-bonding capability, lipophilicity and flexibility was introduced on the phenyl group. They all led to inactive compounds except for compound **9** substituted with a methyl in the 5' position (42% inhibition at 1 μ M, Table 2). Obviously substitution at the phenyl ring is not tolerated by the enzyme. A lack of space might be responsible for this decrease or loss in activity.

Table 2

Furthermore, the naphthalene ring of compound **3** was substituted in positions 1 (compounds **13-18**), 3 (compound **19**), 4 (compound **20**) and 8 (compound **21**). The structures and biological activities are shown in Table 2. Amide groups (compounds **13** and **14**) and a sulfone (compound **16**) were introduced in position 1 of the naphthalene in an attempt to establish H-bond contacts with the two polar amino acids Tyr218 or Ser222. A decrease of activity was observed when the amide moiety was directly linked to the scaffold (22% and 40% inhibition at 100 nM for **13** and **14**, respectively, versus 91% for the unsubstituted **1**) as well as when the amide group was attached via a flexible spacer (compound **15**, no inhibition at 100 nM). From this, it can be concluded that H-bond interactions with polar amino acids are most likely not achieved. On the other hand, introduction of a bromine (compound **17**) or a phenyl (compound **18**) led to highly active compounds, being as active as **3** (83% and 76% inhibition for **17** and **18**, respectively). Compound **19** substituted in position 3 of the naphthalene with an amide group and compound **21** bearing a cyano group in position 8 turned out to be inactive at a concentration of 100 nM. In contrary, introduction of a nitrile group in position 4 led to the highly active **20** (73% inhibition at 100 nM). In order to compare the potency of the most active compounds (**3**, **17**, **18** and **20**), IC_{50} values were determined (Table 3). Compound **18**, substituted in position 1 with a phenyl group turned out to be the most potent inhibitor of this class of compounds with an IC_{50} of 20 nM (against 116 nM for **3**, 40 nM for **17** and 39 nM for **20**). Substitution of the naphthalene moiety is well tolerated in position 1 and 4, position 1 offering a large space for introduction of big substituents.

In order to get an insight in the oral bioavailability of the most potent compounds, derivatives **3**, **18** and **20** were investigated using the Caco-2 cell line. These cells exhibit morphological and physiological properties of the human small intestine (Hidalgo, 2001) and are generally accepted to be an appropriate model for the prediction of peroral absorption. Depending on the P_{app} data obtained, compounds can be classified as low (P_{app} (10^{-6} cm/sec) < 1), medium ($1 < P_{app} < 10$) or highly permeable ($P_{app} > 10$). Compounds **3**, **18** and **20** showed medium cell permeation (P_{app} = $8.9 \cdot 10^{-6}$ cm/sec for **3**, $9.3 \cdot 10^{-6}$ cm/sec for **18** and $4.4 \cdot 10^{-6}$ cm/sec for **20**) indicating that these compounds are able to penetrate the cell membranes, they should show a reasonable oral bioavailability.

All the compounds described so far present a rather flat geometry (naphthalene and phenyl rings being almost in the same plane), similar to the steroid. In order to test the impact of this planarity on activity, a non planar disubstituted compound was synthesized, compound **22**, bearing in addition to the two hydroxy moieties a methyl in 2' position of the phenyl ring and a bromine in the 1 position of the naphthalene ring (Table 2). A decrease in activity for **22** (15% inhibition at 100 nM compared to 83% for compound **17** without 2'-Me) was observed indicating that the flatness of the molecule is an important prerequisite for high activity. It might be interpreted that the 3'-hydroxy group of **22** is located in another region of the active site compared to **3**, not allowing H-bond interactions with His221/Glu282 or Ser142/Tyr155. This result again can be explained by a reduced flexibility of the enzyme active site. It could also be hypothesized that the 2'-methyl group is disfavoured in this region of the enzyme as an hydrophobic group is not well tolerated.

The additional substituent R, described in the pharmacophore model, increases the activity of the inhibitor if it is located in position 1 or 4 of the naphthalene ring. Position 1 offers space for a large group, the 1-phenyl derivative **18** being the most potent compound identified in this series of inhibitors.

4. Selectivity

To assess the selectivity of the most interesting compounds, inhibition of 17 β -HSD2 and affinity to the estrogen receptors α and β were determined.

Since 17 β -HSD2 catalyses the oxidative transformation of E2 into E1, thus "inactivating" E2, inhibition of this enzyme is not desired for the treatment of estrogen-dependent diseases. The inhibition assay of 17 β -HSD2 was performed according to a procedure similar to the 17 β -HSD1 inhibition assay. IC₅₀ values for 17 β -HSD1 and 17 β -

HSD2 as well as selectivity factors were calculated for selected compounds (Table 3). The 1-substituted compounds **17**, **18** and **20**, the most active compounds identified in this report (IC_{50} = 40, 20 and 39 nM for **17**, **18** and **20**, respectively) also show reasonable selectivity toward 17 β -HSD2 (selectivity factor of 16, 27 and 12, respectively, the IC_{50} of compound **20** for 17 β -HSD2 being 473 nM) while the non-substituted compound **3** is less potent (116 nM) but more selective toward the type 2 enzyme (selectivity factor: 48). Surprisingly, compound **1**, structurally similar to compound **3**, is more selective for 17 β -HSD2 (IC_{50} = 302 nM).

Table 3

As inhibitors of 17 β -HSD1 should have low or no affinity for the estrogen receptors α and β (ER α and β) to avoid their stimulation counteracting the therapeutic concept of 17 β -HSD1 inhibition, binding affinities were determined for the most interesting compounds. The binding affinity of compounds **1**, **3**, **17**, **18** and **20** to the ER α and ER β was determined according to a procedure described by Zimmermann et al. (Zimmermann et al., 2005) using recombinant human proteins. Compounds **17** and **18** turned out to be the most selective derivatives showing very little affinity to the ERs (Table 3) and reasonable selectivity toward 17 β -HSD2.

5. Discussion and conclusion

The short SAR study performed highlights the reduced flexibility of the enzyme active site. Compound **3** presents the best geometry. Any changes concerning the position of the hydroxy groups led to a complete loss of activity (in case of **1** and **2**, when the OHs are located into the same plane) or a dramatic drop of activity (for **22**, when one OH is moved in another plane). The enzyme active site might not be able to adjust its geometry to the inhibitors.

The binding of the inhibitors is stabilized in the active site by hydrophobic interactions (between Met147, Leu149, Pro187, Val225 and the phenylnaphthalene scaffold) and hydrogen bond interactions (between His221/Glu282 and Ser142/Tyr155 and the hydroxy groups). Previous docking studies of the phytoestrogens coumestrol **23** and genistein **24** (Hoffrén et al., 2001) in the active site of 17 β -HSD1 revealed Tyr218 as a possible partner for H-bond interaction (with the oxygen present in the rings of **23** and **24**). The scaffolds of compound **6**, coumestrol **23** and genistein **24** are closely related (Chart 3): they mainly differ in the nature of the heteroatom in the ring (nitrogen in case of the quinoline **6** and oxygen in case of the isoflavone **24** and **23**). It was therefore

expected that the quinoline **6** could also interact with the polar amino acids Tyr218 or Ser222. From the biological results obtained and the modelling studies performed, it is unlikely that a H-bond is formed between Tyr218 and the nitrogen of **6** or **7**. In addition, very recently, a computationally generated pharmacophore model of 17 β -HSD1 was reported (Schuster et al., 2008), the Tyr218 is therein not mentioned as partner for potential interactions. It is striking that these two polar amino acids, which do not interact with the steroid, are present in the active site. However, they might be involved in the stabilization of the three dimensional structure of the protein and thus might not be free to establish another interaction.

Within the class of the phytoestrogens, coumestrol **23** and genistein **24** are the most potent inhibitors of 17 β -HSD1 (IC_{50} = 0.2 μ M for **23** (Poirier, 2003) and 2.2 μ M for **24** (Schuster et al., 2008)). Compound **6** with an IC_{50} of 2.5 μ M, is equally potent to genistein **24** but it is less potent than the naphthalene **3** (IC_{50} = 116 nM). The equipotency of compounds **6** and **24** is certainly due to their structural similarities. Coumestrol **23** differs from **6** by the presence of a fused furan moiety and the displacement of the carbonyl group. These changes might have a positive impact on the electronic distribution on the whole molecule and increase its activity.

Chart 3

Furthermore, our results indicate that position 1 of the naphthalene ring is appropriate for the introduction of large substituents and that aromatic groups are better tolerated than amides or sulfone.

This study also improves our pharmacophore model: A non polar scaffold having a phenolic group and a hydroxy moiety as well as an aromatic substituent are the most important features for high activity. The third polar group C (realized by the N) is not well tolerated. The introduction of an additional substituent R only improves the activity when it is located on the naphthalene core, in position 1 or 4, whereas it is detrimental for activity when present on the phenyl ring. The space available in position 1 for the substituent is quite large and a group in this position capable to establish π - π stacking interactions seems to be appropriate. A pharmacophore model, taking these observations into consideration, is depicted in Chart 4. It can serve as basis for the design of new potent 17 β -HSD1 inhibitors.

Chart 4

In this report, we described the investigation of a new class of potent inhibitors of 17 β -HSD1, the 6-(3'-hydroxyphenyl)-2-naphthols. At first the optimized positions for OH-

groups were identified and in a second step the best position for the introduction of a substituent was determined. A new, potent and selective 17 β -HSD1 inhibitor, compound **18**, was discovered. It contains a phenyl group at the 1 position of the naphthalene. It is highly selective toward 17 β -HSD2 and the ERs α and β . It can be used in an *in vivo* disease-oriented model to further validate the concept of 17 β -HSD1 being a promising target for the treatment of estrogen-dependent diseases.

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Chart 1: Pharmacophore model

Chart 2: Designed compounds

Chart 3: Structure comparison of compound **6**, coumestrol **23** and genistein **24**

Chart 4: Revised pharmacophore model

Table 1: Inhibition of human 17 β -HSD1 by compounds **1-7**

Table 2: Inhibition of human 17 β -HSD1 by compounds **8-22**

Table 3: IC₅₀ values and selectivity data for selected compounds

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Chart 1: Pharmacophore model

A: Hydrogen bond donor or acceptor mimicking steroid-O3
 B: Hydrogen bond donor or acceptor mimicking steroid-O17
 C: Polar group for interaction with Tyr218 and Ser222
 R: Additional substituent
 A-B, A-C, B-C: distance about 11, 5 and 7 Å, respectively

Chart 2: Designed compounds

R1: H, CONHR, CH₂CH₂CONHR, Br, Ph
 R2: H, CH₃, CONHR, CH₂CH₂CONHR
 X: CH, N

Chart 3: Structure comparison of compound **6**, coumestrol **23** and genistein **24** **Chart 4:** Revised pharmacophore model

Table 1: Inhibition of human 17 β -HSD1 by compounds **1-7**

Compound	Structure	% Inhibition ^a 17 β -HSD1	
		100 nM	1 μ M
1		n.i.	55
2		n.i.	n.i.
3		91	94
4		26	61
5		n.i.	n.i.
6		24	57
7		14	63

^a Recombinant human 17 β -HSD1, substrate [³H]-E1 [30 nM], NADPH [1 mM]; mean value of 2 determinations, relative standard deviation <20%, Procedure A; n.i. = no inhibition (inhibition <10%).

Table 2: Inhibition of human 17 β -HSD1 by compounds **8-22**

Compd	R1	R2	% Inhibition ^a 17 β -HSD1	
			100 nM	1 μ M
8	H	4'-NHCOMe	n.i.	n.i.
9	H	5'-Me	14	42
10	H	5'-(<i>p</i> OH)Ph	n.i.	n.i.
11	H	5'-CONHMe	n.i.	n.i.
12	H	5'-CH ₂ CH ₂ CONHMe	n.i.	n.i.
13	1-CONHMe	H	22	76
14	1-CONHPh	H	40	80
15	1-CH ₂ CH ₂ CONHMe	H	n.i.	80
16	1-SO ₂ Ph	H	n.d.	33 ^b
17	1-Br	H	83	88
18	1-Ph	H	76	89
19	3-CONHMe	H	n.i.	18
20	4-CN	H	73 ^b	99 ^b
21	8-CN	H	n.i.	53 ^b
22	1-Br	2'-Me	15 ^b	53 ^b

^a Recombinant human 17 β -HSD1, substrate [³H]-E1 [30 nM], NADPH [1 mM]; mean value of 2 determinations, relative standard deviation <20% Procedure A, ^b Human placental cytosolic 17 β -HSD1, substrate [³H]-E1 [500 nM], NADH [500 μ M], mean value of 3 determinations, relative standard deviation <10% except for **16**: 17%, Procedure B; n.i. = no inhibition (inhibition <10%), n.d. = not determined.

Table 3: IC₅₀ values and selectivity data for selected compounds

Compd	Structure	IC ₅₀ (nM) ^a		Selectivity factor ^d	RBA ^e	
		17β-HSD1 ^b	17β-HSD2 ^c		ERα	ERβ
1		2425	302	0.1	5	5
3		116	5641	48	0.1<RBA<1	0.1<RBA<1
17		40	639	16	0.01<RBA<0.1	0.01<RBA<0.1
18		20	540	27	0.01<RBA<0.1	0.01<RBA<0.1

^a Mean value of 3 determinations, relative standard deviation <20%, ^b Human placental cytosolic 17β-HSD1, substrate [³H]-E1 [500 nM], NADH [500 μM] Procedure B, ^c Human placental microsomal 17β-HSD2, substrate [³H]-E2 [500 nM], NAD⁺ [1500 μM] Procedure C, ^d IC₅₀ (17β-HSD2)/ IC₅₀ (17β-HSD1), ^e RBA: Relative Binding Affinity, E2: 100%.