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Review

17β-Hydroxysteroid dehydrogenases (17β-HSDs) as therapeutic targets: Protein structures, functions, and recent progress in inhibitor development

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are oxidoreductases, which play a key role in estrogen and androgen steroid metabolism by catalyzing final steps of the steroid biosynthesis. Up to now, 14 different subtypes have been identified in mammals, which catalyze NAD(P)H or NAD(P)$^+$ dependent reductions/oxidations at the 17-position of the steroid. Depending on their reductive or oxidative activities, they modulate the intracellular concentration of inactive and active steroids. As the genomic mechanism of steroid action involves binding to a steroid nuclear receptor, 17β-HSDs act like pre-receptor molecular switches. 17β-HSDs are thus key enzymes implicated in the different functions of the reproductive tissues in both males and females. The crucial role of estrogens and androgens in the genesis and development of hormone dependent diseases is well recognized. Considering the pivotal role of 17β-HSDs in steroid hormone modulation and their substrate specificity, these proteins are promising therapeutic targets for diseases like breast cancer, endometriosis, osteoporosis, and prostate cancer. The selective inhibition of the concerned enzymes might provide an effective treatment and a good alternative to the existing endocrine therapies. Herein, we give an overview of functional and structural aspects for the different 17β-HSDs. We focus on steroidal and non-steroidal inhibitors recently published for each subtype and report on existing animal models for the different 17β-HSDs and the respective diseases.

Article from the Special issue on Targeted Inhibitors.

Contents

Abbreviations: HSD, hydroxysteroid dehydrogenase; E1, estrone; E2, estradiol; T, testosterone; PGF_2, prostaglandine F_2; SDR, short chain dehydrogenase/reductase; AKR, aldo/keto-reductase; TIM, triosephosphate isomerase; COF, cofactor binding site; SUB, substrate binding site; QSAR, quantitative structure activity relationship; ER, estrogen receptor; MEP, molecular electrostatic potential; MD, molecular dynamic; TG, transgenic; Aβ, beta-amyloid; AD, Alzheimer's disease.

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1. Introduction

Hydroxysteroid dehydrogenases (HSDs) belong to the NADPH/NAD$^+$-dependent oxidoreductases, which interconvert ketones and the corresponding secondary alcohols. As their names imply, they regio- and stereo-selectively catalyze the oxidoreduction in different positions of steroidal substrates (3α-, 3β-, 11β-, 17β-, 20α- and 20β-position). The steroid-converting HSDs play central roles in the biosynthesis and inactivation of steroid hormones, but some of them are also involved in the metabolism of diverse non-steroidal compounds [1–3].

In the classical view, hormones are synthesized and excreted by endocrine glands and act on target cells or target tissues via a receptor mechanism after being transported by the circulatory system ("endocrinology"). In 1988, Labrie et al. [4] introduced a new term called "intracrinology" for describing the fact that "locally produced androgens and/or estrogens exert their action in the cells where synthesis took place without release in the extracellular space" [5].

The HSDs are integral parts of systemic (endocrine) and local (intracrine) mechanisms. In target tissues, they convert inactive steroid hormones to their corresponding active forms and *vice versa*, thus modulating the transactivation of steroid hormone receptors [6,7] or other elements of the non-genomic signal transduction pathways [8,9]. Therefore, HSDs act as molecular switches allowing for pre-receptor modulation of steroid hormone action [10,11].

Exploiting the idea of the molecular switch concept promising therapeutic approaches become feasible, *i.e.* the blockade of specific steroidogenic enzymes by potent and selective inhibitors. This has been successfully realized for non-HSD-enzymes, which also influence the concentrations of steroid hormones locally; examples are the inhibition of aromatase [12–16] (in postmenopausal women) and 5α-reductase [17–22], key enzymes of the estrogen and androgen biosynthesis, respectively.

The present review focuses on 17β-HSDs, which gained major interest as potential drug targets for the treatment of sex steroid hormone-related diseases in recent years. On the one hand this is due to their tissue specificity. On the other, 17β-HSDs often catalyze the final step in steroid hormone biosynthesis. Therefore, selective inhibition should result in fewer side effects compared to inhibition of preceding steps. Furthermore, the rational design of selective inhibitors is facilitated as particular 17β-HSDs have been identified and characterized. Moreover, 3D-structures of important members are available.

Most 17β-HSDs belong to the short chain dehydrogenase/reductase (SDR) protein family [23]. The only exception known so far is 17β-HSD5 (AKR1C3) which belongs to the aldo-ketoreductases (AKR) [24]. In addition to a structured nomenclature for AKRs, the international SDR-initiative recently proposed a new gene-based nomenclature for enzymes of the SDR-family [25]. The first number indicates one of the 48 families, the following letter stands for the SDR type (C, E, I, D, X, A) and the last number notifies the members of the neighboring genes present in that type. The two main types are "classical" (C) and "extended" (E) differing in subunit size and sequence patterns. The identities of 17β-HSDs, the nomenclature according to SDR-Initiative and their gene assignments are given in Table 1. In this review, we will follow the old nomenclature for the sake of clarity.

2. Functional aspects of 17β-HSDs

2.1. 17β-HSD subtypes and multifunctionality

To date, fourteen different mammalian 17β-HSDs have been identified [26–28] and twelve subtypes have been found in human tissues [26]. They differ in tissue distribution [26], sub-cellular localization, and catalytic preference (oxidation or reduction using the cofactor NAD(H) and NADP(H), respectively).

In vitro all 17β-HSD subtypes are able to transform steroid hormones. Actually, the name 17β-HSD has been routinely assigned to many enzymes that convert 17-ketosteroids to 17-hydroxysteroids without attention to what their major substrates may be. In fact, *in vivo*17β-HSDs have diverse substrate specificities as they also catalyze the conversions of other substrates than steroids as for example lipids or retinoids. Until recently, besides 17β-HSD3 and

Table 1
Human 17β-hydroxysteroid dehydrogenases.

Type	Gene	SDR or AKR nomenclature	Function	Disease-association	References
1	HSD17B1	SDR28C1	Steroid (estrogen) synthesis	Breast and prostate cancer, endometriosis	[129,130]
2	HSD17B2	SDR9C2	Steroid (estrogen, androgen, progestin) inactivation	Breast and prostate cancer, endometriosis	[129,130]
3	HSD17B3	SDR12C2	Steroid (androgen) synthesis	Pseudohermaphroditism in males associated with obesity	[131]
4	HSD17B4	SDR8C1	Fatty acid β-oxidation, steroid (estrogen, androgen) inactivation	D-specific bifunctional protein-deficiency, prostate cancer	[132]
5	AKR1C3	AKR1C3	Steroid (androgen, estrogen, prostaglandin) synthesis	Breast and prostate cancer	[133,134]
6	HSD17B6	SDR9C6	Retinoid metabolism, 3α-3β-epimerase, steroid (androgen) inactivation?		[135]
7	HSD17B7	SDR37C1	Cholesterol biosynthesis, steroid (estrogen) synthesis	Breast cancer	[23,136]
8	HSD17B8	SDR30C1	Fatty acid elongation, steroid inactivation, estrogens, androgens	Polycystic kidney disease	[137,138]
9	HSD17B9	SDR9C5-2	Retinoid metabolism		[139]
10	HSD17B10	SDR5C1	Isoleucine, fatty acid, bile acid metabolism, steroid (estrogen, androgen) inactivation	X-linked mental retardation MHBD deficiency Alzheimer's disease	[140] [141]
11	HSD17B11	SDR16C2	Steroid (estrogen, androgen) inactivation, lipid metabolism?		[142]
12	HSD17B12	SDR12C1	Fatty acid elongation, steroid (estrogen) synthesis		[118,143]
13	HSD17B13	SDR16C3	Not demonstrated		[144]
14	HSD17B14	SDR47C1	Steroid (estrogen, androgen?) inactivation, fatty acid metabolism	Breast cancer, prognostic marker	[29,145]

17β-HSD14, 17β-HSD1 and 2 were thought to be exclusively converting sex steroids (Fig. 1) [26,29]. However, new results suggest the participation of the two latter enzymes also in retinoic acid metabolism [30,31]. Indeed, recombinant human 17β-HSD1 converts *all-trans* retinal to retinol with a catalytic efficiency only a few-fold lower than photoreceptor-associated retinol dehydrogenase (prRDH) [30]. Other 17β-HSD types were already known to be multifunctional and some of them play important roles in different metabolic pathways [26,32]. 17β-HSD7 is mainly involved in cholesterol synthesis, 17β-HSD4 is implicated in β-oxidation of fatty acids, 17β-HSD5 participates in both prostaglandin and steroid metabolism, and 17β-HSD12 is required in fatty acid elongation. 17β-HSD10 catalyzes the oxidation of short chain fatty acids. 17β-HSD6 and 9 play a role in retinoid conversion. For some 17β-HSDs the physiological function is not yet clear (Table 1).

2.2. 17β-HSDs as potential drug targets

For several types of 17β-HSDs their participation in the pathophysiology of human diseases has been postulated. Additionally, for some of them, their expression level can be used as prognostic marker in breast or prostate cancer (Table 1). The specificity of each 17β-HSD subtype for a preferred substrate together with distinct tissue localization, suggests that inhibition of these enzymes might be a good approach for the treatment of steroid dependent diseases.

2.2.1. 17β-HSD1

17β-HSD1 catalyzes the activation of estrone (E1) to the most potent estrogen estradiol (E2), which is known to have a crucial role in the development of estrogen-dependent diseases (Fig. 1). An increased E2/E1 ratio as well as high levels of 17β-HSD1 mRNA point out the pivotal role of 17β-HSD1 in breast cancer [33,34], ovarian tumor [35], endometriosis [36], endometrial hyperplasia [37], and uterine leiomyoma [38]. Consequently, inhibition of 17β-HSD1 is considered as a valuable therapeutic approach for their treatment. Since 17β-HSD1 and 2 may have opposing action, 17β-HSD1 inhibitors must be screened for selectivity toward 17β-HSD2.

2.2.2. 17β-HSD2

Osteoporosis occurs in elderly people when the level of active sex steroids decreases. Estrogen replacement therapy is beneficial for the treatment of osteoporosis [39,40], but it is no longer recommended because of adverse effects (breast, endometrial and ovarian cancers, stroke, thromboembolism). Since 17β-HSD2 oxidizes E2 into E1 (Fig. 1), decreasing the amount of E2 in bone cells, inhibition of this enzyme [41] is a promising approach for the treatment of this disease.

2.2.3. 17β-HSD3

17β-HSD3 activates Δ4-androstene-3,17-dione into testosterone (T) (Fig. 1). The enzyme is present exclusively in the testis and its mRNA is over-expressed in prostate cancer tissues. As T is known to be responsible for cell proliferation in androgen dependent diseases [42], 17β-HSD3 inhibitors – exerting effects equivalent of chemical castration – could be therapeutics for the treatment of diseases like prostate cancer.

2.2.4. 17β-HSD5

17β-HSD5 is up-regulated in breast and prostate cancer. It has a high prostaglandin synthase activity. The produced prostaglandin PGF2 can lead to cell proliferation in both hormone-sensitive and -insensitive diseases [24]. 17β-HSD5 inhibition, blocking both intratumoral androgen and PGF2 synthesis, is therefore an attractive approach, not only for the treatment of steroid-sensitive, but also -insensitive tumors.

2.2.5. 17β-HSD10

Type 10 17β-hydroxysteroid dehydrogenase (17β-HSD10) has a very broad substrate profile. It has been reported to be involved in the metabolism of 3-hydroxyacyl-CoAs, isoleucine, and sex- and neurosteroids [43]. Interestingly, it has been proposed that

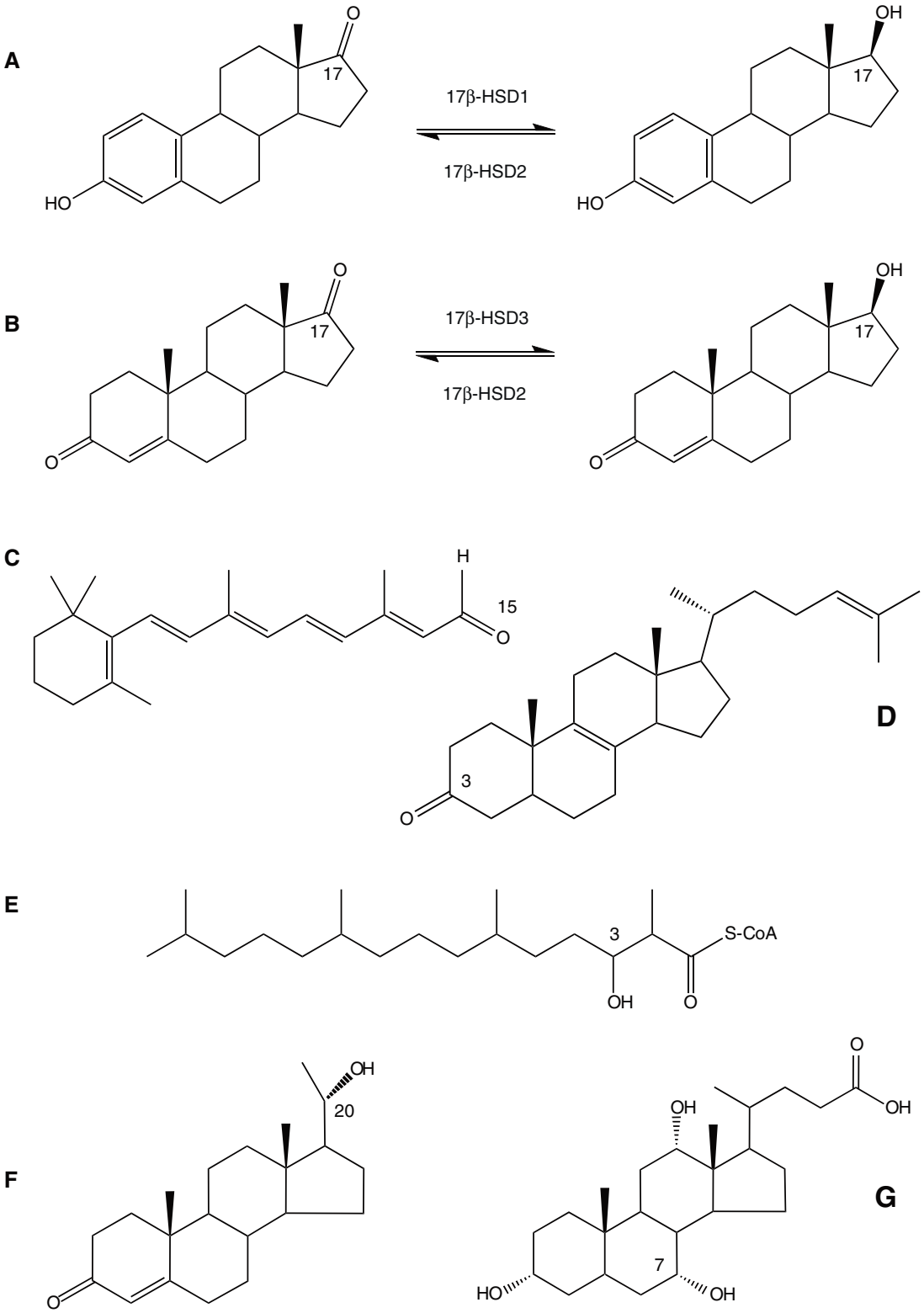

Fig. 1. Conversions catalyzed by different 17β-HSDs. (A) Conversion of estrone and 17β-estradiol, (B) conversion of Δ4-androstene-3,17-dione and testosterone, (C) retinal is converted to retinol by 17β-HSD1, (D) zymosterone is converted to zymosterol by 17β-HSD7, (E) pristanoyl-CoA is a substrate of 17β-HSD4, (F) conversion of 20α-hydroxyprogesterone is catalyzed by 17β-HSD2, (G) cholic acid is converted by 17β-HSD10. For references see Table 1. Numbers depict conversion position.

this enzyme plays an important role in the pathological processes of Alzheimer's disease (AD), initially mainly because 17β-HSD10 binds to amyloid-β [44] and appears to be up-regulated in patients suffering from this disease. The mechanism by which 17β-HSD10 contributes to the pathology of AD, however, is still not completely understood. The protein-protein interaction of 17β-HSD10 with amyloid-β appears to inhibit the enzymatic activity of 17β-HSD10 [45] by preventing the binding of its cofactor NAD^+ [46]. Recent *in vitro* studies with a potent 17β-HSD10 inhibitor [47] have shown that inhibition of this enzyme can prevent its interaction with the

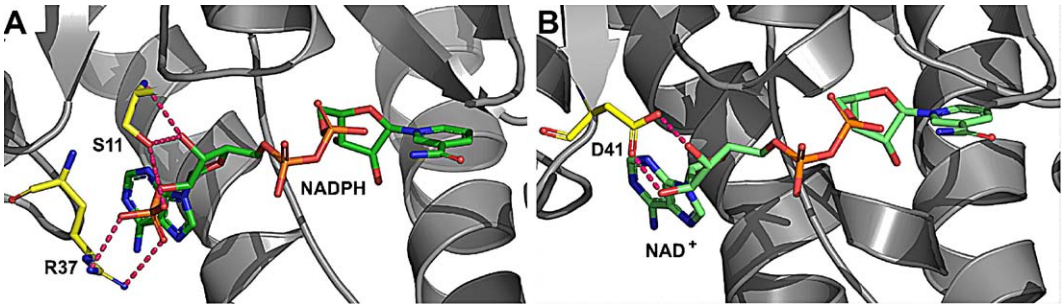

Fig. 2. Insights into the cofactor binding sites (COF) of reductive and oxidative 17β-HSDs. (A) COF of the reductive 17β-HSD1 with NADPH and Arg37 and (B) COF of the oxidative 17β-HSD10 with NAD^+ and Asp41, rendered as sticks and labelled. The dotted lines indicate H-bonds. Figure rendered with Pymol (http://www.pymol.org/).

amyloid-β peptide, suggesting 17β-HSD10 as a potential target for the treatment of AD.

3. Catalytic process and structural aspects

3.1. Unidirectional steroid flux in living cells driven by cofactor abundance

The different 17β-HSDs are characterized by binding either the phosphorylated (NADP(H)) or the non-phosphorylated nicotinamide adenine dinucleotide (NAD(H)) cofactors [48]. It has been elucidated by structural and mutagenesis studies that in the Rossmann fold [49] of 17β-HSD1 and 17β-HSD3, a positively charged amino acid is able to form a salt bridge with the 2′-phosphate group of the cofactor NADP(H) (Arg37 in 17β-HSD1 [50], see Fig. 2A; Arg80 in 17β-HSD3 [51]). As a consequence, these enzymes preferably bind the phosphorylated cofactors. In the case of 17β-HSD2 and 17β-HSD4 the positively charged amino acid residue is replaced by a negatively charged one (Glu116 in 17β-HSD2 [52]; Asp41 in 17β-HSD4 [53], see Fig. 2B). Accordingly, the latter 17β-HSDs bind the non-phosphorylated cofactors. *In vitro* it has been shown that HSDs are able to catalyze both the oxidation and the reduction, depending on which redox state of cofactor is added. However, under *in vivo* conditions, as in living cells, the catalysis is unidirectional due to the fact that $NADP^+$ is preferably abundant in its reduced form ($NADPH/NADP^+ > 500$), whereas NAD^+ is mainly present in its oxidized form ($NAD^+/NADH > 700$). These ratios are homeostatically maintained by glucose metabolism and O_2 supply [52]. Hence, the directionality of the catalysis by 17β-HSDs is driven by the cellular abundance of the cofactor redox forms. In the past, 17β-HSDs have been erroneously designated as oxidative or reductive *per se*.

3.2. Catalytic mechanism of 17β-HSDs

Although different kinetics for HSDs are described in the literature (*e.g.* for 17β-HSD1 see [6], for 17β-HSD5 see [54]), they have the following chemical mechanism in common: a reversible hydride transfer from NADPH to a ketosteroid or a hydride transfer from a hydroxysteroid to NAD^+, which is attended by a proton shift for charge equalization. Based on structure analysis and mutagenesis studies, as well as sequence alignment, common features have been identified to be essential for the catalytic process: three conserved amino acid residues, Ser142, Tyr155 and Lys159 ("catalytic triad") and a water molecule [55]. Further investigations showed, that an additional conserved water molecule stabilized by a H-bond interaction with an Asn residue (together with the "catalytic triad" forming a "catalytic tetrad") plays a critical role in the enzymatic process for HSDs [56,57]. Three catalytic mechanisms are proposed for 17β-HSD1 [6,58]: one concerted (simultaneous transfer of hydride and proton, not shown) and two stepwise. The latter differ in the intermediate presence of either an oxyanion or a carbocation and are depicted in Fig. 3A and B. Although numerous structural information is available, it has not yet been clarified which mechanism is the most likely one.

3.3. Sequence and structural analysis of human 17β-HSDs

As already mentioned above, 17β-HSDs belong to the short-chain dehydrogenase/reductase (SDR) family, except 17β-HSD5, which is an aldo/ketoreductase (AKR) family member. SDRs and AKRs differ in their folding pattern (Rossmann fold *vs.* triosephosphate isomerase (TIM) barrel), in the stereospecificity of hydride transfer (*pro*-S *vs. pro*-R from the nicotinamide C4), and in the quaternary structure of their active form (dimer/tetramer *vs.* monomer) [59]. They share a common pattern of catalytic residues (Tyr and Lys), which are adjacent in space. These amino acids are close in linear sequence for the SDRs, but more distant for the AKRs. Since only 17β-HSD5 belongs to the AKR-family and has been comprehensively characterized recently by Penning et al. [60], we focus here on the sequential and structural analysis of the SDR-family members.

3.3.1. Characteristics from primary sequences and 3D-structures

The human 17β-HSDs of the SDR-family have a very low overall sequence identity (<25%), although some characteristic amino acid motifs are highly conserved, such as the T-G-xxx-G-x-G motif ("cofactor binding site"), the Y-xxx-K sequence ("active center") and the N-A-G motif (Fig. 4). Tyr155 and Lys159, together with Asn114 and Ser142 (numbering according to human 17β-HSD1), are fundamental for catalysis [55,61], while the N-A-G motif is responsible for stabilization of the protein 3D-structure. Other conserved residues are Arg or Asp, determinant for cofactor-specificity, *i.e.* reductive or oxidative reactions as mentioned in Section 3.1 (Fig. 2). Aside from that, substrate specificity is controlled by additional amino acids at different positions. In human 17β-HSD1, for example, Leu149 is responsible for C18/C19-steroid discrimination [62,63] and Gly145 in the vicinity of the catalytic Tyr155 plays a fundamental role in steroid/retinoid discrimination [30]. The 17β-HSDs are either membrane-bound or cytosolic. For six out of the thirteen SDR members, 3D-structures were resolved, namely for the subtypes 1, 4, 8, 10, 11 and 14. They all have a Rossmann fold that consists of a seven-stranded, parallel β-sheet core surrounded by six parallel α-helices. Their cofactor binding sites (COFs) (Fig. 4), which comprise the Rossmann fold and the T-G-xxx-G-x-G motif, are conserved and rigid, but differences can be seen in the 3D-structures of the F/G-segment and the C-terminal part, both delimiting the substrate binding sites (SUBs) (Figs. 5 and 6).

The crystal structures of the 17β-HSDs of the SDR-family are characterized by wide, easily accessible active sites, reflected by a rather broad tolerance for structurally different substrates. An exception is 17β-HSD1, as it displays a high substrate selectivity.

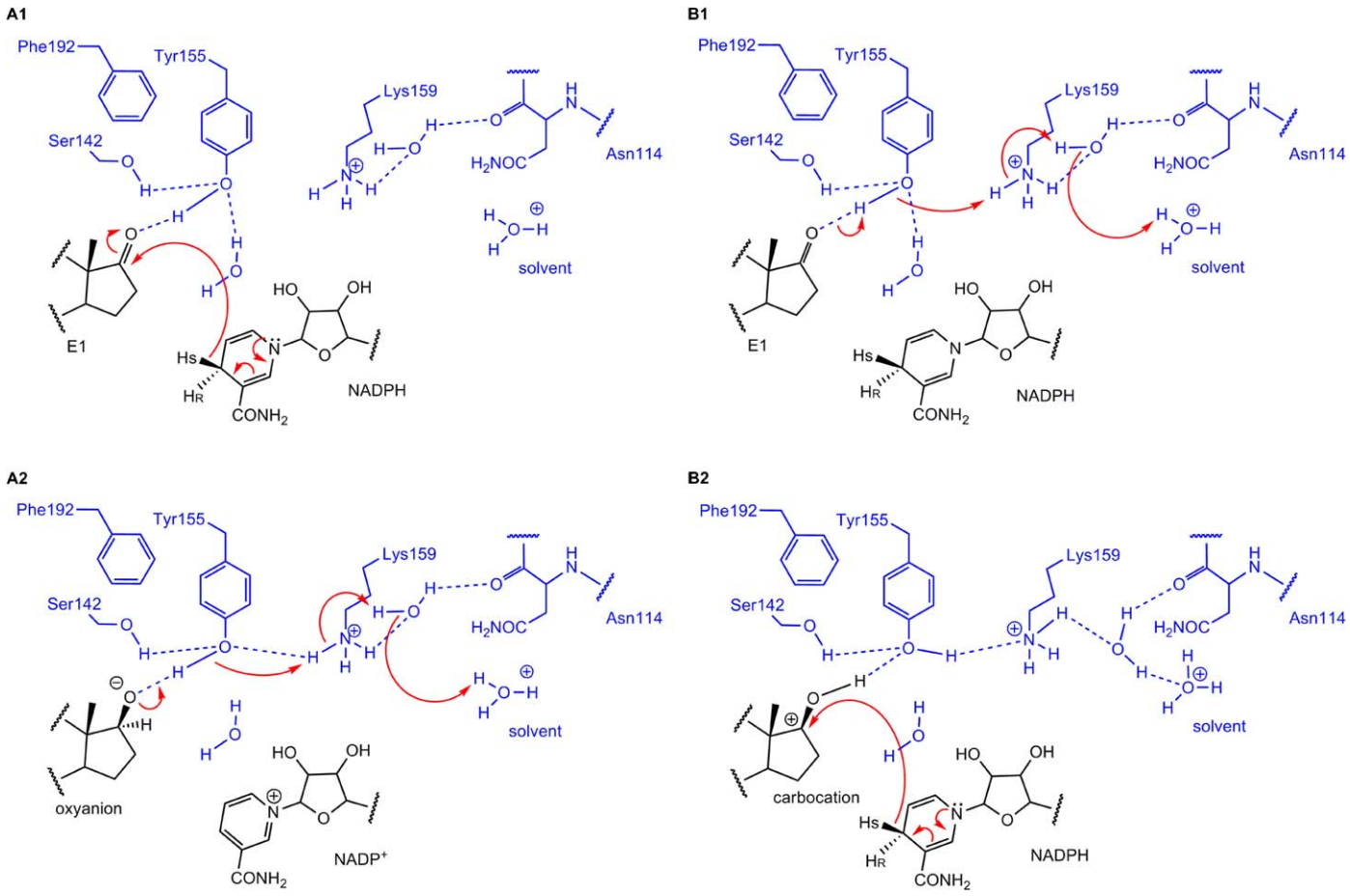

Fig. 3. Two possible stepwise catalytic mechanisms for 17β-HSD1. (A) In the first step the *pro*-S hydride of NADPH is transferred to the α-face of E1 at the planar C17 carbon (A1), resulting in an energetically favorable aromatic system; subsequently the resultant oxyanion is protonated by the acidic OH group of Tyr155 (A2). (B) In the first step the keto oxygen of E1 is protonated by the acidic OH of Tyr155 (B1); then the resultant carbocation accepts the *pro*-S hydride of NADPH at the α-face (B2). The proton relay is facilitated by a H-bond network involving Lys159, two water molecules and Asn114, an electrostatic interaction between the protonated side chain of Lys159 and the phenyl ring of Tyr155 [58] as well as T-stacking between Phe192 and Tyr155 [66]. Hydrogen bonds are represented in dashed lines. For the sake of clarity π–π-interactions are not depicted.

This different behavior is due to the fact that the other 17β-HSDs – compared to 17β-HSD1 – have shorter F/G-segments and disordered or missing C-terminal parts (except 17β-HSD4), rendering their active sites sterically less restricted (Figs. 5 and 6B, C, E, F). Interestingly, when comparing the tertiary structures of the types 4, 8, 10, 11, and 14, different cofactor dependent orientations of the F/G-segment are observed (Fig. 7). In the presence of cofactor, the two helices form a V-shaped structure, whereas in absence of cofactor, they flip upward into a more linear orientation, revealing easily accessible COFs.

The C-terminal region of the crystallized 17β-HSDs varies in length and form, resulting in a SUB with the form of a cleft (17β-HSD4, 8, 10, 11, 14) or of an internal cavity for 17β-HSD1 (Fig. 6A–F). 17β-HSD4 has a very long and folded C-terminal region, including multiple-recognition and catalytic sites, in accordance to its extraordinary specificity for diverse substrates [26]. Unique

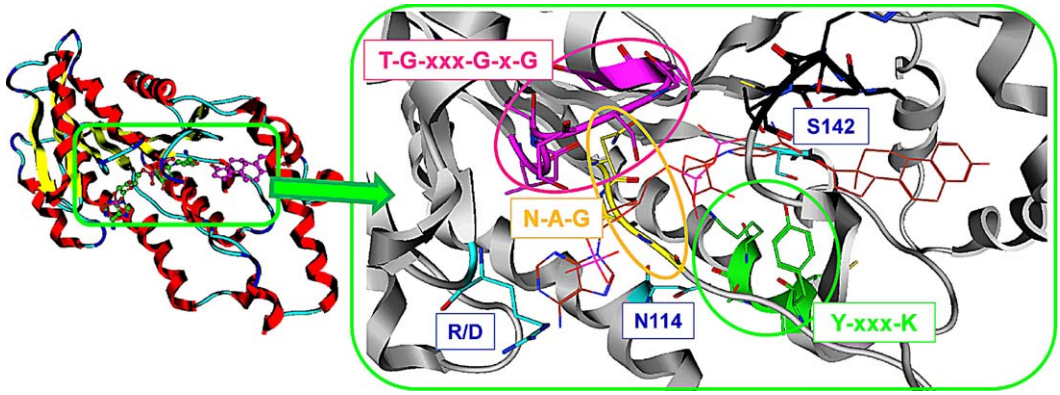

Fig. 4. Insights into conserved regions among the 17β-HSD subtypes. Tertiary structure of 17β-HSD1, rendered as cartoon, with a zoom into the transitional zone between COF and SUB, where the conserved regions T-G-xxx-G-x-G, N-A-G and Y-xxx-K, as well as S142, N114 and R37 (or D41) are located. Figure rendered with MOE (http://www.chemcomp.com/).

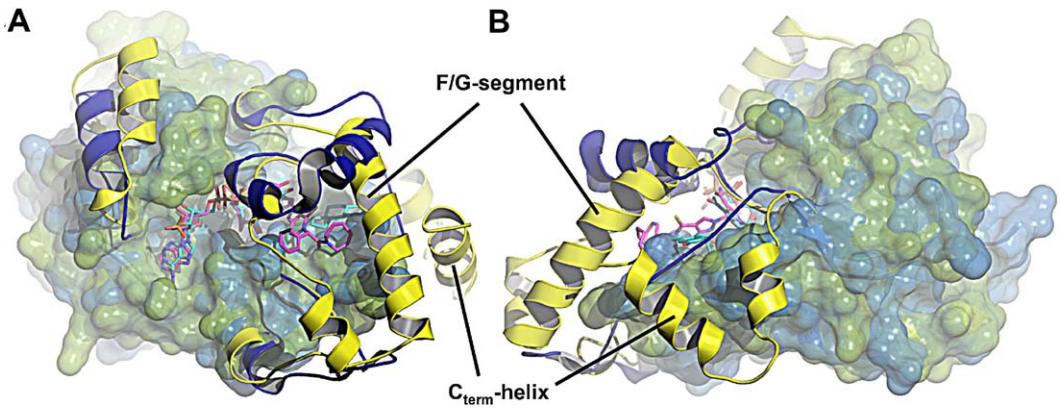

Fig. 5. Front (A) and side view (B) of the superimposed crystal structures of 17β-HSD1 (1a27; yellow) and 17β-HSD10 (1u7t; blue). F/G-segment and C-terminal region are rendered as cartoon, highlighted and labelled; the rest of the proteins is rendered as molecular surface. These areas show a marked mismatch between 17β-HSD1 and all other 17β-HSDs crystallized so far. Figure rendered with Pymol (http://www.pymol.org/).

domains discriminate 17β-HSD1 from the other crystallized 17β-HSDs. These are a longer βFαG′-loop, an αG′-helix and a C-terminal helix, which modulate the solvent accessibility of COF and SUB (Fig. 6A and D).

3.3.2. *17β-HSD1 crystal structures*

To date 20 crystal structures of 17β-HSD1 are available in the protein data bank (PDB) as: apoform (1bhs), holoform (1fdv, 1qyv), binary complex with E2, androgens or inhibitors (1fds, 1fdw, 1dht, 3dhe, 1jtv, 1iol, 3dey, 1i5r, 3hb4, 3klm) and ternary complex with cofactor and E2 or inhibitors (1fdt, 1equ, 1fdu, 1a27, 1qyw, 1qyx, 3hb5). Remarkably, no crystal structure with the substrate E1 exists.

All crystals reveal an overall identical tertiary structure: a rigid COF and a narrow, hydrophobic SUB, which constitutes a "substrate recognition domain" delimited by the C-terminal region [64,65]. E2 is stabilized by hydrogen bonds between the O3 and His221/Glu282, as well as between the O17 and Tyr155/Ser142 [64]. Major differences in the structures have been identified only for the highly flexible βFαG′-loop. This loop is not resolved in twelve crystal structures and can occupy three possible orientations, dependent on the presence of cofactor and ligands, an opened, a semi-opened and a closed enzyme conformation [66].

For some steroidal inhibitors the binding mode is known as they are co-crystallized in complex with 17β-HSD1 (1equ, 3hb5, 1i5r). The data reveal the importance of a defined βFαG′-loop conformation for compound binding. Since no protein structure complex with non-steroidal inhibitors exists, computational studies have been performed to investigate their binding. These studies showed that the choice of the crystal structure was determinant for the identification of a binding mode and that the latter was strongly dependent on the loop conformation [67].

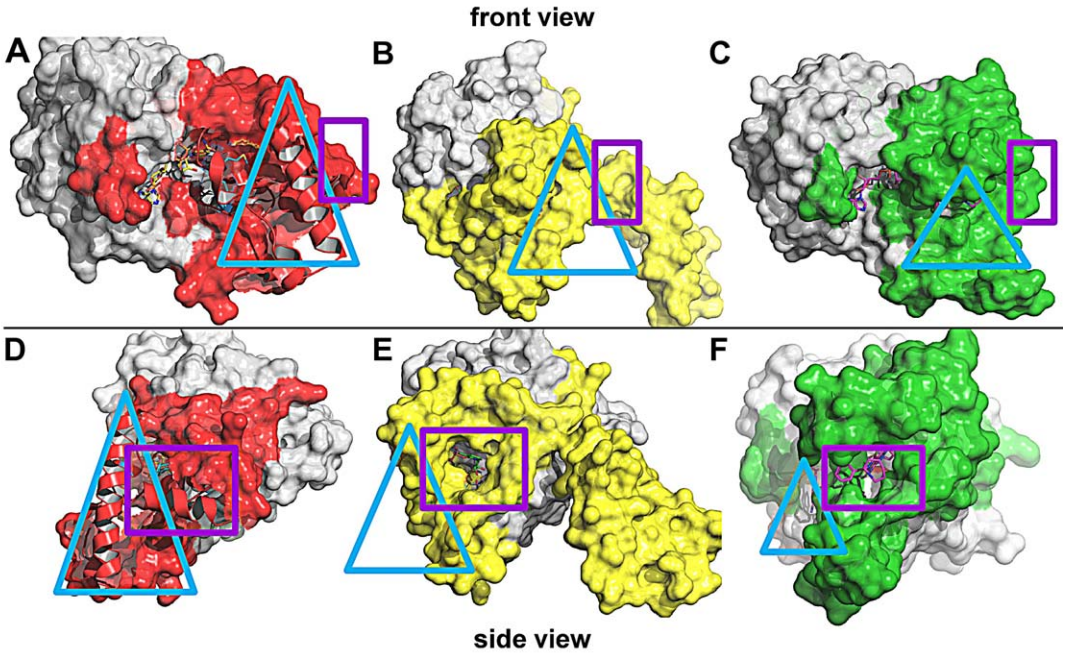

Fig. 6. Different steroid active site forms and volumes. The colored areas of the molecular surfaces of 17β-HSD1 (A, D; 1a27; red), 17β-HSD4 (B, E; 1zbq; yellow) and 17β-HSD10 (C, F; 1u7t; green) indicate the mismatch regions between 17β-HSD1 and all the other family members. These three types reveal different SUB shapes: 17β-HSD1 (A, D) shows an internal cavity (the surface of F/G-segment and C-terminal part have been replaced with cartoons to allow an insight into 17β-HSD1 SUB), while 17β-HSD4 (B, E) and 17β-HSD10 (C, F) exhibit a wide, easily accessible and a narrow, shielded cleft, respectively. The triangle (F/G-segment) and the square (C-terminal) represent the missing parts of 17β-HSD4 and 17β-HSD10 with respect to 17β-HSD1 (triangle and square in A and D as reference). The size of square and triangle in B, C, E and F is scaled to the absent areas. Figure rendered with Pymol (http://www.pymol.org/).

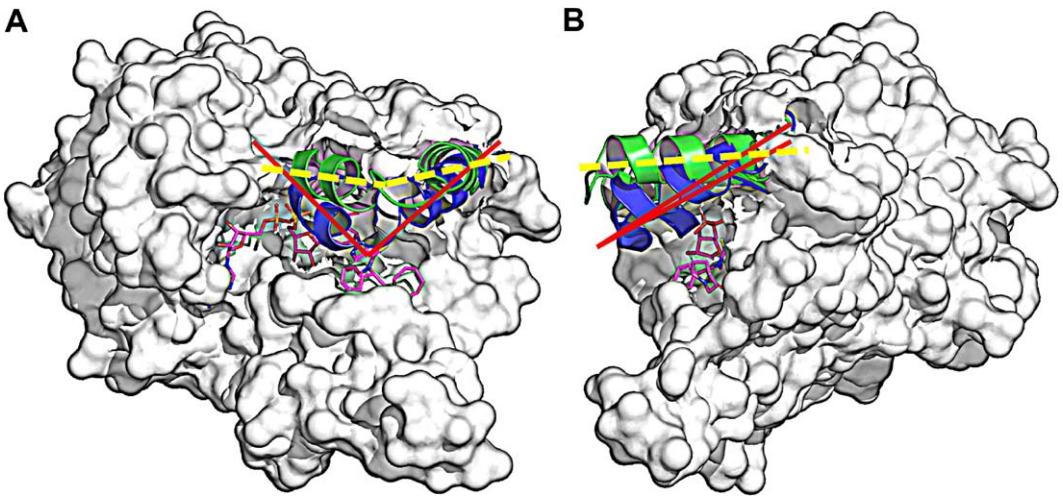

Fig. 7. Cofactor-dependent differences in the tertiary structure of the F/G-segment of 17β-HSDs (except 17β-HSD1; (A) front and (B) side view). The F/G-segments are rendered as cartoons (17β-HSD10 – 1u7t, blue; 17β-HSD14 – 1yde, green), whereas the rest of the proteins as white surface or, like NAD^+ of 17β-HSD10, as magenta sticks. When cofactor is present, as for 17β-HSD10, the F/G-segment flips down folded to a V (red lines), and blocks the cofactor in the COF. On the contrary, in 17β-HSD14 the F/G-segment is almost straight (yellow dotted lines), revealing a very easily accessible active site (both COF and SUB), probably not suitable to bind the cofactor tightly enough for catalysis. Figure rendered with Pymol (http://www.pymol.org/).

From the crystal structures, two small apolar subpockets can be identified at both ends of the hydrophobic, tunnel-like SUB. The first one is situated next to the catalytic tetrad. It is delimited by Gly94, Leu95, Leu96, Asn152, Tyr155, Tyr218, Phe192, Met193, and Val196 (the orientations of the latter three residues, however, depend on the investigated crystal structure). As seen for the crystal structure 3hb5 this pocket can host the benzylamide moiety of the steroidal inhibitor E2B, which forms H-bonds with the polar residues at the bottom (Asn152, Tyr218 and backbone of Leu95). The second hydrophobic subpocket, formed by Met147, Leu149, Phe259, Leu262, Tyr275 and Met279, is located close to the C-terminal region and might be suitable to host substituents in 2-position of the steroidal scaffolds.

3.4. 17β-HSD interspecies analyses

Interspecies sequence analysis is crucial for the determination of appropriate animal models to study the efficacy of inhibition of a given enzyme *in vivo*.

3.4.1. 17β-HSD1

The sequence-identities between human 17β-HSD1 and 17β-HSD1 of other species range from 51% (zebrafish) to 99% (chimpanzee) and homologies from 70% to 100%, respectively. The biggest differences are located in the F/G segment (residue 191–230), which is lining the hydrophobic SUB, and in the C-terminal region (Fig. 8).

The moderate interspecies homology leads to difficulties in identifying a suitable species in order to establish a proof of concept model to test inhibitors active on the human enzyme. For example, 17β-HSD1 of mouse and rat are 83% homologous to the human (sequence identity 74% and 75%, respectively) in the first 287 amino acids. As a consequence of this, different substrate affinities are observed and moreover the rodent 17β-HSD1 also converts androgens efficiently (*i.e.* androstenedione to testosterone) in contrast to the human enzyme [68–70]. Different classes of non-steroidal inhibitors – potent for human 17β-HSD1 and selective toward 17β-HSD2 – were tested in rat liver preparations [71]. They showed moderate activity and low selectivity suggesting that the rat is not a suitable animal model for testing the efficacy of potential drugs against human steroid converting enzymes. In a similar approach Möller et al. evaluated the inhibitory potency of E2/E1 derivatives on 17β-HSD1 of several species recombinantly expressed in *Escherichia coli*. The compounds showed similar inhibitory activity on the human, marmoset (*Callithrix jacchus*) and pig (*Sus scrofa*) enzymes, while they were mostly inactive with the rat and mouse 17β-HSDs type 1 [72]. In *in vitro* experiments with marmoset placenta preparations, we recently identified some highly potent

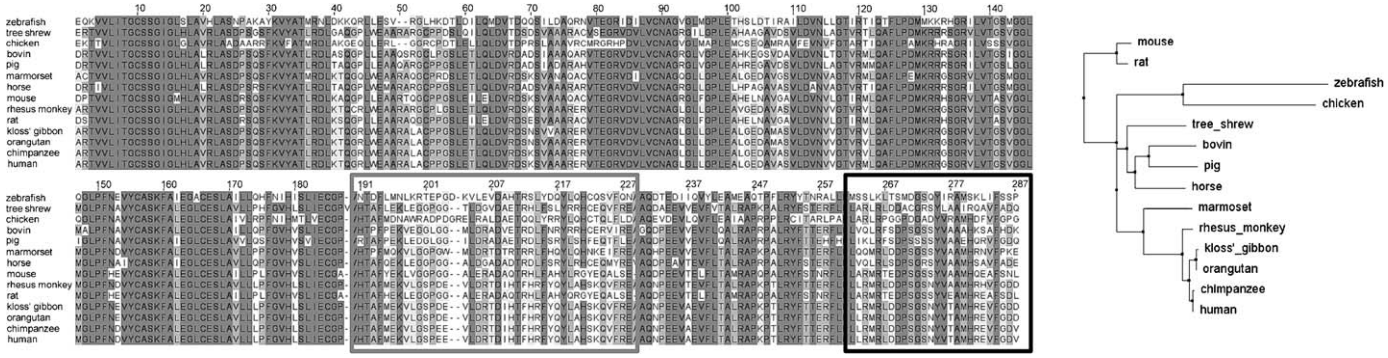

Fig. 8. Multiple sequence alignment of 17β-HSD1 of various species. The dark and the fair boxes in the lower sector represent the F/G-segment and the C-terminal region, respectively; in these areas most of the primary as well as tertiary structure differences among the species are observed. The numbering above the sequences refers to human sequence numbers. On the right side a neighbor joining tree is represented, clustering the closest species. The alignment was performed by using the MAFFT multiple sequence alignment webserver [127] using Jalview 2.4.0.b2 [128].

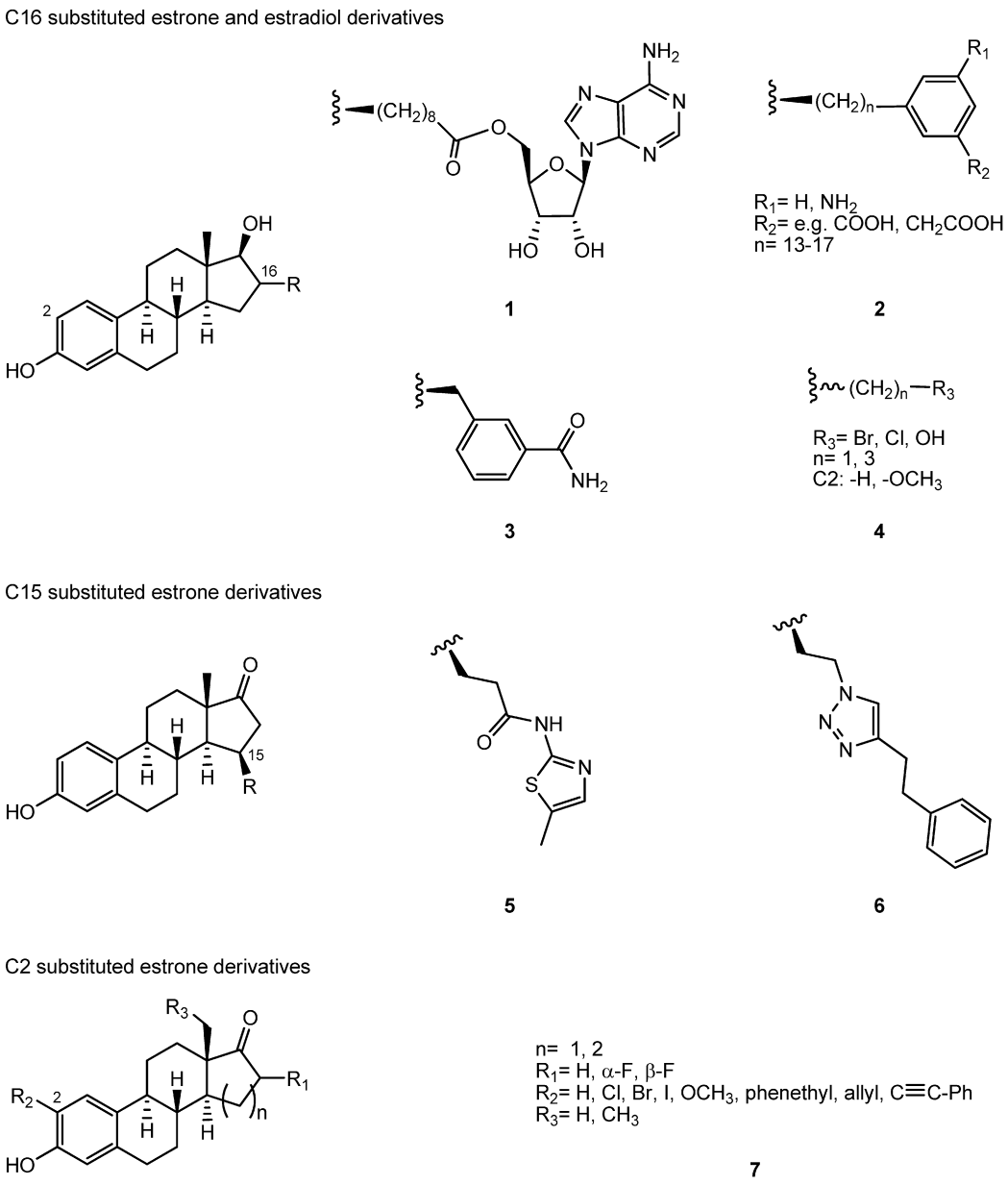

Fig. 9. Steroidal inhibitors of 17β-HSD1.

non-steroidal inhibitors, with comparable inhibitory efficiency to that on the human enzyme (data not published). Therefore, the choice of an animal model for pharmacological testing is of major importance. If the tests on lead compounds are not verified in humanized models or with recombinant human proteins some potentially efficient small molecules could be dropped off the drug development pipeline.

3.4.2. 17β-HSD3

Compared to 17β-HSD1, the human 17β-HSD3 presents a higher homology/similarity toward the enzymes of rat and mouse (17β-HSD3 95% *vs.* 17β-HSD1 83%), while the sequence identity is similar (74%) [73].

4. Inhibitors of 17β-HSDs

17β-HSD inhibitors have been reviewed by Poirier [74,75], Brožič et al. [76] and Day et al. [77]. In the following we focus on new structures that have not been covered by these reviews. Very recently one more review article was published [78].

4.1. Inhibitors of 17β-HSD1

Steroidal and non-steroidal inhibitors are described to bind into SUB and/or COF. For phytoestrogens, however, competitive NMR-experiments recently suggested that these inhibitors interact neither with SUB nor with COF. The dimer interface of 17β-HSD1 was proposed to be a possible binding site by docking studies [79].

4.1.1. Steroidal compounds

4.1.1.1. C16 substituted E1 and E2 derivatives. Already in 2002, the adenosine derivative **1** (Fig. 9) was designed and synthesized as a bifunctional inhibitor. Crystallization (1i5r) confirms this compound to occupy the SUB and COF, mimicking E2 and parts of the cofactor [80]. Recently the synthesis of the corresponding estrone derivative was performed and resulted in a comparably strong inhibitor in a cell-free assay (4 nM *vs.* 12 nM) [81].

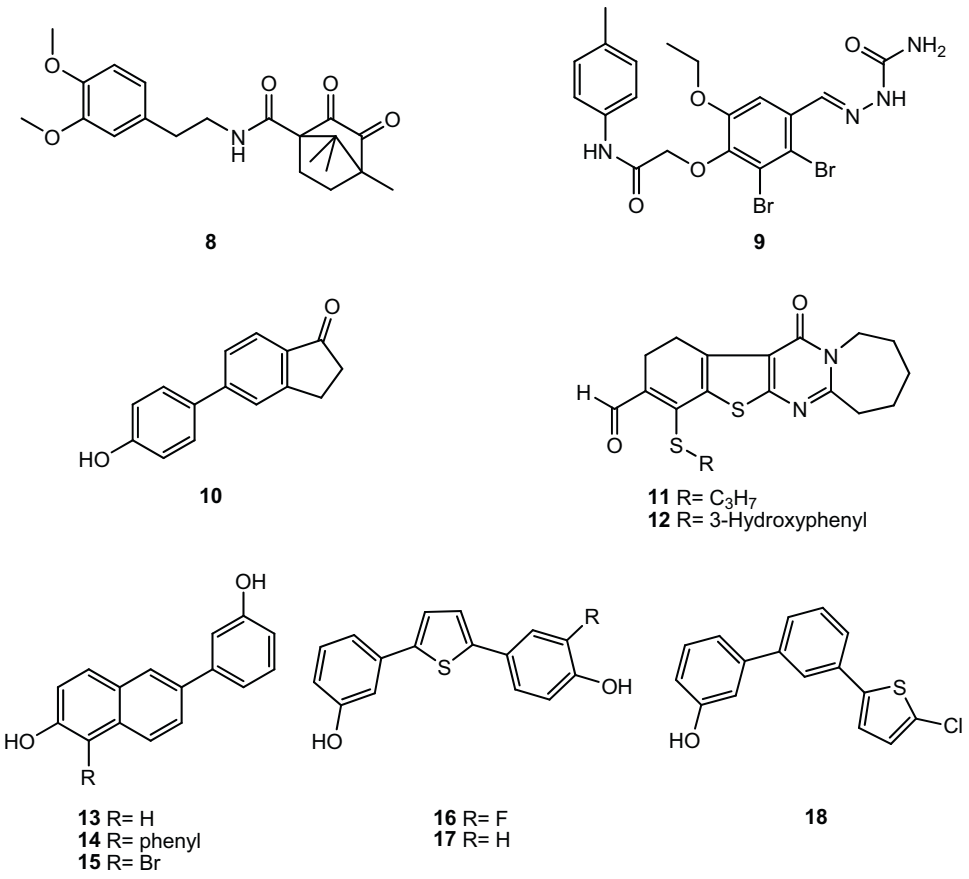

Fig. 10. Non-steroidal inhibitors of 17β-HSD1.

Two strategies were followed to simplify the complex structure of the adenosine derivatives. The first led to a series of interesting compounds **2** with the adenosine moiety replaced by smaller substituents *e.g.* carboxy- and amino-substituted phenyl-rings [81]. In a second approach diverse libraries of 16β-substituted estradiol derivatives were synthesized, in which the alkyl spacer was partially replaced by a peptide linker bearing an aromatic capping group [82]. However, none of these attempts resulted in compounds, which could equal the activity of **1**.

Since the dual site inhibitors are not able to penetrate the cell membrane, an additional series of C16-substituted estradiol and estrone derivatives was synthesized. Introduction of a *m*-carbamoylbenzyl substituent in 16β-position led to highly active derivatives of estrone and estradiol (**3**) with IC_{50} values in T-47D cells of 171 and 44 nM, respectively. The compounds showed selectivity toward 17β-HSD2 (in HEK-293 cells over-expressing the enzyme), but also estrogenic potency in a cellular proliferation assay in T-47D and MCF-7 cells. However, compound **3** was also able to inhibit the E1 mediated proliferation to some extent. Its binding mode was elucidated by X-ray crystallography (3hb4, 3hb5). While the steroidal core occupies the SUB, the substituent enters an additional subpocket [83].

Besides introduction of a benzyl side chain, the same group also studied small C16α/β-halogen- or hydroxy-alkyl analogs of estradiol with additional substituents in 2-position (compounds **4**) for reduction of estrogenic activity [84]. This approach led to compounds with comparably weak inhibitory activity on 17β-HSD1 (IC_{50} > 1 μM).

4.1.1.2. C15 substituted E1 derivatives. A different approach for the design of 17β-HSD1 inhibitors was performed by Messinger et al. [85–87]. The authors describe the modification of estrone in 15-position with polar substituents linked by an alkyl spacer (**5**, **6**). The most active compound was a methylthiazolyl-propanamide substituted estrone derivative **5** showing an IC_{50} of 4 nM in a cell-free inhibition assay (substrate E1 30 nM).

4.1.1.3. C2 substituted E1 derivatives. Modifications in the 2-positions of E1 and D-homo-estrone derivatives were performed and resulted in highly active compounds **7**. Computational studies suggested these substituents to target a lipophilic subpocket close to the C-terminal helix [88]. The 2-phenethyl-D-homo-estrone as the most active compound showed an IC_{50} of 15 nM (inhibition of the reduction of 15 nM E1 in *E. coli* homogenate over-expressing His-tagged 17β-HSD1).

4.1.2. Non-steroidal compounds

4.1.2.1. Candidates identified by virtual screening. Using a pharmacophore model that describes the spatial arrangement of structural features required for 11β-HSD1 inhibition Schuster et al. [89] identified some compounds which also showed moderate activity for 17β-HSD1. A glycyrrhetinic acid derivative and compound **8** (Fig. 10) showed IC_{50} values around 20 μM. Based on two pharmacophore models that were built on crystal structures from 17β-HSD1 containing steroidal ligands, they found a series of active compounds, the best of which was **9** (IC_{50} 5.7 μM). Docked in the active site of 17β-HSD1 (1equ), **9** occupied the SUB as well as parts of the COF.

4.1.2.2. Phenyl ketones, imidazoles and cinnamic acid ester derivatives. Phenyl ketones, biphenyl ketones, and phenylalkylimidazoles were synthesized and tested for 17β-HSD1 inhibition

[90,91]. However, they showed low activities (10–68% inhibition at 100 μM) in a cell-free assay using rat testicular microsomes. Originally developed as inhibitors of 17β-HSDcl (from fungus *Cochliobolus lunatus*), two cinnamic acid ester derivatives were tested on recombinant human 17β-HSD1 enzyme and revealed only very poor activity of 32% and 35% inhibition at an inhibitor concentration of 6 μM [92].

4.1.2.3. Biphenyl mimics of E1. Based on the skeleton of E1 biphenyl ethanone and indanone/tetralone derivatives were synthesized as potential inhibitors of 17β-HSD1 [93]. In a cell-based assay using T-47D human breast cancer cells and a low substrate concentration (2 nM), compound **10** (Fig. 10) was the most active one (IC_{50} 1.7 μM).

4.1.2.4. Thiophenepyrimidinones. Very good inhibition of 17β-HSD1 was reported by Lilienkampf et al. [94] for thiophenepyrimidinones (Fig. 10). The most potent inhibitors in this class were the alkylderivative **11** (73% inhibition at 0.1 μM) and the 3-hydroxyphenyl derivative **12** (94% inhibition) tested in a cell-free recombinant human 17β-HSD1 assay at low E1-concentration (30 nM). Only compound **11** showed a strong inhibition of 74% at high inhibitor (1 μM) and low substrate concentration (2 nM) in human MCF-7 breast cancer cells transfected with cDNA encoding for human 17β-HSD1. Combining docking, molecular dynamic simulation and 3D-QSAR studies, Karkola et al. identified these compounds to occupy the SUB, but not mimicking E2 [95].

4.1.2.5. (Hydroxyphenyl)naphthols. A pharmacophore model, developed by the Hartmann group [96,97], was based on the steroidal oxygens at C3 and C17 and a hydrophobic core. Two inhibitor classes were derived from it, consisting of two hydroxy groups in a distance of about 12 Å.

Frotscher et al. reported on a group of (hydroxyphenyl)naphthalene and -quinoline derivatives (Fig. 10) [96]. In this series, 6-(3-hydroxyphenyl)-2-naphthol **13** showed the best inhibitory activity (IC_{50} 116 nM; IC_{50} for 17β-HSD2 5641 nM). Position 1 of **13** was identified to be appropriate for introduction of phenyl (**14**, IC_{50} 20 nM) or bromo (**15**, IC_{50} 40 nM) substituents. The compounds showed a good inhibitory profile, turned out to be selective toward 17β-HSD2, and displayed negligible affinities to the estrogen receptors ER α and β (relative binding affinities <0.1% compared to E2) [97,98]. Molecular docking studies revealed an E2 mimicking binding mode with the substituent in 1-position directed toward NADPH.

4.1.2.6. Bis(hydroxyphenyl) substituted arenes. The second inhibitor class consisting of bis(hydroxyphenyl)arenes (Fig. 10) was developed by Bey et al. [67,99–101]. In an extensive structure–activity study, it was found that the exchange of the middle ring can turn an inactive compound into a highly active and selective inhibitor of 17β-HSD1. A possible explanation for the observed sharp structure-activity relationships could be identified in the different molecular electrostatic potential (MEP) maps of these compounds [99]. MEP maps indicate reactive sites of a molecule. They are a valid tool for the interpretation and prediction of molecular interactions and recognition processes [102]. Supportive for their significance in this case is the fact that rather small substituents (especially fluorine) and their positions have a major impact on 17β-HSD1 inhibitory activity. Introduction of fluorine (**16**) led to an increase in activity and selectivity (IC_{50} 8 nM, IC_{50} 17β-HSD2 940 nM) compared to its non-substituted analog **17** (IC_{50} 69 nM, IC_{50} 17β-HSD2 1950 nM). An ensemble docking approach, based on the crystal structures 1a27, 1fdtA, 1fdtB and 1i5r, was performed. It revealed two plausible binding modes: a steroid-mimicking for the enzyme in its closed state (1fdtB, 1a27) and an alternative one for opened enzyme conformers only (1fdtA, 1i5r) in which the inhibitors are shifted beneath the cofactor. Interestingly, the latter was substantiated by MEP distribution analysis and MD simulations [67].

4.1.2.7. Heterocyclic substituted biphenylols. Heterocyclic substituted biphenylols and their aza-analogs bearing only one OH-group [103], were recently found to inhibit 17β-HSD1 (Fig. 10). Computational analysis suggests that the 1,3-disubstituted compounds, due to their structure, interact in a slightly different binding mode compared to the steroid. The chlorothiophene part of compound **18** (IC_{50} 560 nM, IC_{50} 17β-HSD2 2366 nM) points to a predominantly hydrophobic sub pocket as mentioned in 3.2.2 and described for C-16 substituted estrogens [83].

Further biological evaluation of compounds from the latter three classes was performed using a screening system [71,104], which included several assays for the evaluation of the following parameters: (1) activity (non-cellular assay at a substrate (E1) concentration of 500 nM; cellular assay in T-47D at a substrate concentration of 50 nM), (2) selectivity (17β-HSD2 at 500 nM E2; ERα; ERβ; proliferation assay; hepatic CYP-enzymes; metabolic stability), and (3) the *in vivo* profile of the compounds. Some of the compounds not only showed high activity and selectivity, but also favorable pharmacokinetic properties. Moreover, *in vitro* proof of concept was accomplished with several compounds inhibiting E1-induced T-47D cell proliferation [71].

4.2. Inhibitors of 17β-HSD2

Several inhibitors of 17β-HSD2, including spirolactones, fluorinated estratriene derivatives, and pyrrolidinones were reviewed recently [74,75].

Al-Soud et al. [101] and Bey et al. [100] identified bis(hydroxyphenyl)triazoles and (is)oxazoles **19**–**22** (Fig. 11) as inhibitors of 17β-HSD2. Compounds **19** and **20** showed 42–44% inhibition of E2 oxidation by 17β-HSD2 at a concentration of 1 μM using human placental microsomes. Under the same conditions, compounds **21** and **22** turned out to be potent inhibitors with IC_{50} values of around 0.25 μM.

Interestingly, (hydroxyphenyl)naphthols can be selective for either 17β-HSD1 or 17β-HSD2 depending on the hydroxy substitution pattern. For instance, compound **23** (Fig. 11) showed a better inhibition of the 17β-HSD2 enzyme compared to 17β-HSD1 (IC_{50} 17β-HSD2 302 nM *vs.* 17β-HSD1 2425 nM), whereas compound **13** (Fig. 10) was specific for the 17β-HSD1 (IC_{50} 17β-HSD1 116 nM *vs.* 17β-HSD2 5641 nM) [99]. A change in selectivity preference induced by minor structural modifications was also observed in the class of heterocyclic substituted biphenylols. The 17β-HSD2 inhibitor **24** (IC_{50} 17β-HSD2 470 nM *vs.* 17β-HSD1 2380 nM) was transformed into a 17β-HSD1 inhibitor by changing the position of the nitrogen [103].

4.3. Inhibitors of 17β-HSD3

In a high throughput screening Vicker et al. [105] discovered the diphenylether **25** (Fig. 12) as a hit compound (IC_{50} 700 nM). In an optimization process using an in-house homology model of 17β-HSD3 based on the crystal structure of 7α-HSD [105], compound **26** was identified as a novel potent and selective inhibitor with an IC_{50} around 200 nM (293-EBNA cells, expressing human 17β-HSD3).

Recently, Harada et al. [106] developed novel 17β-HSD3 inhibitors with a 7-hydroxycoumarin core structure substituted in 4-position. In particular, compounds bearing a pyridine moiety (**27** and **28**, Fig. 12) exhibited good potency. In a cell-based assay (human enzyme over-expressed in HeLa-cells) they showed IC_{50} values of 3 and 1.5 nM, respectively. Compound **28**, being the most

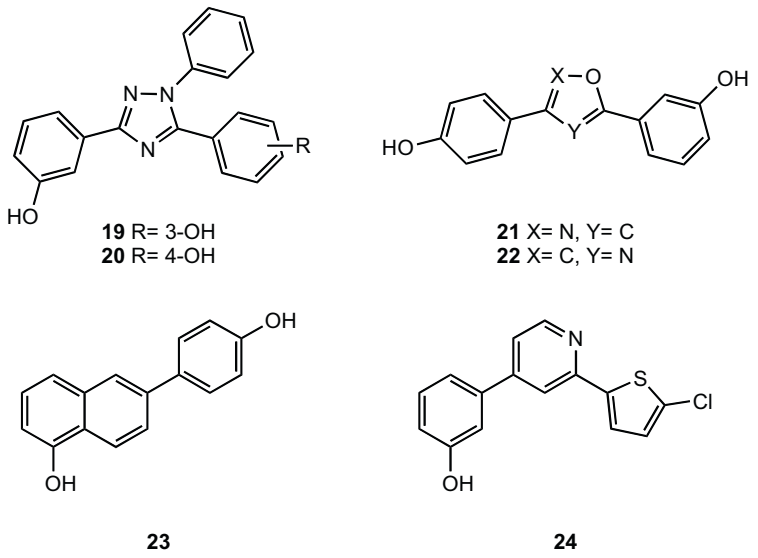

Fig. 11. Inhibitors of 17β-HSD2.

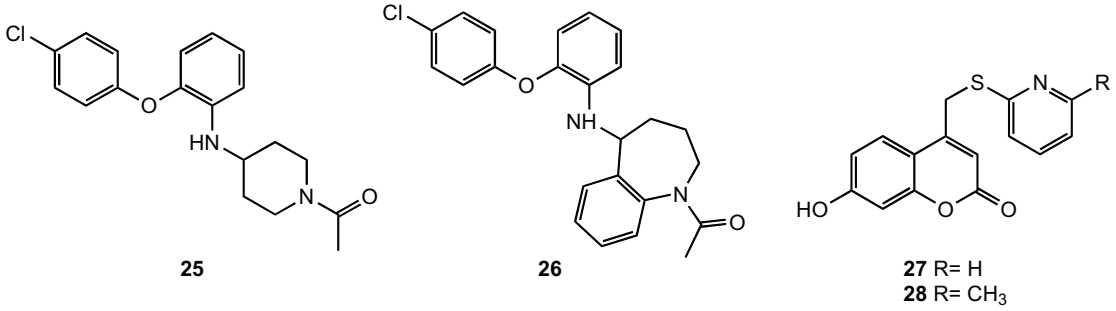

Fig. 12. Inhibitors of 17β-HSD3.

potent inhibitor of 17β-HSD3 identified in this series, exhibited good selectivity toward 17β-HSD1 and 17β-HSD2.

Perfluorinated aliphatic acids classified as persistent organic pollutants were recently described to inhibit human 17β-HSD3 weakly [107].

4.4. Inhibitors of 17β-HSD5

Bydal et al. tested a series of C18-steroid lactones, originally designed as inhibitors of 17β-HSD2 [108], for their activity toward 17β-HSD5 [109]. Compound **29** (Fig. 13) was found to be the most potent inhibitor with an IC_{50} value of 2.9 nM in HEK-293 cells over-expressing human 17β-HSD5 [109]. It showed selectivity toward 17β-HSD2 and no binding affinity to estrogen, androgen, progestin and glucocorticoid receptors. The benzimidazole derivative **30** was reported to show high potency (IC_{50} 40 nM) and selectivity toward 17β-HSD3 [110]. A third class of 17β-HSD5 inhibitors (sulfonylindoles) was discovered by Niimi et al. [111]. The best compound of this series **31** showed a good potency (IC_{50} 69 nM) and was orally available.

4.5. Inhibitors of 17β-HSD7

Although the physiological role of 17β-HSD7 is not well understood, potent and selective inhibitors of this enzyme have been developed [112]. In HEK-293 cells over-expressing 17β-HSD7, compounds **32** and **33** (Fig. 14) showed IC_{50} values of 230 nM and 458 nM, respectively.

5. Animal models

With the aim of gaining further insight into the physiological role of different 17β-HSDs, as well as for inhibitor testing, several animal models were developed recently. Some models with genetically inactivated 17β-HSDs like that for types 7 and 12 were

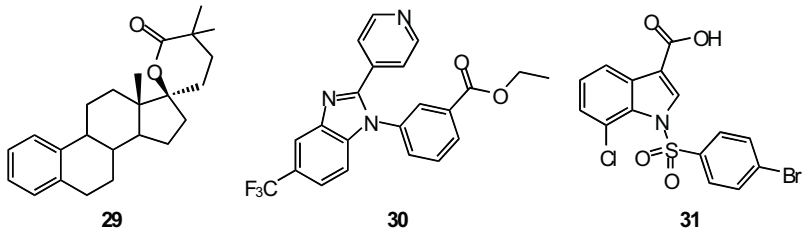

Fig. 13. Inhibitors of 17β-HSD5.

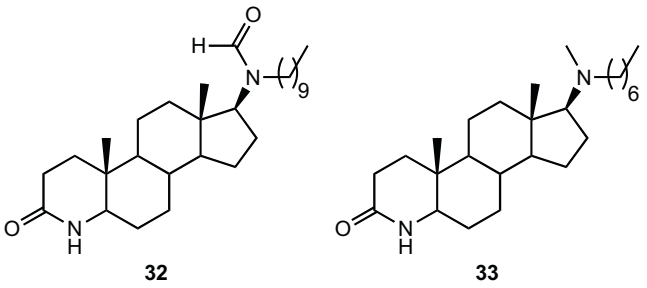

Fig. 14. Inhibitors of 17β-HSD7.

homozygous lethal and could not be characterized in respect to physiological role [113,114].

5.1. 17β-HSD1

In vivo evaluation of 17β-HSD1 inhibitors is complicated by the fact that the rodent enzymes only show moderate homology/identity to the human one (cf. Section 3.4.1). Due to these species differences there is a high probability that inhibitors optimized for activity toward human 17β-HSD1 do not inhibit the rodent enzyme. In addition, rodents and humans vary considerably in enzyme distribution in the different tissues. Attempts to overcome these problems include xenograft models using nude mice.

In one case MCF-7 cells stably expressing recombinant human 17β-HSD1 were inoculated in immunodeficient mice [115–117]. Several compounds were able to inhibit the E1 stimulated tumor growth. A similar approach is described by Day et al. [118], who used the T-47D breast cancer cell line. A decrease of tumor growth could be demonstrated by application of the 17β-HSD1 inhibitor STX1040. As both xenograft models are using established cell-lines, the question arises whether they provide more information than respective *in vitro* cell-culture experiments in combination with pharmacokinetic data.

A xenograft endometriosis model based upon tissue instead of cell-lines was established by Grümmer et al. [119] using nude mice implanted with endometrial tissue from human donors. In this model, expression of steroid hormone receptors and steroid converting enzymes as well as the proliferation of the ectopic tissue were analyzed. Several 17β-HSD1 inhibitors [120] induced changes in the expression levels of these enzymes.

Experiments with genetically altered animals (*e.g.* transgenic mice) bearing and expressing the human HSD17B1 gene are an alternative approach concerning *in vivo* evaluation of the therapeutic concept (proof of concept). Such a transgenic mouse was developed by Lamminen et al. [121]: using male and female mice moderately expressing human 17β-HSD1 in different tissues, several compounds were shown to inhibit E1 conversion by 85% in males whereas in females only an inhibition of 33% was observed. This difference might be due to higher expression levels of the wild type enzyme in the female mice. Interestingly, endometrial hyperplasia of transgenic mice ubiquitously expressing high levels of human 17β-HSD1 could be reversed by application of a steroidal 17β-HSD1 inhibitor [37].

The marmoset monkey *C. jacchus* was used as a disease model for endometriosis. Endometriotic lesions were induced either non-invasively by endometrial reflux or invasively by laparatomy [122]. The comparatively high degree of phylogenetic proximity of marmoset and human makes this model very interesting and certainly more significant than rodent models. Moreover, this is the least artificial model by comparison. Although potent inhibitors of human 17β-HSD1 displaying strong activity toward the marmoset enzyme could be identified (cf. Section 3.4.1), no *in vivo* application has been reported so far.

5.2. 17β-HSD2

Transgenic mice expressing human 17β-HSD2 (HSD17B2TG) [31,123] showed growth retardation, delayed eye opening and disrupted spermatogenesis. Since part of these effects could be rescued by a synthetic retinoic acid receptor agonist, it can be speculated that 17β-HSD2 plays a role in the action of retinoids. Surprisingly, the HSD17B2TG mice did not show a significant rise in the tumor incidence which was expected according to the protective hypothesis for 17β-HSD2. In a second study the same group showed that the over-expression of human 17β-HSD2 led to disturbances in the skeletal development of male mice [124]. Studies with the HSD17B2TG suggested a sex-steroid independent role for 17β-HSD2 besides its steroid converting function. To clarify this point a mouse bearing two genetic alterations was generated, ubiquitously expressing both human 17β-HSD1 and human 17β-HSD2 [124]. Using this "bi-TG mouse" the involvement of 17β-HSD2 in sex steroid independent pathways could be proven.

Ovariectomized cynomolgus monkeys were used as an osteoporosis model to evaluate the efficacy of 17β-HSD2 inhibitors. Bagi et al. [41] showed that oral application of a highly potent pyrrolidinone 17β-HSD2 inhibitor led to a decrease in bone resorption and maintenance of bone formation.

5.3. 17β-HSD3

Day et al. [125] developed the first xenograft model in castrated mice, using LNCaPwt cells to evaluate 17β-HSD3 inhibitors. Compound **26** strongly inhibited the tumor growth by 81%, demonstrating that 17β-HSD3 inhibition might be an efficient strategy for the treatment of hormone dependent prostate cancer.

5.4. 17β-HSD10

Transgenic mice over-expressing human 17β-HSD10 showed a lower neurologic deficit score with increased brain ATP compared to non-transgenic mice, suggesting that inhibition of 17β-HSD10 protects from cerebral infarction and ischemia [126]. Another transgenic mouse over-expressing 17β-HSD10 and beta-amyloid (Aβ) was established as a model for Alzheimer's disease (AD) [46]. The interaction of 17β-HSD10 and Aβ in mitochondria in AD promotes dysfunction of mitochondria and cell death. Inhibition of this interaction might be an interesting approach for the treatment of AD.

6. Conclusion

17β-HSDs are multifunctional enzymes, but some of them, such as type 1, 2, and 3, play a key role in androgen and estrogen biosynthesis, as they catalyze in the target cell the interconversion of low bioactive 17-ketosteroids into their active 17β-hydroxyforms and *vice versa*. 17β-HSDs are therefore key enzymes involved in the development, growth, and function of all reproductive tissues in both males and females. They are also suitable targets to modulate the concentration of the potent E2 and testosterone in case of steroid-dependent diseases. As they could act selectively in an intracrine manner, inhibitors of these enzymes might be superior to the existing endocrine therapies (*e.g.* aromatase inhibitors, antiestrogens and selective estrogen receptor modulators, GnRH analogs) regarding the off-target effects. For patients over-expressing the target enzyme in the diseased tissues, such an approach could lead to a straightforward personalized treatment.

A large number of 17β-HSD inhibitors, both steroidal and non-steroidal, has been described in recent years (especially for type 1, 3 and 5). The knowledge of the protein 3D-structure facilitated the inhibitor design.

Some of the most active and selective inhibitors were investigated *in vivo* in animal disease-oriented models. They showed efficacy, validating the target enzymes but none of them reached the clinical trial stage. One reason for this might be the difficulty to identify an appropriate species to conduct the functional assays: due to little sequence homology very potent inhibitors of the human enzyme show little activity toward 17β-HSDs of other – especially rodent – species. Furthermore it is difficult to find a quick, easy and robust readout (biomarker) for the determination of clinical efficiency.

The highly active inhibitors recently discovered could not only be used as therapeutics, but also as diagnostic tools to identify the presence of a specific 17β-HSD and therefore help the physicians to choose the appropriate treatment for the patient without use of invasive techniques.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.12.013.

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