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Mini-review

Design and synthesis of bisubstrate inhibitors of type 1 17β-hydroxysteroid dehydrogenase: Overview and perspectives

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Abstract

Type 1 17β-hydroxysteroid dehydrogenase (17β-HSD1) is a key steroidogenic enzyme that catalyses the reduction of steroid estrone into the most potent endogenous estrogen estradiol using the cofactor NAD(P)H. Bisubstrate inhibition is a good way to enhance the potency of inhibitors of cofactor-assisted enzymes. The design of a bisubstrate inhibitor of 17β-HSD1, the estradiol/adenosine hybrid EM-1745, is reviewed and strategies for future designs of inhibitors are proposed.
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Keywords: Inhibitor; Enzyme; Hydroxysteroid dehydrogenase; Steroid; Cancer; Estradiol

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women, representing about one-third of all cases, according to both the American Cancer Society and the National Cancer Institute of Canada. In 50–75% of these cancers, the estrogen receptor (ER) is present and estrogens play a key role in tumour growth. Thus in addition to surgery, radiotherapy and classical chemotherapy, hormone therapy has been used with success in the treatment of ER^+ breast cancer, using either an antagonist of the estrogen receptor [1] or an inhibitor of aromatase [2], one of the enzymes involved in the biosynthesis of estrogens (Fig. 1) [3–5].

Other key enzymes involved in steroidogenesis are 17β-hydroxysteroid dehydrogenases (17β-HSDs). This enzyme family controls the formation and inactivation of the most potent endogenous estrogen estradiol (E_2) and androgen testosterone. Thus this family, of which 12 members are known to date [6], constitutes an interesting therapeutic target in the treatment of estrogen- and androgen-sensitive diseases, including breast and prostate cancers [7,8]. Although these enzymes have shown both oxidative and reductive activities in cell homogenates, it has been demonstrated that each isoform has an almost exclusively unidirectional activity in intact cells [9]. This selectivity is thought to originate in the preferred binding of the enzyme to either NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) or NAD^+(nicotinamide adenine dinucleotide, oxidized form) cofactor (Fig. 2).

Thus it is observed that enzymes binding preferably to NADPH are reductive, and those which prefer NAD^+ are oxidative [9]. Human 17β-HSD types 1, 3, 5, 7 and 12 are reductive enzymes, whereas types 2, 4, 8, 10 and 11 are oxidative. The conversion of estrone (E_1) to E_2 is the most important activity observed for types 1, 7 and 12 17β-HSDs. Even though type 1 is not observed in all breast tumours, its presence seems to be correlated with poor prognosis [10], as it is the most active of all three known estrogenic types [11]. 17β-HSD1 remains therefore an interesting target in breast cancer hormonal therapy.

Because of the therapeutic interest in its inhibition and of the fact that it has been known for a long time, the work on

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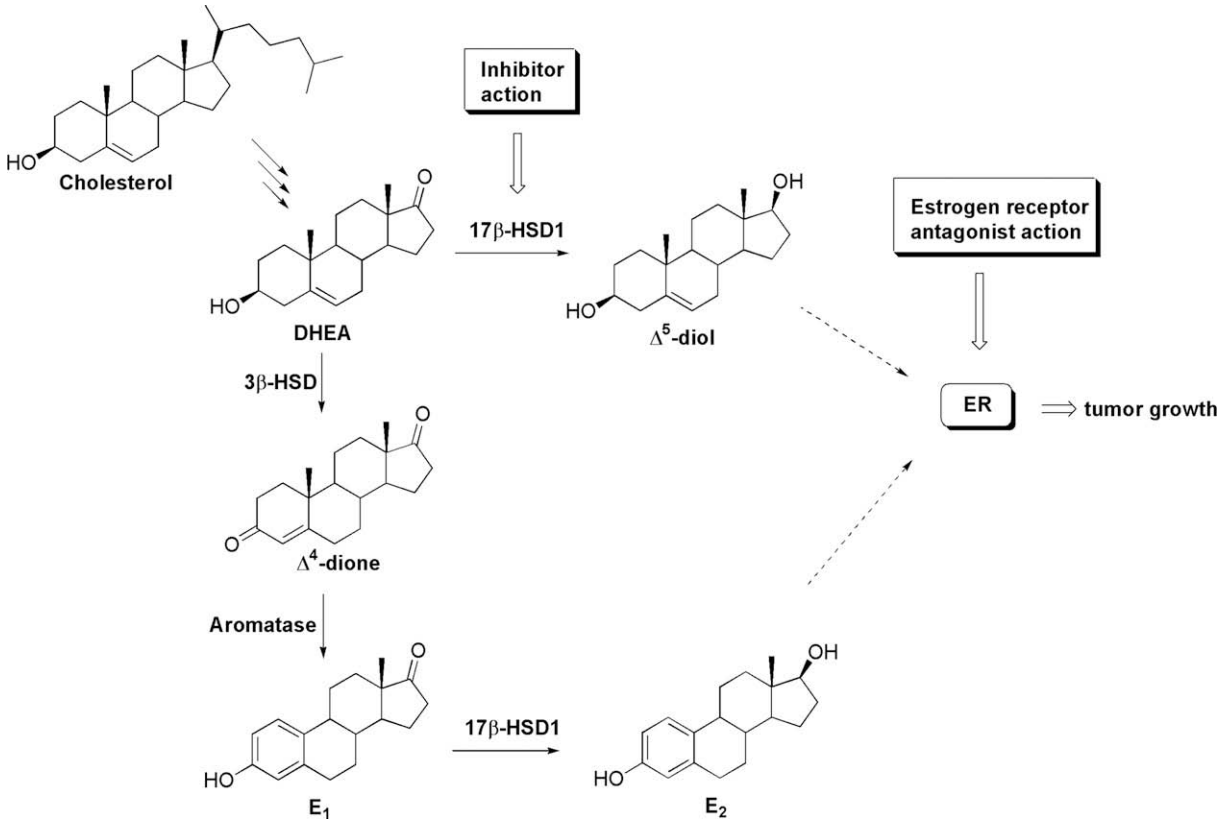

Fig. 1. Summary of the biosynthesis (steroidogenesis) of estrogens estradiol (E_2) and androst-5-ene-3β,17β-diol (Δ^5-diol) from cholesterol. For an overview of steroidogenic enzymes, see Ref. [3]; DHEA: dehydroepiandrosterone; androst-4-ene-3,17-dione (Δ^4-dione); E_1: estrone; 17β-HSD1: 17β-hydroxysteroid dehydrogenase type 1; 3β-HSD: 3β-hydroxysteroid dehydrogenase; ER: estrogen receptor.

17β-HSD1 inhibitors is the most extensive in the 17β-HSD family [7]. Among these early efforts, our laboratory contributed both alkylating-agent type irreversible inhibitors (compounds **1** and **2**) [12] and C6β-substituted reversible inhibitors (compounds **3**, **4** and **5**, Fig. 3) [13,14]. However, inhibitors of low-nanomolar potency are more recent, earlier compounds rarely reaching sub-micromolar activity.

One known way to increase the potency of an inhibitor is the so-called ''hybrid'' or ''bisubstrate'' inhibitor concept [15–17], in which a cofactor (or cofactor mimic) is covalently linked to a substrate (or substrate mimic). The resulting compound, when optimized, has the potential to have a binding free energy equal to the sum of the binding energies of the substrate and cofactor. The resulting affinity would therefore be equal to the product of the substrate and cofactor binding affinities. Moreover, this compound would have the required high specificity afforded by its substrate-binding moiety, and to some extent, by its specific linker conformation and length. Although the concept has been mainly used in structural probes of enzymatic mechanisms [18–20], it has led to some very potent therapeutic compounds, such as Mupirocin [21], a femtomolar-range inhibitor of bacterial isoleucyl-tRNA synthetase that is used as a topical antibiotic. With this in mind, one such compound, EM-1745 (compound **6**, Fig. 3), was designed as inhibitor of 17β-HSD1 [22–24]. In addition to report all data we previously published, this review article also reports unpublished data thus giving a final overview for this first E_2-adenosine hybrid compound inhibiting 17β-HSD1.

2. The design of EM-1745

Since the complexity of the NADPH cofactor (Fig. 2) is quite forbidding for chemical synthesis, an effort was made, via preliminary binding studies, to find out which part of the cofactor is essential in binding to 17β-HSD1. It was observed that adenosine diphosphate (ADP) and NADPH had similar binding affinities to 17β-HSD1. ADP constitutes a subpart of NADH without the nicotinamide ring and the second ribose molecule. Furthermore, in the 17β-HSD1/E_2/$NADP^+$ complex structure [25], the nicotinamide ring has a weaker electron density than the rest of the cofactor, which can be ascribed to a lack of direct interactions with the active site. It was thus assumed that the adenosine moiety was in itself sufficient to mimic the cofactor, since no other important interactions were visibly taking place elsewhere within the cofactor binding site, and that 17β-HSD1 can accommodate both NADPH and NADH, although it shows higher affinity for NADPH [26].

Having established the structure of the new cofactor moiety, and knowing that E_2 is a good steroidal substrate of 17β-HSD1, 3D structures of the 17β-HSD1/E_2 complex [27] and of the apoenzyme [28] obtained from X-ray diffraction

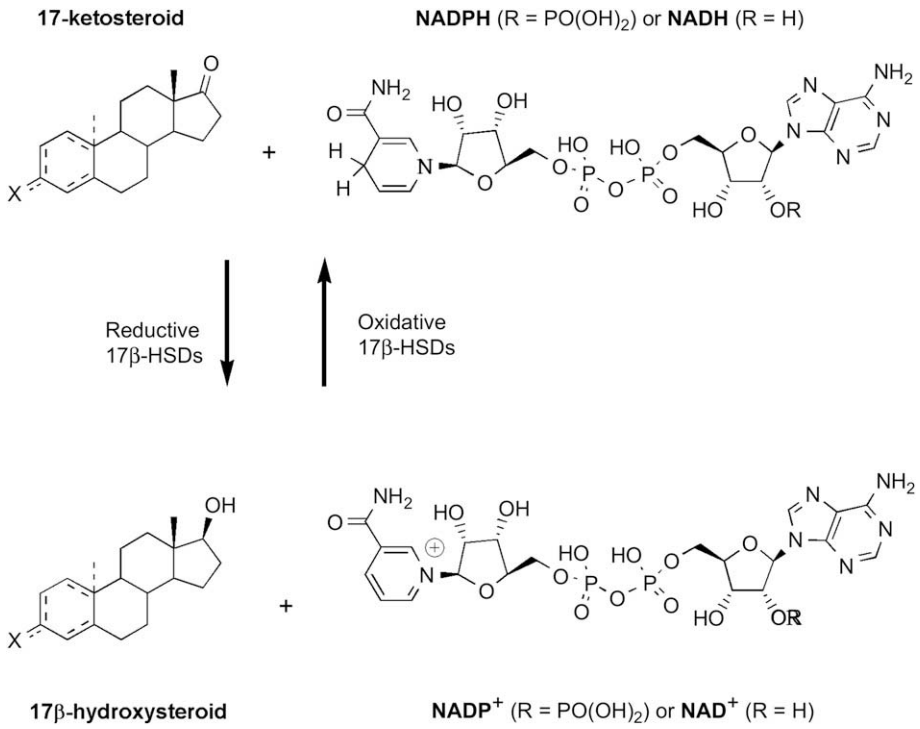

Fig. 2. The key role of cofactors NADPH and NAD^+ in the interconversion of 17-ketosteroids into 17β-hydroxysteroids.

of protein crystals were used in the design of the hybrid inhibitor. The adenosine moiety of the NADH cofactor and substrate E_2 were linked with an alkyl carbonyl linker attached to the C16β-position of the steroid and to the 5′-carbon of the adenosyl ribose. Inhibitors with linker chain lengths of 5, 6, 7, 8, 9 and 12 methylene groups were thus generated, submitted to an energy minimization procedure, and their energies examined. It was found that the lowest minimum energy was for the linker length of 7 methylenes, although the differences between 7, 8 and 9 were minimal. When the ability of the candidate molecules to fit into the active site was examined for the degree of fit into the active site using molecular surfaces, 8 and 9 methylenes seemed to afford the best fit. Modeling thus predicted that the ideal linker length for the E_2-adenosine hybrid inhibitor would be 8 or 9 methylenes. In parallel with the modeling study, chemical synthesis was made, focusing on linker chain lengths between 6 and 11 methylenes. Enzymatic assay in cell homogenates using NADH as cofactor showed that a linker chain length of 8 methylenes gave the best 17β-HSD1 inhibition, with an IC_{50} of 52 nM (Fig. 4) for the transformation of E_1 to E_2 [24].

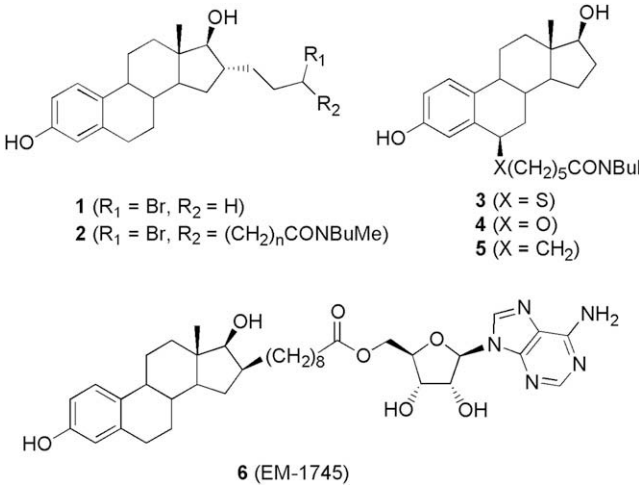

Fig. 3. Some 17β-HSD1 inhibitors.

Kinetic studies of the oxidative reaction using the purified enzyme with $NADP^+$ as cofactor showed that the inhibitor has a competitive behaviour towards E_1, as expected, with an apparent K_i of 3 nM for the transformation of E_2 into E_1 (Fig. 5) [22].

The structure–activity relationship (SAR) study of a series of EM-1745 analogues also clearly established the key role of both substrate and adenosine parts to generate potent inhibition [24]. It has shown that when adenosine was replaced with another group while keeping the E_2 moiety, inhibiting potency against 17β-HSD1 dropped considerably. Similar results were obtained when assaying a steroid-less molecule consisting the esterified adenosine attached to the linker alkyl chain. Finally, the hypothetic interactions of the substrate and cofactor parts with the enzyme were confirmed with an X-ray crystallographic structure of the complex (Fig. 6) [22].

3. The simplified hybrid inhibitors

Despite its high inhibitory activity on purified 17β-HSD1 and in cell homogenates, we identified two major drawbacks when using EM-1745 in intact cells or in vivo models. The

E_2-$(CH_2)_n$-Adenosine

n	Carbon 16 side - chain orientation	IC_{50} (nM) Assay 1	Assay 2
6	beta	–	430
7	beta	–	93
8 (EM-1745)	beta	–	52
9	beta	140	–
10	beta	120	–
10	alpha	310	–
11	beta		1000
E_1	–	600	810

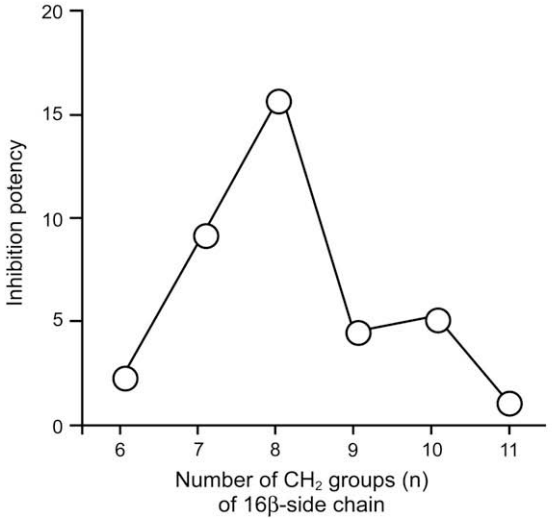

Fig. 4. Optimization of linker chain length between the E_2 substrate and adenosine cofactor moiety. The inhibition potency of hybrid compounds (IC_{50} of unlabeled E_1/IC_{50} of inhibitor) was reported relatively to the activity of E_1 used itself as an inhibitor for the transformation of [^{14}C]-E_1 into [^{14}C]-E_2 in presence of NADH as cofactor.

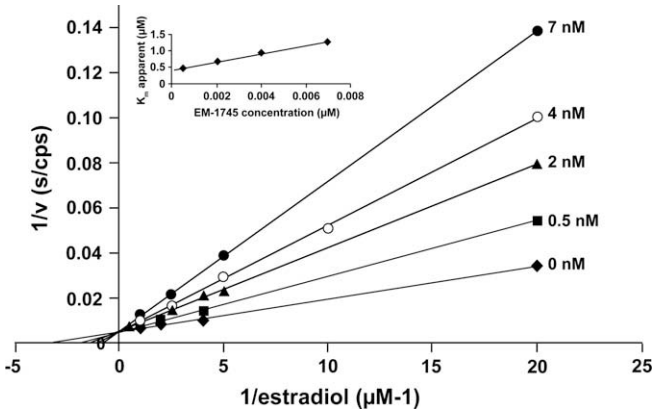

Fig. 5. Double reciprocal Lineweaver–Burk plot of the oxidative reaction (transformation of E_2 to E_1) showing competitivity of EM-1745 against E_2 in the presence of $NADP^+$ [22].

inhibitor might not penetrate the cellular membrane, and the inhibitor might be somehow metabolized, most probably at the ester bond site. To overcome this problem, a new, simplified hybrid inhibitor was then designed and synthesized [30]. This compound contains a *meta*-substituted aniline as a mimic of the adenosine moiety of EM-1745 and the ester bond was replaced with a carbon–carbon bond, much more resistant to metabolism (Fig. 7). The hope was for an aniline cofactor mimic to conserve interactions with the side chains of Asp65 and Ser11 which are present in the 17β-HSD1/EM-1745 complex. Longer linker chain lengths of 13–15 methylenes were used in this design, making up for the loss of the ester bond and ribose unit. Substituents included methyl ester, carboxylic acid, alcohol and bromomethyl groups (compounds **7**). To verify the relevance of both substituents, derivatives lacking the amino group substituent on the phenyl ring, and a simple unsubstituted aniline, were also synthesized. Enzymatic assay using a transfected human embryonic kidney (HEK)-293 cell homogenate showed a significantly lower potency of compounds **7**–**9** in inhibiting 17β-HSD1 when compared to EM-1745 (**6**) [31]. The optimal linker chain length was thus found to be 13 methylenes and the best substituent was the carboxylic acid group. Surprisingly, far from being beneficial, the presence of the amino group on the cofactor mimic (compound **8**) was detrimental to inhibition. In fact, compounds **8** and **9** at a concentration of 0.1 μM inhibited 27 and 49%, respectively, the transformation of E_1 (100 nM) into E_2 by 17β-HSD1 [31]. To better understand these phenomena, a structural study of the simplified hybrid inhibitors in the enzyme active site was necessary.

4. Crystallization of the compound 8/17β-HSD1 complex

Crystallization of a compound **8**/17β-HSD1 complex was attempted using the standard soaking method developed in Dr. Lin's laboratory [22] for steroidal ligands in 17β-HSD1. In short, a previously formed apoenzyme crystal is soaked in a concentrated buffered aqueous solution of the chosen ligand. This was attempted on several occasions, with the highest safe concentrations of ligand possible without endangering the crystal. When diffracted, no crystal showed any visible ligand electronic density. This could be due to low ligand affinity for the enzyme, or to an excessive static or dynamic mobility of ligand atoms (B-factor) caused by its high flexibility. As an alternative structural study method, it was decided to perform a molecular dynamics simulation of the complex.

5. Molecular dynamics study of the simplified hybrid inhibitors

Because molecular dynamics simulation is a quite computationally expensive procedure, the compounds submitted have to be chosen carefully. In our case, the compounds most likely to yield valuable information were those which had the best linker chain length and substituent group for inhibition and,

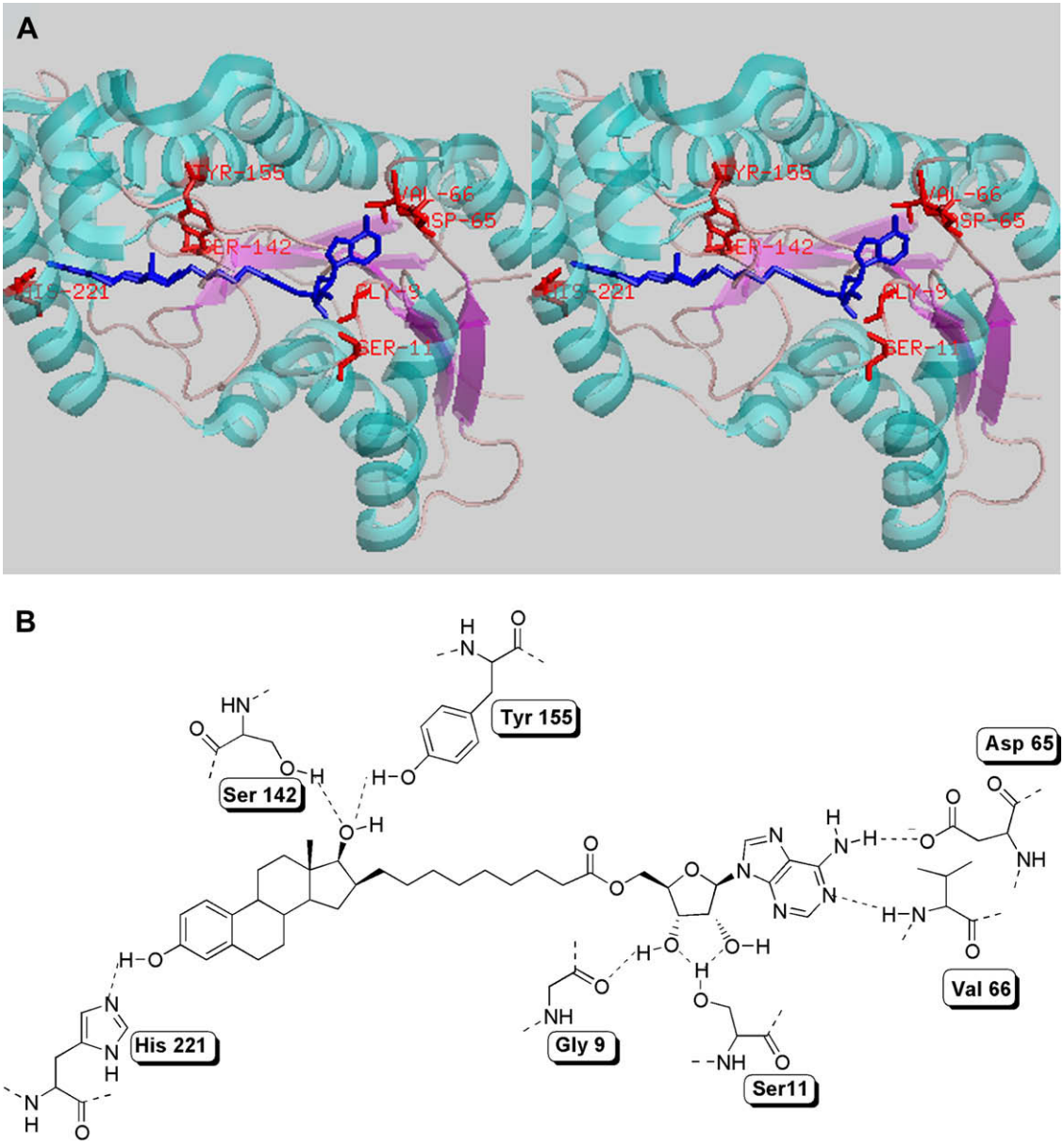

Fig. 6. A: Stereo representation of crystal structure of 17β-HSD1 complexed with EM-1745 generated using the PyMOL software [29]. B: Schematic diagram of the most important interactions between EM-1745 and the two binding sites: substrate (left side) and cofactor (right side). For a more detailed description of interactions see Fig. 7 of Ref. [22].

since we wanted to understand why the amino group was detrimental to inhibition, we had to compare inhibitor **9** (with the carboxylic acid only) with the one containing the amino group (compound **8**, Fig. 7). We also had to include one reference system, and the best choice for us was EM-1745 itself. Since automated docking of the simplified hybrid inhibitors is difficult due to the high number of torsions in the long alkyl chain, the molecules, compounds **8** and **9**, were built into the enzyme structure starting from the available 17β-HSD1/EM-1745 structure (PDB ID 1I5R) [22]. Topologies for the ligands were built using the server-based software PRODRG [32]. The carboxylic acid moiety was considered as ionized (carboxylate), since it would probably be mostly in that form in physiological conditions, given the pK_a of similar acids. Using the open-source molecular dynamics package GROMACS [33], these structures were solvated in a truncated octahedral box of single point charge (SPC) water molecules with walls 0.85 nm away from the protein. Sodium counterions were generated using the GROMACS program *genion* when needed to neutralize the negative charge. Using periodic boundary conditions (PBC) and the particle mesh Ewald (PME) method for long-distance electrostatic energy calculations, the systems were submitted to the steepest descents minimization method until convergence at $F_{max} < 1500\ kJ\ mol^{-1}\ nm^{-1}$. The minimized structures were then submitted to a "soaking" procedure in which the molecular dynamics is run for 20–100 ps using a time step of 2 fs with a harmonic restraint on the protein. This allows to relieve bad contacts between the solvent

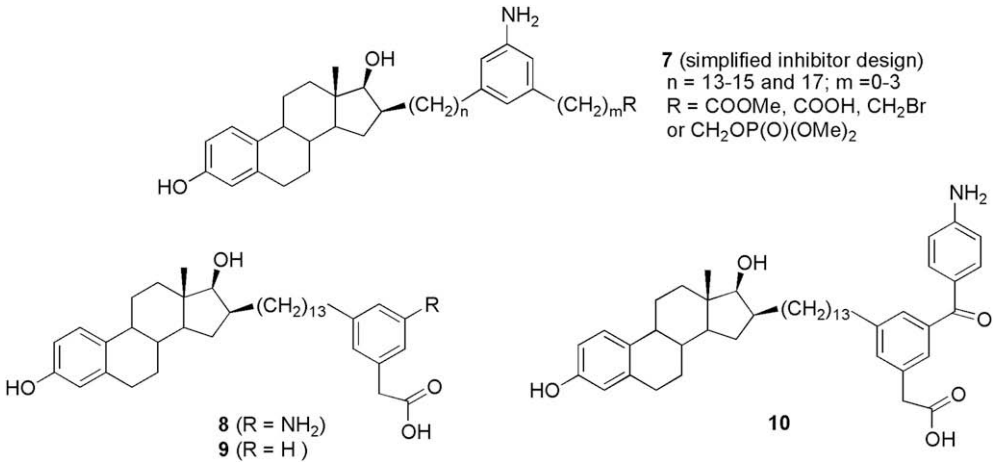

Fig. 7. The simplified hybrid inhibitors of first (compounds **7**–**9**) and second (compound **10**) generation.

(or inhibitor) and protein without undue deformation of the protein. The systems were then ready for the full, unrestrained 1 ns molecular dynamics simulation using constant temperature, pressure and molecule number (canonical or NPT ensemble) conditions at 300 K and 1 atm with Berendsen-type temperature and pressure coupling schemes and a time step of 2 fs.

The simulation results were analyzed using analysis tools available in GROMACS or with the visualization software VMD [34]. Our main interest was in the protein-inhibitor interactions, mainly in the formation and breaking of hydrogen bonds, as compared with what is observed in the reference inhibitor EM-1745 and also in the 17β-HSD1/E_2/$NADP^+$ complex [25]. Analysis of the compound **8** system trajectory showed that at no moment in the 1 ns simulation the amino group of the cofactor mimic was forming an H-bond with Asp65, or with any other residue. However, the carboxylic acid group formed a hydrogen bond with Ser11 after 800 ps, which is also an interaction present in the 17β-HSD1/EM-1745 complex [22]. In the case of compound **9**, the carboxylic acid group formed a stronger electrostatic interaction with Arg67/Arg37. This salt bridge corresponds to the one observed for the 3′-phosphate in the 17β-HSD1/E_2/$NADP^+$ complex, which confirmed that the carboxylate group could in that case be a bioisostere of the phosphate group.

Visual inspection of the 3D structure of compound **8** complexed with 17β-HSD1 showed the reason for the lack of interaction with the amino group. When the carboxylate group is in a proper position to interact with Ser11 or Arg67/Arg37, the amino group on the same aromatic ring, i.e., on the same plane, is simply not in the appropriate range or orientation for a hydrogen bond. To improve the hybrid inhibitor design, an alternate structure represented by compound **10** (Fig. 7), was generated and submitted to the same procedure as described previously. This inhibitor takes into account that in the substituted aniline design, interaction of the amino group with Asp65 cannot take place simultaneously with the one between the carboxylic acid group and Ser11. Indeed, in the adenosine of EM-1745, the two equivalent interacting groups are not situated in the same plane. To correct this problem, two different aromatic cycles are used in the hypothetic compound **10**.

The analysis of the trajectory showed the expected interactions between Asp65 and the amino group of **10** and between Arg67 and the carboxylate group of **10**. The graph of the distances between the interacting groups versus time (Fig. 8) shows that the amino group and the carboxylate group are simultaneously within hydrogen-bonding distances (applying a cut-off of 3.5 Å) with their intended target side chains from 400 ps until the end of the simulation.

A supplementary interaction between the keto group linking the two aromatic rings and the backbone of Ser11 was also observed in the compound **10**/17β-HSD1 complex (Fig. 9). Qualitative analysis of the molecular dynamics trajectories of the simplified hybrid inhibitor complexes of compounds **8** and **9** with 17β-HSD1 allowed us to conclude that the proposed cofactor mimics could not conserve all the interactions of the adenosine moiety of EM-1745 in the enzyme,

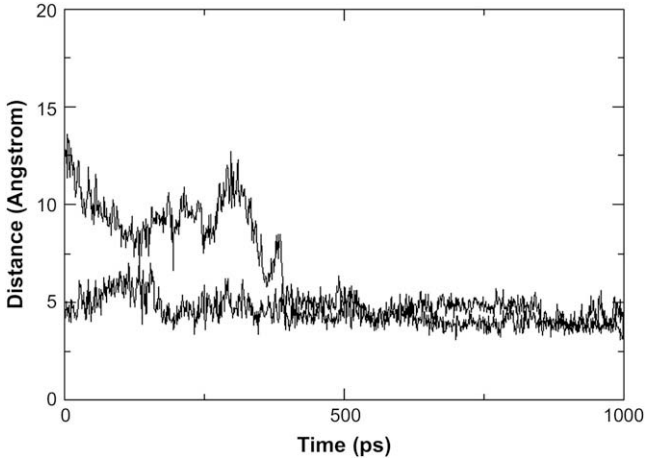

Fig. 8. Graph of distance versus time for the key elements of compound **10** interacting with 17β-HSD1. The upper curve represents the distance between the amino group and Asp65 whereas the lower curve is for the carboxylate group and Arg67.

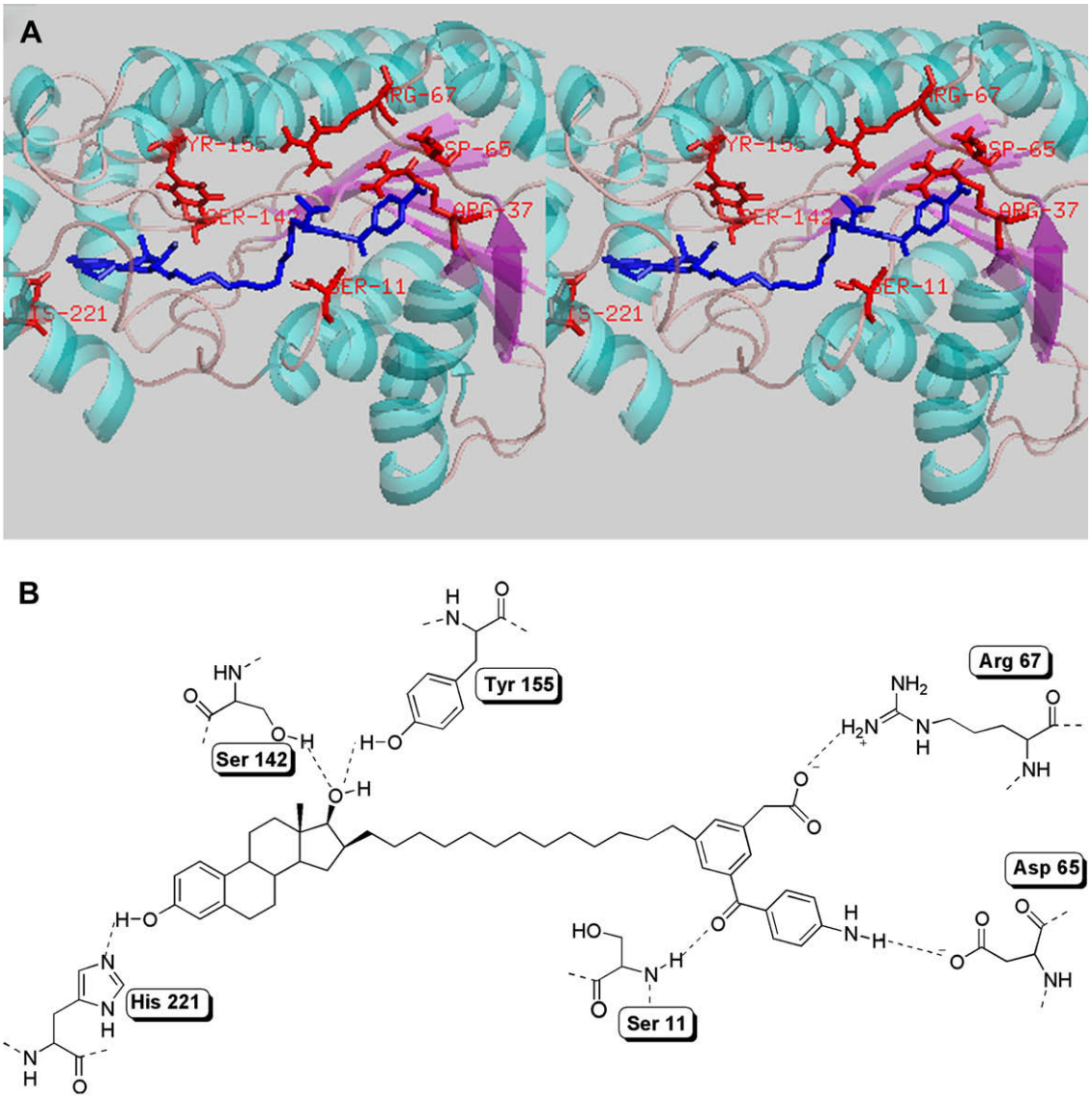

Fig. 9. A: Stereo representation of the 17β-HSD1/compound **10** complex. The structure was obtained from the last frame in the 1 ns molecular dynamics simulation and generated using PyMOL [29]. B: Schematic diagram of the most important interactions between 17β-HSD1 and compound **10**.

thus explaining the lower potency of **8** and **9** when compared to EM-1745 (compound **6**) [31]. The alternate inhibitor design (compound **10**) seems to be a better cofactor mimic but binding free energy calculations would be needed to estimate its potency as an inhibitor of 17β-HSD1 when compared with adenosine.

6. Inhibitory potency of EM-1745 in intact and homogenated cells

For a compound to be used therapeutically against a cytosolic enzyme such as 17β-HSD1, it must be able to cross the cellular membrane to reach its target. Also, for our purpose, which is hormone therapy, the product must not be cytotoxic. The use of intact cells in the inhibition assay is thus a good way to insure that a compound has a good potential to be used as a drug. Even when assayed in intact HEK-293 transfected cells, for which no exogenous cofactor is necessary, EM-1745 still retains some inhibiting power, albeit considerably lower (Fig. 10A). This means that it, or one of its metabolites which has a lower inhibiting potency, can penetrate the cellular membrane.

The preference of 17β-HSD1 for NADPH over NADH being known [9], we thought it would be interesting to compare the inhibitory activity of EM-1745 with NADPH versus NADH as exogenous cofactor in a cell homogenate assay. As expected, inhibitory potency of EM-1745 is lower when NADPH rather than NADH is used as cofactor (Fig. 10B). From these data, we estimated an IC_{50} of ~8 μM for EM-1745 when tested for the transformation of E_1 into E_2 in presence of NADPH. Furthermore, the results obtained in cell homogenates using NADPH are comparable to those observed in the intact cell assay. Considering how the different cofactors are distributed in intact cells [9], it is very probable that the

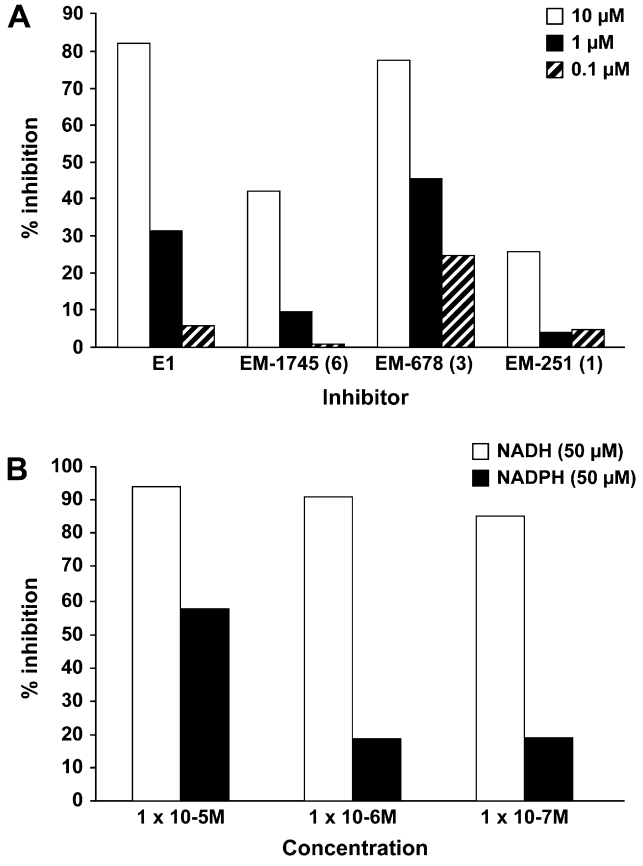

Fig. 10. A: Inhibition potency of various inhibitors (compounds **1**, **3**, **6** and E_1) in intact HEK-293 cells overexpressing 17β-HSD1 at three concentrations (0.1, 1 and 10 µM). The transformation of [^{14}C]-E_1 (60 nM) into [^{14}C]-E_2 was determined and the inhibition percentage calculated as previously reported in literature [14]. B: Inhibition potency of EM-1745 (**6**) versus NADH or NADPH in homogenated HEK-293 cells overexpressing 17β-HSD1. The transformation of [^{14}C]-E_1 (100 nM) into [^{14}C]-E_2 was determined and the inhibition percentage calculated as previously reported in literature [35].

enzyme will have to use NADPH for the reduction of E_1 in this system, and that itself could explain the lower potency of the hybrid inhibitor **6** when compared to the results obtained in cell homogenates.

7. Considerations on the mechanism of action of EM-1745

From the structural data of the 17β-HSD1/EM-1745 complex [22], it appears that the formation of a tertiary complex involving the enzyme/bisubstrate inhibitor/substrate or enzyme/bisubstrate inhibitor/cofactor is not possible since both cofactor and substrate-binding sites are occupied by the bound bisubstrate inhibitor EM-1745 in a closed active site (Fig. 6). However, the adenosine moiety of EM-1745 does not bind the cofactor binding site of 17β-HSD1 as strongly as the phosphated adenosine moiety of NADPH, and thus the bisubstrate inhibitor EM-1745 (without a phosphate group) cannot compete efficiently enough against the cofactor NADPH (with a phosphate group) as illustrated in Fig. 10B. The kinetic mechanism of human 17β-HSD1 was reported to be of random type [36], which means that either the cofactor or the substrate can randomly bind first to the enzyme. However, this result seems to be an exception since many of the HSDs which belong to the short-chain dehydrogenases/reductases (SDR) and aldo-keto reductases protein families appear to have an ordered mechanism [37]. Furthermore, the recent crystallographic and kinetic studies of Drosophila alcohol dehydrogenase suggest an ordered binding mechanism for this SDR enzyme [38]. Because the exact kinetic mechanism of human 17β-HSD1 remains controversial, we considered both kinetic mechanisms in our next discussion using the theory of bisubstrate inhibition, which has recently been described in detail as a diagnostic tool for mechanisms [39]. When deriving the kinetic equations of a random bi–bi mechanism for a bisubstrate inhibitor such as EM-1745 (no tertiary complex possible), it is clear that pure competitive behaviour would be observed not only for the substrate, but also for the cofactor. In the case of an ordered bi–bi reaction mechanism in which the cofactor binds first, the inhibitor still competes against the cofactor. Whatever the enzymatic mechanism, random or ordered, the affinity of the EM-1745 cofactor-mimic moiety is a key factor in the overall potency of a bisubstrate inhibitor targeting 17β-HSD1.

8. Perspectives for the design of 17β-HSD1 inhibitors

It cannot be concluded that EM-1745 is metabolized or incapable of crossing the cellular membrane, but it is clear that it is much less efficient against NADPH, the natural 17β-HSD1 cofactor most likely to be present in physiological conditions, than against NADH. For this reason, all future 17β-HSD1 inhibitors should be screened against exogenous NADPH in cell homogenates or pure enzyme. Indeed, in intact cells, it has been shown that endogenous NADPH is the prevalent cofactor for 17β-HSD1 [9]. In the case of EM-1745, the IC_{50} value of 52 nM was obtained against NADH, and does not necessarily reflect the reality of the natural environment of the enzyme. From the data presented here, we can infer that an increase of the EM-1745 hybrid inhibitor's potency against 17β-HSD1 could be achieved through an improvement of the affinity of its cofactor moiety. One way to do this would be to add a phosphate group on the 3′-carbon of the adenosine moiety, as present in NADPH. Although not easy, the chemical synthesis of a steroidal derivative of adenosine phosphate was recently reported in literature [40]. Another way would be to use a more efficient cofactor mimic such as the one in the compound **10** design (Fig. 7), in which the bothersome phosphate group is adequately replaced with a more chemically manageable carboxylic acid group. Other reported Rossman-fold-targeting compounds, such as gossypol derivatives, have also been tested for 17β-HSD1 inhibition [41]. They have been shown to be competitive against the oxidative cofactor NAD^+ with a K_i value as low as 2.2 µM [41], as compared to 250 µM for the adenosine esterified with the alkyl side chain spacer of EM-1745 [22]. The size and complexity of EM-1745 do not make it a very drug-like compound, but bigger compounds of higher complexity have been used in the past as drugs. However, in order to design smaller drug-like inhibitors, another strategy in the design of 17β-HSD1 inhibitors

could be to look for synergy with the cofactor rather than to compete against it.

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