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Review

Contribution to the development of inhibitors of 17β-hydroxysteroid dehydrogenase types 1 and 7: Key tools for studying and treating estrogen-dependent diseases

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (17β-HSDs) belong to a group of key enzymes involved in the biosynthesis of steroidal hormones by catalyzing the reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids. From three members known in the early nineties, the 17β-HSD functional family has grown to 15 members over the last 20 years. This growing number of 17β-HSD isoforms questioned the importance of each member, especially in their implication in estrogen- and androgen-dependent diseases, such as breast and prostate cancers. One of the strategies used to address the physiological importance of 17β-HSDs is to use potent and selective inhibitors. Furthermore, enzyme inhibitors could also be of therapeutic interest by reducing the level of estradiol (E2). Focusing on estrogens, we targeted 17β-HSD types 1 and 7, two enzymes able to transform the weak estrogen estrone (E1) into the potent estrogen E2. The present review article gives a description of different classes of inhibitors of 17β-HSD1 (C6-derivatives of E2, C16-derivatives of E2 as alkylating and dual action compounds, E2-adenosine hybrids, E2-simplified adenosine hybrids, and C16-derivatives of E1 or E2) and of inhibitors of 17β-HSD7, all these inhibitors developed in our laboratory. The chemical structures and inhibitory activity of these steroidal inhibitors, their potential as therapeutic agents, and their use as tools to elucidate the role of these enzymes in particular biological systems will be discussed.

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1. Introduction

Steroid hormones are produced from cholesterol by successive transformations involving different enzymes [1]. Among these enzymes, 17β-hydroxysteroid dehydrogenases (17β-HSDs) catalyze the stereoselective reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids, with nicotinamide adenine dinucleotide (phosphate), reduced or not, as cofactor (Fig. 1) [2–5]. Certain members of this enzyme functional family also catalyze, albeit to a lesser extent, the reduction and the oxidation at steroidal position 3. Non-steroidal compounds are also transformed by certain 17β-HSDs. While only three 17β-HSDs (types 1–3) were known at the beginning of the nineties [2], sev-

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Reductive 17β-HSDs (NADPH as cofactor)
Inhibitor Action
Oxidative 17β-HSDs (NAD^+ as cofactor)
17-ketosteroid (less potent steroid receptor ligand)
17β-hydroxysteroid (more potent steroid receptor ligand)

Fig. 1. Reversible transformations (reduction and oxidation) catalyzed by 17β-HSDs in presence of cofactor NAD(P)H or $NAD(P)^+$. Typical steroid substrates are C18-steroid estranes (X = OH) and C19-steroid androstanes (X = O or OH). *Note*: The stereochemistry at steroid carbons 5 (androstanes), 8, 9 and 14 was not shown in all drawings, but they are known to be 5α, 8β, 9α and 14α. Alpha (α) and beta (β) refer to the positioning of an hydrogen or another group under (α) or over (β) the steroidal plane.

eral studies have demonstrated the existence of other isoforms (fifteen until now [6–10]). Tissue distribution, substrate affinity and availability of substrates and cofactors thus contribute to direct the enzymatic transformation (i.e. the carbonyl reduction or the alcohol oxidation). Being able to convert inactive or less active steroid hormones into more potent ones and *vice versa*, 17β-HSDs play a key role in hormonal regulation and function in the human. A huge amount of knowledge on 17β-HSDs was published since the first report of a 17β-HSD activity by Ryan and Engel [11]. Different topics related to 17β-HSDs, such as multiple types (isoforms), nomenclature, characteristics, role, function, mechanism, 3D-structure, therapeutic applications and inhibitors, were however covered by a series of review articles [2–10,12–20]. These articles are recommended to readers who wish to increase their knowledge of the 17β-HSDs and their inhibitors.

Sexual hormones act via specific nuclear receptors, such as estrogen receptor (ER) and androgen receptor (AR), and they are known to respectively play important roles in the regulation of the growth of human breast and prostate cancers [21–28]. The inhibition of reductive 17β-HSDs is an attractive strategy for reducing the level of hydroxysteroids, such as estradiol (E2), 5-androstene-3β,17β-diol (5-diol) and testosterone (T), whereas the inhibition of oxidative 17β-HSDs may help maintain physiological levels of E2 and T (Fig. 2). So, there is a therapeutic potential to use a potent inhibitor of 17β-HSDs. Furthermore, from a research point of view, inhibitors of 17β-HSDs are also useful tools to elucidate the role of these enzymes in certain particular biological systems. Interested in the regulation of the formation of estrogens, we targeted the well known 17β-HSD1 and the more recently reported 17β-HSD7, two enzymes able to transform the weak estrogen estrone (E1) into the potent estrogen E2.

The development of potent inhibitors of aromatase and 5α-reductase [29], two key steroidogenic enzymes, and their use in clinical applications thereafter, stimulated the research on inhibitors of other key steroidogenic enzymes. We started a research program in 1991 aimed at developing inhibitors of 17β-HSDs. Focusing initially on 17β-HSD1, we gradually extended our program by targeting additional isoforms such as 17β-HSD2, 17β-HSD3, 17β-HSD5, and 17β-HSD7. The present manuscript summarizes our contribution to the development of steroidal inhibitors of 17β-HSD1 and 17β-HSD7.

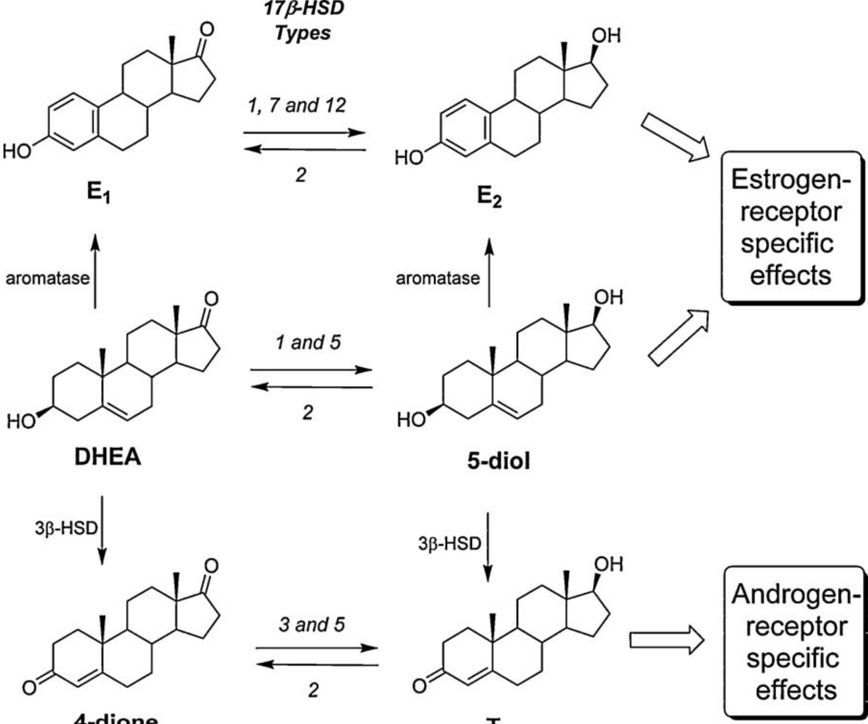

Fig. 2. Partial representation of substrates transformed by 17β-HSDs. For a more exhaustive representation of substrates, see Fig. 2 of Ref. [12].

1 (X = OH; Y = S; Z = CONBuMe; 6α/β; n = 2)
2 (X = OH; Y = S; Z = CONBuMe; 6α/β; n = 5)
3 (X = OH; Y = S; Z = CONBuMe; 6α/β; n = 7)
4 (X = OH; Y = S; Z = CONBuMe; 6α/β; n = 10)

5 (X = OH; Y = S; Z = CONBuMe; 6α; n = 5)
6 (X = OH; Y = S; Z = CONBuMe; 6β; n = 5)

7 (X = OH; Y = O; Z = CONBuMe; 6α; n = 5)
8 (X = OH; Y = O; Z = CONBuMe; 6β; n = 5)

9 (X = H; Y = S; Z = CONBuMe; 6α; n = 5)
10 (X = H; Y = S; Z = CONBuMe; 6β; n = 5)

11 (X = OH; Y = S; Z = CH_3; 6α; n = 5)
12 (X = OH; Y = S; Z = CH_3; 6β; n = 5)

13 (R = H; X = O; 6α)
14 (R = H; X = O; 6β)

15 (R = H; X = β-OH; 6α)
16 (R = H; X = β-OH; 6β)

17 (R = OCH_3; X = β-OH; 6β)

Fig. 3. C6-derivatives of E2 (compounds **1**–**17**) as reversible inhibitors of 17β-HSD1.

2. Inhibitors of 17β-HSD1

The first isoform of the 17β-HSD functional family reported in the literature, type 1 or human placenta estradiol dehydrogenase, catalyzes the reductive transformation of the less potent estrogen, E1, into the most potent one, E2. This enzyme also catalyzes, although at a lower rate, the transformation of dehydroepiandrosterone (DHEA) into 5-diol. This latter C19-steroid constitutes a weak estrogen [30], but its importance increases in postmenopausal women in which it becomes the major estrogen. Being involved in the formation of estrogens, 17β-HSD1 plays a key role in estrogen-dependent diseases, especially breast cancer, and this is the reason why we started developing inhibitors of this enzyme.

2.1. C6-derivatives of E2

Compounds **1**–**4** (Fig. 3) were originally designed as antiestrogens [31], but a screening study of their potency to inhibit the human placental cytosolic (17β-HSD1) transformation of E1 (3.6 nM) into E2 showed an interesting level of inhibition (IC_{50} = 0.26–11.2 μM), and the study was extended in order to optimize and rationalize this promising result [32]. The optimized compound **6** (EM-678), bearing a five methylenes 6β-alkylamide side chain, is a reversible inhibitor of the synthesis of E2 from E1 catalyzed by 17β-HSD1, whereas the corresponding 6α-epimer, compound **5**, is inactive (IC_{50} = 0.17 and 12.0 μM, respectively). Such dependence upon the stereochemistry (side-chain orientation at position 6) might indicate selective interactions between this type of compound and the enzyme. Unfortunately, an estrogenic activity was observed for compound **6**, as witnessed by its proliferative activity on two estrogen-sensitive (ER^+) cell lines (T-47D and ZR-75-1) in culture [32]. The instability of the thioether bond was hypothesized to be responsible for this effect. Thus, the cleavage of the thioether bond was suspected to cause the formation of estrogenic $\Delta^{6,7}$-E2.

Extending our work on these C6-derivatives of E2 bearing a *N*-methyl, butyl thiaheptanamide side chain at position 6, Tremblay et al. [33] tested three strategies to modify the biological profile (estrogenicity and inhibitory potency) of the lead compound **6** by synthesizing the E2 derivatives **7**–**12**. The study confirmed that the β-configuration of the side chain in position 6 led to a much better inhibition than the α-configuration. The replacement of the 3-OH by a hydrogen atom (**9** and **10**) as well as that of the amide group by a methyl (**11** and **12**) was clearly unfavorable for the inhibition of 17β-HSD1. Changing the thioether for an ether bond (**7** and **8**) decreased the estrogenicity by 10-fold on T-47D ER^+ cells of the lead compound while the inhibitory potency on 17β-HSD1 was only 5-fold lower for the transformation of E1 (100 nM) into E2 by homogenated HEK-293 transfected cells.

In another strategy, Cadot et al. [34] replaced the C–S bond by a C–C bond. For the inhibitory activity on 17β-HSD1, a slightly better enzyme inhibition was obtained for compounds having a 17β-hydroxy (**15** and **16**), when compared to 17-ketone analogues (**13** and **14**) and the 6β-configuration give a better inhibitory potency. The use of a more stable C–C alkylamide side chain is not detrimental for enzyme inhibition in T-47D cells and allowed the synthesis of a 2-methoxy (MeO) analogue **17** (which was not possible for the C–S lead compound **6**). However, the presence of the MeO group reduced the inhibitory activity on 17β-HSD1 (**17** *versus* **16**). For the proliferative activity on ER^+ cell lines, the C–C inhibitors tested were clearly less estrogenic than the C–S lead compound **6**. The results were less impressive in MCF-7 cells but followed the same pattern observed in T-47D. At 1 μM, the 2-MeO analogue **17** exerted a weak inhibition of 17β-HSD1, but no estrogenic activity in both ER^+ cell lines. Furthermore, at 1 μM, compound **16** inhibited 82% of 17β-HSD1 activity (82% for **6**) in intact T-47D breast cancer cells and it is 2.1-fold less estrogenic than compound **6** in the same cell line. Compound **16** did not inhibit the two other estrogenic 17β-HSDs, types 7 and 12, at 1 μM.

2.2. C16-derivatives of E2 which have dual action: 17β-HSD1 inactivators and ER antagonists

Based in part on previous studies on affinity labeling agents, several E2 derivatives bearing a short (three-carbon) side chain in position 17α or 16α were synthesized and tested for their ability to inhibit the transformation of E1 into E2 by 17β-HSD1 (cytosolic fraction of human placenta) [35,36]. Among several chemical groups used in this study, better enzyme inhibition was obtained

Fig. 4. C16-derivatives of E2 (compounds **18**–**24**) as irreversible inhibitors of 17β-HSD1. Those with an alkylamide side chain are estrogen-receptor antagonists.

for compounds having a good leaving group at the end of a C16α-side chain. Thus, 16α-bromopropyl-E2 (**18**) (Fig. 4) efficiently inhibits 17β-HSD1 transforming E1 (2–6 nM) into E2 with an IC_{50} value of 0.46 μM. Its 17-keto analogue similarly inhibits the enzyme activity. Since this kind of compound inhibits 17β-HSD1 in a time-dependent manner and since enzymatic activity cannot be restored later, we concluded that this was an irreversible inhibitor (inactivator type). This conclusion is in accordance with the correlation observed between the ability of the tested leaving groups (F, Cl, Br, I) to dissociate and their potency to inhibit 17β-HSD1 when added at the end of the 16α-propyl chain [36]. Addition of untritiated E1 protects the enzyme against the inactivation caused by **18** (EM-251) suggesting a competitive inhibitor. The inhibitor **18** was shown to be inactive against types 2 and 3 isoforms [37,38]. These results proved that selectivity could be obtained with this type of inhibitor in spite of their high reactivity toward nucleophilic residues of amino acids in the active site of the enzyme. Moreover, it was shown that the 16-position of E2 was in a good location to introduce a pharmacophore responsible for the selective inhibition of 17β-HSD1. The bromoalkyl inhibitory group was then selected for an optimization study focusing on the side-chain length and orientation (α and β). In this study [39], the most potent inhibitory effect was observed when the length of the side chain was 3 or 4 carbons. However, the 16β-bromopropyl easily undergoes cyclization with 17β-OH and its inhibitory effect on the enzyme is then lost. Consequently, 16α-bromopropyl-E2 (**18**), 16α-bromobutyl-E2 (**19**), and 16β-bromobutyl-E2 (**20**) were the best inhibitors obtained from this study.

Since the representative inhibitor **18** was found to be agonist on the estrogen-sensitive (ER^+) human breast cancer cell line ZR-75-1, three strategies were designed to eliminate their intrinsic estrogenicity. In the first one, a structurally modified antiestrogen was designed containing a bromobutyl side chain at the 16α-position [40]. Biological results revealed that compound **21** showed a lower potency than **18** (IC_{50} = 16 and 0.41 μM, respectively) to inhibit the human placental 17β-HSD1 transforming E1 (3.6 nM) to E2. Interestingly, it did not display estrogenic effects and retained satisfactory antiestrogenic properties as demonstrated on ZR-75-1 cells [40]. Nevertheless, it appeared from these studies that the bulky alkylamide side chain at the 7α-position in combination with the 16α-bromobutyl side chain was responsible for the lower 17β-HSD1 inhibition. In the second approach to eliminate the residual estrogenic activity of **18**, new potential dual-action inhibitors were designed by substituting the 16α-position of E2 with various bromoalkylamide side chains containing two pharmacophores within the same chain. After optimization [41–44], the inhibitory potency of compounds **22** and **23** was evaluated by measuring the reduction of E1 (1 nM) into E2 using both pure and transfected 17β-HSD1. Disappointing results were however obtained when they were tested in homogenated HEK-293 cells overexpressing 17β-HSD1 (IC_{50} = 13 and 38 μM, respectively for **22** and **23**) [40]. Fortunately, compound **22** did not display estrogenic effects and did show 74% of antiestrogenic activity at 1 μM when tested on ZR-75-1 cells [43]. In fact, it inhibited the E2 (0.1 nM)-induced cell proliferation close to the level reached by untreated (control) cells. Compound **23** showed a slight agonist activity and weak antagonist effects at the same concentration [40]. In conclusion, the best dual-action inhibitor synthesized, compound **22**, showed an IC_{50} of 13 μM for 17β-HSD1, while displaying antiestrogenic activity at 1 μM. In the third approach to block the estrogenicity of **18**, we synthesized its 2-MeO analogue, compound **24** [45]. This compound is clearly a less potent inhibitor than **18** (IC_{50} = 5.8 and 1.0 μM, respectively) for the reduction of E1 (100 nM) into E2 by 17β-HSD1 overexpressed in HEK-293 cells (homogenated cells), but also less estrogenic when tested on ER^+ T-47D cells. Contrary to **18**, no estrogenic-like effect was observed for **24** in the estrogen-sensitive tissues (uterus and vagina weight) of ovariectomized mice when using 10 μg of inhibitor injected subcutaneously twice daily for 9 days.

2.3. E2-adenosine hybrid compounds

Based on an initial idea to make use of the binding energies of both the substrate and cofactor sites [46], structure–activity relationship studies [40–44] and 3D-structure of 17β-HSD1 [47–49], a series of E2-adenosine hybrid compounds **25**–**34** (Fig. 5) were synthesized as enzyme inhibitors [50–52]. These compounds were designed to interact with two binding sites of the enzyme: the adenosine moiety to interact with the cofactor binding site and the E2 moiety to interact with the substrate binding site. The E2 and adenosine components are linked by an alkyl-chain spacer

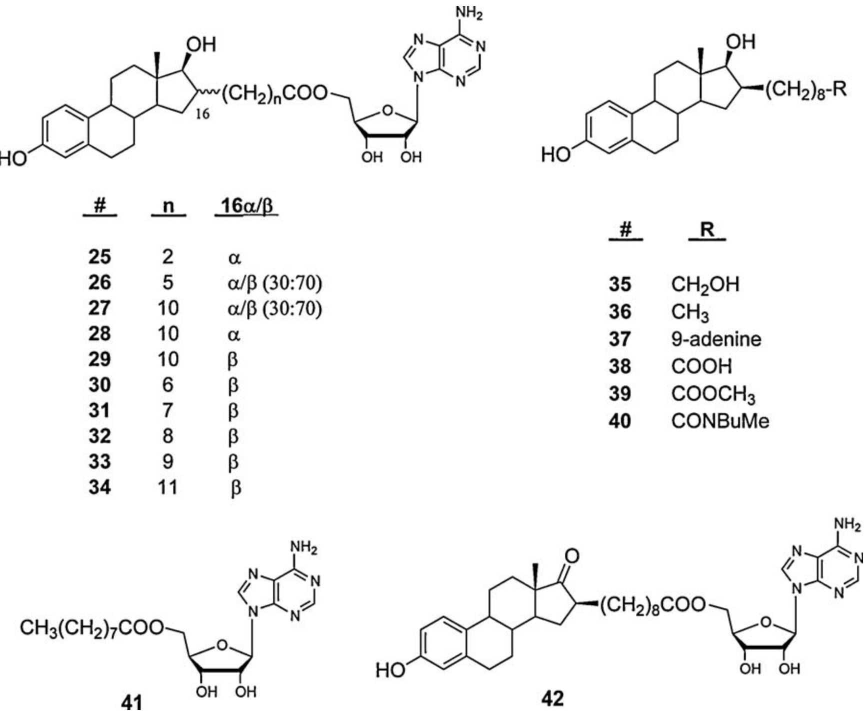

Fig. 5. C16-derivatives of E2 (compounds **25**–**42**) as reversible dual-site inhibitors of 17β-HSD1 (E2/adenosine hybrid compounds). They are competitive inhibitors *versus* both the enzyme substrate E1 and the cofactor NADH.

($(CH_2)_n$). The cytosolic fraction of transfected HEK-293 cells was the first source of 17β-HSD1 used to test the synthesized compounds. The E2-adenosine hybrids were found to inhibit the enzyme reduction of E1 (100 nM) into E2 with IC_{50} values ranging from 0.052 to 13.5 μM, depending on the length of the spacer (n) between the two components (n = 2–11) [51]. The optimal spacer length was determined as being 8 methylene groups (compound **32**; EM-1745), which corresponds pretty well to the length that was predicted by modeling [52]. By synthesizing and testing analogues of **32** having no adenosine moiety (compounds **35**–**40**) or no E2 nucleus (compound **41**), it was clearly demonstrated that both components (E2 and adenosine) are crucial for inhibitory activity [51]. A new and more efficient synthesis of **32** as well as its C17-ketone analogue **42** was next reported and, contrary to what was expected, the C17-ketone **42** was a less (3 times) potent inhibitor than the C17-alcohol **32** [53]. The mode of inhibition and K_i value of the most efficient hybrid inhibitor was further studied. The Lineweaver–Burk plots for E2 oxidation by purified human 17β-HSD1 indicated a typical reversible competitive inhibition against the substrate E2 with a calculated K_i of 3.0 nM [52]. The crystal structure of the inhibitor **32**/17β-HSD1 complex at a resolution of 1.6 Å provides evidence of strong interactions between the steroid (E2) and cofactor moiety (adenosine) and some amino acids of the enzyme [52]. These last results confirmed that the hybrid inhibitor **32** works by interacting with the two binding sites (substrate and cofactor).

2.4. E2-simplified adenosine hybrid compounds

Despite its high inhibitory activity on purified 17β-HSD1 and in cell homogenates, we identified some major drawbacks when using EM-1745 (**32**) in intact cells [54]. In fact, the lack of potent inhibition in intact cells made be attributed to the possibility that, it does not penetrate the cell membrane, does not compete well against NADPH due to the lack of a phosphate group or it is metabolized rapidly. To overcome these problems, the adenosine moiety of **32** was replaced and the ester bond eliminated. Hybrid inhibitors of second generation were then designed and synthesized using different approaches.

In the first approach, seventeen simplified hybrids represented by general structure **43** (Fig. 6) contain a meta substituted aniline as a partial mimic of the adenosine moiety of the potent inhibitor **32**, whereas the ester bond was replaced by a carbon–carbon bond, resistant to metabolism [55,56]. The aim was to generate an aniline based cofactor mimic that would interact with the side chain of Asp65 or Ser11, which interactions are present in the **32**/17β-HSD1 complex. Longer spacer chain lengths of 13–15 and 17 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, bromomethyl, and phosphotriester groups. To verify the relevance of both phenyl substituents, derivatives **44** and **45**, respectively lacking the amino group or the CH_2COOH group on the phenyl ring, were also synthesized. Enzymatic assay using a transfected HEK-293 cell homogenate showed a significantly lower potency of **43**–**45** in inhibiting 17β-HSD1 when compared to **32** [56]. The optimal linker chain length was found to be 13 methylenes and the best substituent was the carboxylic acid group (compound **46**). Surprisingly, the presence of the amino group on the partial cofactor mimic was detrimental to inhibition. In fact, compounds **46** and **44** at a concentration of 0.1 μM inhibited 27 and 49%, respectively, the transformation of E1 (100 nM) into E2 by 17β-HSD1 overexpressed in HEK-293 cells (homogenated) [56]. Crystallization of **46**/17β-HSD1 complex was attempted using a soaking method developed by Dr. Lin's laboratory for steroidal ligands [52]. When diffracted, no crystal showed any visible ligand electronic density [54]. This could be due to low ligand affinity for the enzyme, or to an excessive static or dynamic mobility of ligand atoms caused by its high flexibility. Alternatively, a molecular dynamics simulation of **46** complexed with 17β-HSD1 [54] demonstrated that both NH_2 and CH_2COOH groups cannot interact simultaneously with the targeted amino acids, suggesting that the positioning of these two groups should be optimized before obtaining a better enzyme inhibition.

43
n = 13, 14, 15 or 17;
m = 0, 1, 2 or 3;
R = $COOCH_3$, COOH, CH_2OH, CH_2Br or $CH_2OP(O)(OCH_3)_2$

44 (R_1 = H, R_2 = CH_2COOH)
45 (R_1 = NH_2, R_2 = H)
46 (R_1 = NH_2, R_2 = CH_2COOH)

Fig. 6. C16β-derivatives of E2 (compounds **43**–**46**) as reversible dual-site inhibitors of 17β-HSD1 (E2/simplified adenosine (phenyl derivative) hybrid compounds).

In the second approach, Tremblay et al. [57] hypothesized that molecules containing both hydrophobic (steroid) and hydrophilic (amino acid side chain) components could be interesting candidates to interact with both the steroid binding site and some amino acid residues of the cofactor binding site of the enzyme. In an exploratory study, nine E2 derivatives bearing various polar chemical groups (one, two or three amino acids) and represented by **47** (Fig. 7) were synthesized as prototypes of such inhibitors. The enzymatic screening revealed that none of the novel compounds can inhibit the reductive activity of 17β-HSD1. This result was quite predictable considering that the side chain length and diversity of the amino acids was not adapted to mimic the right part of **32** (EM-1745). In fact, the aim of this exploratory study was first to synthesize and characterize such kind of peptidosteroids.

In the third approach, libraries of C16-derivatives of E2 were designed and prepared by solid-phase synthesis to target both the substrate and cofactor binding sites. After first investigating the effectiveness of five different linkers to link the phenol of a 16β-(azidopropyl)-E2 to polystyrene resin and performing solid-phase chemistry [58,59], we decided to develop a new linker. The versatility of this multidetachable sulfamate linker is its ability to generate two kinds of compounds depending on the chosen cleavage method (Fig. 8) [60]. Thus, acidic conditions provide an aryl sulfamate derivative whereas nucleophilic conditions provide a phenol derivative; the former is useful for generating steroid sulfatase inhibitors [61,62] and carbonic anhydrase inhibitors [63], and the latter is useful for generating 17β-HSD1 inhibitors as well as ER agonists and ER antagonists [64]. The new linker can be used for the solid-phase synthesis of steroidal and non-steroidal compounds, thus adding to its potential [65–67]. Ciobanu and Poirier [68] thus synthesized two libraries of 16β-aminopropyl-E2 derivatives **48** (48 sulfamates and 48 phenols) designed with the aim of targeting mainly the enzyme steroid sulfatase and, in addition, the enzyme 17β-HSD1. Selected members of the phenol library developed by this methodology were tested for the inhibition of 17β-HSD1. At concentrations of 1 and 10 μM, they inhibited, respectively 17–42% and 57–80% of the transformation of E1 (100 nM) into E2 in homogenated HEK-293 cells overexpressing 17β-HSD1. While hits from this series were less potent than a reference inhibitor known from literature, inhibitory activity was however detected and further optimization of results by broadening the selection of building blocks (amino acids and carboxylic acids) is possible.

Using the multidetachable sulfamate linker, Bérubé et al. prepared three libraries of E2 derivatives **49** (30, 63 and 25 compounds) with two or three levels of molecular diversity (addition of one or two amino acid(s) followed by adding a carboxylic acid) [69]. For the elaboration of libraries, different kinds of building blocks (amino acids and carboxylic acids) were selected to potentially interact with the cofactor binding site. Members of two libraries

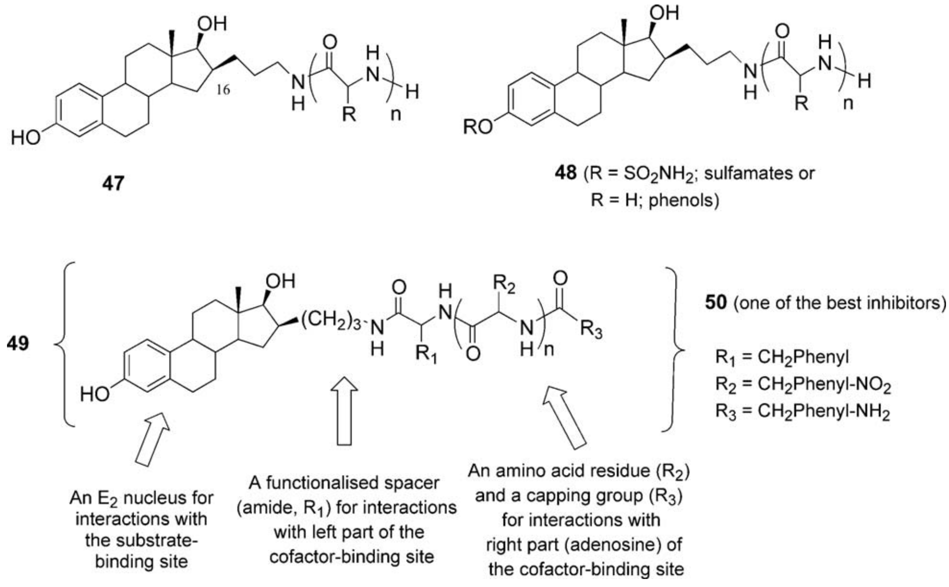

Fig. 7. C16β-derivatives of E2 (compounds **47**–**50**) as reversible dual-site inhibitors of 17β-HSD1 (E2/simplified adenosine (peptide derivative) hybrid compounds).

Fig. 8. The multidetachable sulfamate linker is a versatile tool for generating sulfamate derivatives or phenol derivatives according to the cleavage conditions (acid or nucleophile, respectively). X and Y correspond to the B-ring of a steroid nucleus or a functionalized alkyl group and a hydrogen atom.

were screened on homogenated HEK-293 cells overexpressing 17β-HSD1 (transformation of E1 (100 nM) into E2). E2 derivatives with 3 levels of molecular diversity gave better inhibition than compounds with two levels of diversity. Furthermore, 26 compounds gave better inhibition than **18** (59% at 1 μM), used as a reference compound, and 8 of these compounds gave better inhibition than unlabeled E1 (73% at 1 μM), used as an inhibitor. One of the best results was obtained with compound **50** (82% of inhibition at 1 μM). However, none of the members of both libraries gave better inhibition than **32** (EM-1745), suggesting that key interactions with cofactor binding sites were not optimized. Unfortunately, the members of the third library of E2 derivatives (those suspected to more closely mimic the **32**/17β-HSD1 interactions) were not pure enough to be tested. The studies reported above clearly reveal the complexity of maximizing interactions between the inhibitor and the enzyme. In fact, predicting the structure of a molecule producing strong interactions is not easy to do considering the side-chain flexibility. The use of combinatorial chemistry to generate diversified libraries is an interesting strategy, but it certainly necessitates the synthesis of a huge number of target compounds before being successful.

2.5. C16-derivatives of E1 or E2 (reversible inhibitors)

Extending their work at position C16 of E2 and E1, which resulted in the development of alkylating inhibitors (Section 2.2) and paved the way for the synthesis of E2-adenosine hybrid inhibitors (Section 2.3), Poirier et al. [70] introduced a short side chain (R group) at position C16 in order to potentially generate an interaction with an amino acid of the catalytic triad or with another of the amino acids surrounding the nicotinamide residue of NAD(P)H cofactor. The 24 compounds divided in three series, represented by compounds **51**–**53** (Fig. 9), were tested as inhibitors for the reduction of E1 (100 nM) by purified 17β-HSD1. Better inhibitory activities were obtained with the third series (compound **53**), especially for 16β-phenylmethyl-E2 (**54**) producing IC_{50} values ranging from 0.79 to 1.0 μM. For the aryl (R) derivatives, the inhibitory potency increases in the following order: **51** < **52** < **53**, suggesting that the presence of a more flexible 16β-methylene group allows a better positioning of the aryl moiety (fewer steric interactions with enzyme amino acid residues). Compound **54** with a benzyl group was docked into the 3D-structure of 17β-HSD1 (configuration when in complex with E2) showing that the interactions of the inhibitor with the catalytic triad composed of Tyr155, Lys159 and Ser142 were not affected much by the C16-R group, but the steroid nucleus was shifted from the E2 position to enable the phenyl group to get closer to a hydrophobic pocket formed by residues Leu96 and Val196. Clearly the interaction involving the phenyl could be improved for better enzyme inhibition.

Better enzyme inhibitions were obtained by adding a carbamoyl group ($CONH_2$) on the 16β-benzyl ring of E1 or E2. Since this amide group can be found in the nicotinamide moiety of 17β-HSD1 cofactor (NADPH or NADH), we hypothesized that it could generate key interaction with amino acid neighboring the catalytic site. In fact, our results and those from other researchers [12,20,33,71–73] clearly demonstrated the importance of an alkylamide group for inhibiting some 17β-HSDs. Among the synthesized compounds **55**–**62**, **55** was the most potent inhibitor with an IC_{50} value of 44 nM for the transformation of E1 (60 nM) into E2 by 17β-HSD1 in intact T-47D cells [74]. We also demonstrated that **55** selectively inhibited type 1, because it did not inhibit reductive types 7 and 12 at 10 μM and did not inhibit types 2 and 5 at 1 μM. A meta carbamoylbenzyl group, which probably mimics the nicotinamide moiety of the cofactor, appears to be an important characteristic of this new class of 17β-HSD1 inhibitors. A kinetic study demonstrated that **55** inhibited the reduction of E1 to E2 by purified human 17β-HSD1 with a K_i of 0.9 nM [75]. Under the same conditions, the K_m for this transformation was reported to be 30 nM [76]. The study showed that compound **55** acted as a reversible and competitive inhibitor against E1 [75]. Interestingly, the proliferative activity induced by a physiologic concentration of E1 (0.1 nM) in T-47D ER^+ cells was reduced by 62% using **55**, and this effect cannot be the result of an antiestrogenic activity of **55** (by its direct action on ERα) [74]. The cell growth reduction was not 100% because a weak (38%) estrogenic activity was induced by compound **55** itself, an E2 derivative. As exemplified by the synthesis of compounds **63**–**65**, three strategies were tested to decrease the undesirable residual estrogenic activity. Although the resulting inhibitors **63**–**65** were less estro-

51 (X = O; R = Alkyl, Aryl, etc.)
52 (X = 17β-OH; R = Alkyl, Aryl, etc.)

53 (R = Alkyl, Aryl, etc.)
54 (R = Phenyl)

55 (R_1 = H, R_2 = $CONH_2$, R_3 = H, X = 17β-OH)
56 (R_1 = H, R_2 = $CONH_2$, R_3 = H, X = O)
57 (R_1 = CH_3, R_2 = $CONH_2$, R_3 = H, X = 17β-OH)
58 (R_1 = CH_3, R_2 = $CONH_2$, R_3 = H, X = O)
59 (R_1 = H, R_2 = H, R_3 = $CONH_2$, X = 17β-OH)
60 (R_1 = H, R_2 = H, R_3 = $CONH_2$, X = O)
61 (R_1 = CH_3, R_2 = H, R_3 = $CONH_2$, X = 17β-OH)
62 (R_1 = CH_3, R_2 = H, R_3 = $CONH_2$, X = O)

63 (R_1 = R_2 = R_3 = H)
64 (R_1 = OH, R_2 = OCH_3, R_3 = H)
65 (R_1 = OH, R_2 = H, R_3 = $(CH_2)_{10}CONBuMe$)

Fig. 9. C16-derivatives of E2 (compounds **51**–**65**) as reversible inhibitors of 17β-HSD1.

genic than our lead compound **55**, they exhibited an important drop in inhibitory activity on 17β-HSD1.

In collaboration with Dr. S.X. Lin, crystals of **55**/17β-HSD1 (binary complex) and **55**/17β-HSD1/NADP+ (ternary complex) were obtained and analyzed [75]. The crystals diffracted to a resolution of 2.2 Å and 2.0 Å for the binary complex and ternary complex, respectively. Both hydrogen-bonding as well as van der Waals interactions are present in the two complexes. In addition to weak hydrophobic interactions and a strong hydrogen bond between the O-17 of **55** and the Oγ of Ser142, a π–π interaction is present between the benzylamide ring and Tyr155 in both complexes. There is no interaction between O3 and His221 because the E2 core of **55** is shifted in the structures along the line of the steroid backbone. Based on 3D structures of complexes and kinetic studies, compound **55** does not interfere with the NADP+ binding site, thus differing from the E2-adenosine hybrid inhibitor **32**. Since compound **55** is a more potent inhibitor of 17β-HSD1 than compound **32** in intact cells, and its chemical synthesis is straightforward, it represents a superior drug candidate than compound **32**.

3. Inhibitors of 17β-HSD7

17β-HSD7 is a membrane-associated enzyme expressed in typical steroidogenic tissues such as ovaries and testis, but also in the uterus, placenta, breast, prostate, liver and in several neural tissues [77–80]. It converts E1 into E2, the most potent female hormone, and also deactivates, to the same extent, the powerful androgen dihydrotestosterone (DHT) into 5α-androstane-3β,17β-diol (3β-diol) [79], an androgen metabolite with intrinsic estrogen-like effects [81]. 17β-HSD7 also functions as a zymosterone reductase and is thus involved in postsqualene cholesterogenesis [82–84]. The precise physiological role of human 17β-HSD7 in estrogen biosynthesis is not clear, but its dual enzymatic activity and its widespread distribution indicate a tendency to act as an intracrine regulator of steroid metabolism, increasing the concentration of estrogens in its surroundings. To better understand the biological role of this new steroidogenic enzyme, we started developing inhibitors of 17β-HSD7.

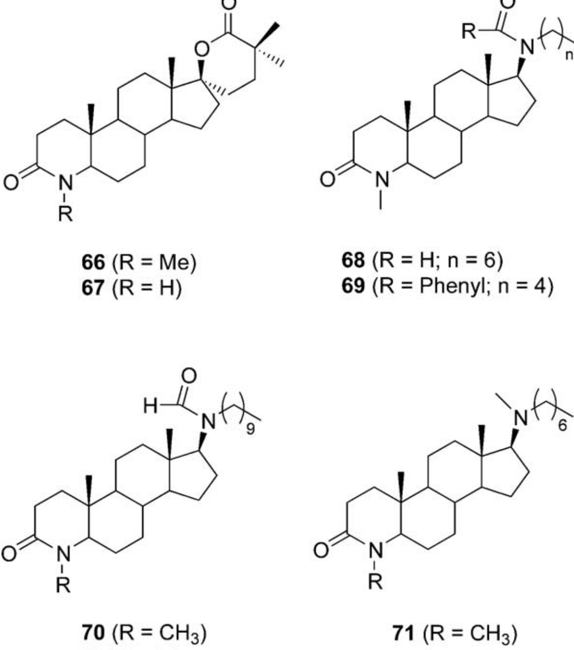

Fig. 10. C17-derivatives of 4-methyl-4-aza-5α-androstan-3-one (compounds **66**–**71**) and 4-aza-5α-androstan-3-one (compounds **72** and **73**) as inhibitors of 17β-HSD7.

From a screening done with a series of 150 compounds belonging to different classes (natural hormones, anti-hormones, phytoestrogens, known inhibitors of other steroidogenic enzymes), Bellavance et al. [85] identified four C-19 steroids (compounds **66**–**69**) (Fig. 10) that were able to decrease the transformation of E1 (100 nM) into E2 in intact human 17β-HSD7 transfected HEK-293 cells. Our preliminary study demonstrated that a 4-aza-5α-androstane nucleus is an important structural feature and that its inhibitory potency is greatly improved by introduc-

Table 1
Characteristics of key inhibitors of 17β-HSD1 and 17β-HSD7 identified from our studies.

Inhibitor #	Chemical structure and number of chemical steps in brackets	Target isoform	Inhibitory potency	Selectivity *versus* other enzymes	Estrogenic activity[a]
6 (EM-678)	HO; OH; S-$(CH_2)_5$-CONBuMe (4 + 2)	17β-HSD1 (reversible)	IC_{50} = 203 nM (T-47D intact cells). IC_{50} = 170 nM (cytosolic fraction of human placenta)	17β-HSDs 7, 12	Less than E2 [32,33]
16	HO; OH; $(CH_2)_6$-CONBuMe (8)	17β-HSD1 (reversible)	IC_{50} = 216 nM (T-47D intact cells)	17β-HSDs 7, 12	Less than **6** [34]
18 (EM-251)	HO; OH; $(CH_2)_3$-Br (6)	17β-HSD1 (irreversible competitive)	IC_{50} = 320 nM (Transf. HEK-293 homogenated cells). IC_{50} = 170 nM (cytosolic fraction of human placenta)	17β-HSDs 2, 3	Less than E2 [40]
22	HO; OH; Br; $(CH_2)_5$CONBuMe (13)	17β-HSD1 (irreversible competitive)	IC_{50} = 13 μM (Transf. HEK-293 homogenated cells)	N/A	Non-estrogenic; antiestrogen [40]
32 (EM-1745)	HO; OH; $(CH_2)_8$COO; O; OH OH; N; N; N; N; NH_2 (9)	17β-HSD1 (reversible dual-site competitive; 3D-structure of Enz–Inh complex)	IC_{50} = 52 nM (Transf. HEK-293 homogenated cells). Weakly active in intact cells. K_i = 3 nM (pure enzyme; E2 to E1)	N/A	Non-estrogenic
55	HO; OH; O; NH_2 (3)	17β-HSD1 (reversible competitive; 3D-structure of Enz–Inh complex)	IC_{50} = 44 nM (T-47D intact cells). K_i = 0.9 nM (pure enzyme; E1 to E2)	17β-HSDs 7, 12	Slightly estrogenic [74]
72	H; O; N; 9; O; N; H (5)	17β-HSD7	IC_{50} = 230 nM (Transf. HEK-293 intact cells)	5α-Rs 1, 2	Non-estrogenic[b] [88]
73	N; 6; O; N; H (5)	17β-HSD7	IC_{50} = 458 nM (Transf. HEK-293 intact cells)	17β-HSDs 1, 5, 12; 5α-Rs 1, 2	Non-estrogenic[b] [88]

[a] The estrogenic activity was assessed by measuring the proliferation of estrogen-sensitive (ER^+) cell lines (ZR-75-1, MCF-7 or T-47D) treated 7 days with the inhibitor.
[b] Non-androgenic when tested on androgen-sensitive (AR^+) Shionogi cells [88].

ing a long alkylamide side chain (compounds **68** and **69**) or a dimethylated spiro-δ-lactone nucleus at position 17 (compounds **66** and **67**). Further investigation, aiming at the improvement of the 17β-substituent, allowed us to identify 17β-formamido, 17β-benzamido as well as 17β-amino derivatives that efficiently inhibit the conversion of E1 into E2 and of DHT into 3β-diol by 17β-HSD7 [86]. According to their IC_{50} values, the best inhibitors for E1 into E2 conversion are the spiro-δ-lactone **66** (116 nM), the 17β-(N-decylformamido) derivative **70** (195 nM) and the tertiary amine **71** (189 nM). Such values are very good considering that the K_m of the enzyme for E1 as substrate is evaluated to be 3.25 μM [79].

When testing their selectivity over 17β-HSD7, it appeared that compound **66**, with a spiro-δ-lactone scaffold [87], inhibited 17β-HSD5 but not compounds **70** and **71**, whereas the 4-methyl-4-aza-5α-androstane nucleus was able to block the enzymatic activity of 5α-reductase type 1, regardless of the nature of the 17β-substituent. Inhibitors **66** and **70** also proved themselves as good inhibitors of 5α-reductase type 2, but not the tertiary amine **71**. Selectivity for 17β-HSD7 was achieved by synthesizing compounds **72** and **73**, the 4-aza-5α-androstane analogues of compounds **70** and **71**, respectively. Compound **72** still presents a good inhibitory potency toward 17β-HSD7 for both enzymatic reactions (E1 conversion: IC_{50} = 230 nM, DHT conversion: inhibition = 40% at 0.3 μM and 86% at 3 μM) when compared to compound **70**. However, compound **73**, although selective, is slightly less potent (E1 conversion: IC_{50} = 458 nM, DHT conversion: inhibition = 29% at 0.3 μM and 74% at 3 μM) than its analogue, compound **71**. Compounds **72** and **73** represent the only known selective inhibitors of 17β-HSD7 able to efficiently block reactions performed by this steroidogenic enzyme. Moreover, these compounds did not stimulate the proliferation of androgen-sensitive (AR^+) Shionogi cells and estrogen-sensitive (ER^+) CAMA-1 cells, which means that these inhibitors are not androgenic nor estrogenic compounds [88]. Compound **73** was tested as inhibitors of 17β-HSD types 1, 2, 5 and 12 and did not show significant inhibitory activity at 1 and 10 μM [89].

4. Using key enzyme inhibitors

From the studies reported in Sections 2 and 3, we have identified key inhibitors of isoforms 1 and 7 of 17β-HSD, and summarized some of their properties in Table 1. At the request of several investigators, certain inhibitors were supplied for use in different studies or simply as reference products [37,38,90–95]. Blomquist et al. [38] used an inhibitor of 17β-HSD1 (compound **18**) and an inhibitor of 17β-HSD2 to confirm the presence of 17β-HSD2 in A431 cells, a cell line derived from an epidermoid carcinoma of the vulva. More recently, he used specific inhibitors of steroid sulfatase and 17β-HSDs, as compound **55**, to evaluate the steroid sulfatase and 17β-HSD activity levels of tumor samples of ovarian carcinoma [90]. In their study of the specific steroidogenic enzymes involved in the transformation of DHEA into E2 in choriocarcinoma JEG-3 cells, Samson et al. [91] determined that 17β-HSD1 is the major enzyme that catalyzes the transformation of E1 in E2 using inhibitor **55**. Compounds **55** and **73** were used by Fournier and Poirier [92] to determine the involvement of reductive 17β-HSD1 and 17β-HSD7, respectively, in the formation of E2 in four cell lines of endometrial cancer and one cell line of cervical cancer. Having in mind a certain controversy regarding which reductive 17β-HSD isoform(s) is(are) involved in breast cancer, types 1, 7 or/and 12, Laplante et al. [89] used selective inhibitors of three 17β-HSDs, type 1 (**55**), type 7 (**73**) and type 12, to investigate the relative importance of each isoform in the formation of E2 in ten human breast cancer cell lines. The results clearly showed a great variability between each cell line. In some cases the formation of E2 was completely inhibited, as when using **55** in T-47D cells, but that was not the result observed in other cell lines. The question now is how to compare the different breast cancer cell lines tested in our study, or used by other researchers, with a mammary tumor tissue, especially regarding the 17β-HSD activity producing the potent estrogen E2. As example, a model of T-47D tumors (xenograft) is an excellent model for testing the potency of a 17β-HSD1 inhibitor, but it is not necessarily representative of breast cancer, especially if other 17β-HSD isoforms are involved in the production of E2. The effect of selective inhibitors of 17β-HSDs in breast cancer tissue samples could be an interesting way to better understand which 17β-HSD isoforms are involved in the formation of E2 in breast cancer. Experiments using the ability of a potent 17β-HSD inhibitor or a combination of inhibitors to block the formation of E2 in breast cancer tissues would be very close to a real case of breast cancer in a woman.

5. Conclusion

We have designed different kinds of steroid inhibitors of 17β-HSD1 mainly using classical structure–activity relationship (SAR) studies and, in some cases, using recently developed methodologies such as molecular modeling (crystallography, docking experiments, molecular dynamics) and solid-phase or solution-phase combinatorial chemistry. These inhibitors having been prepared over a long period of time, they were tested using several assays: inhibition of pure human placental 17β-HSD1, inhibition of 17β-HSD1 in either intact or homogenates of HEK-293 cells transfected with 17β-HSD1, and inhibition of endogenously expressed 17β-HSD1 in T-47D cells. Although not tested in the same enzymatic assay, it is clear that compound **55** (Table 1) is the most interesting regarding its high inhibitory potency in intact cells, its selectivity for other 17β-HSDs and its short efficient chemical synthesis (only 3 steps). Compound **55** is a reversible competitive inhibitor for the substrate only and is not a dual-site (substrate/cofactor) inhibitor. This drug-amenable compound was used as a proof of concept showing that an inhibitor of 17β-HSD1 can block the proliferation induced by E1 in breast cancer cells. Its weak estrogenic activity is problematic for a potential use in breast cancer therapy, but a non-estrogenic version of this inhibitor is underway.

Compound **32** clearly illustrates the concept of a dual-site inhibitor that interacts with both the substrate and cofactor binding sites of 17β-HSD1 (as proved by crystal structure of the complex Enz/Inh, kinetic studies and SAR), and thus having a very high binding affinity for the enzyme when tested in pure enzyme or homogenated cells. However, its weak inhibitory activity in intact cells is a serious drawback that cannot be easily resolved. Modification of the adenosine moiety was explored but did not provide satisfactory results until now.

Compounds **6** and **18** are less potent inhibitors than **55**. They are reversible inhibitors, but we did know if they are competitive or not regarding the substrate and the cofactor. Both are estrogenic compounds although less than E2, but this residual estrogenic activity of the E2 nucleus clearly limits their therapeutic potential. Compounds **18** and **22** are irreversible competitive inhibitors able to inactivate the 17β-HSD1 by formation of a covalent bond as proved by kinetic studies. The inhibitory potency of **18** proximate those of **6** and **16**, but **18** is an estrogenic compound. Unfortunately, the strategy tested to block the estrogenicity greatly reduced the capacity of **22** to inhibit 17β-HSD1 and increased the number of chemical steps for its preparation. However, considering that **22** is an antiestrogen and an inhibitor of 17β-HSD1, it would be interesting to experiment this type of dual-action molecule for breast cancer therapy.

Compounds **72** and **73** inhibit two transformations catalyzed by 17β-HSD7, namely E1 into E2 and DHT into 3β-diol. Being the first inhibitors of this isoform, their mechanism of action (reversible or

not, competitive or not) remains to be elucidated. They are however selective inhibitors of 17β-HSD7 since they did not inhibit the isoforms 1, 2, 5 and 12 of 17β-HSD as well as the isoforms 1 and 2 of 5α-reductase. Interestingly, they did not appear to be estrogenic nor androgenic compounds when tested in vitro (cell proliferation).

Inhibitors **55** and **73** are two promising candidates for mechanistic studies as well as in vitro and in vivo studies aiming to reduce the level of potent estrogen E2, especially in breast cancer models. Although the therapeutic potential of these two inhibitors and those reported in Table 1 were not yet tested, they were used several times as reference compounds and useful tools to elucidate the role of 17β-HSDs.

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