Journal Pre-proof

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PII: S0223-5234(20)30881-3

DOI: https://doi.org/10.1016/j.ejmech.2020.112909

Reference: EJMECH 112909

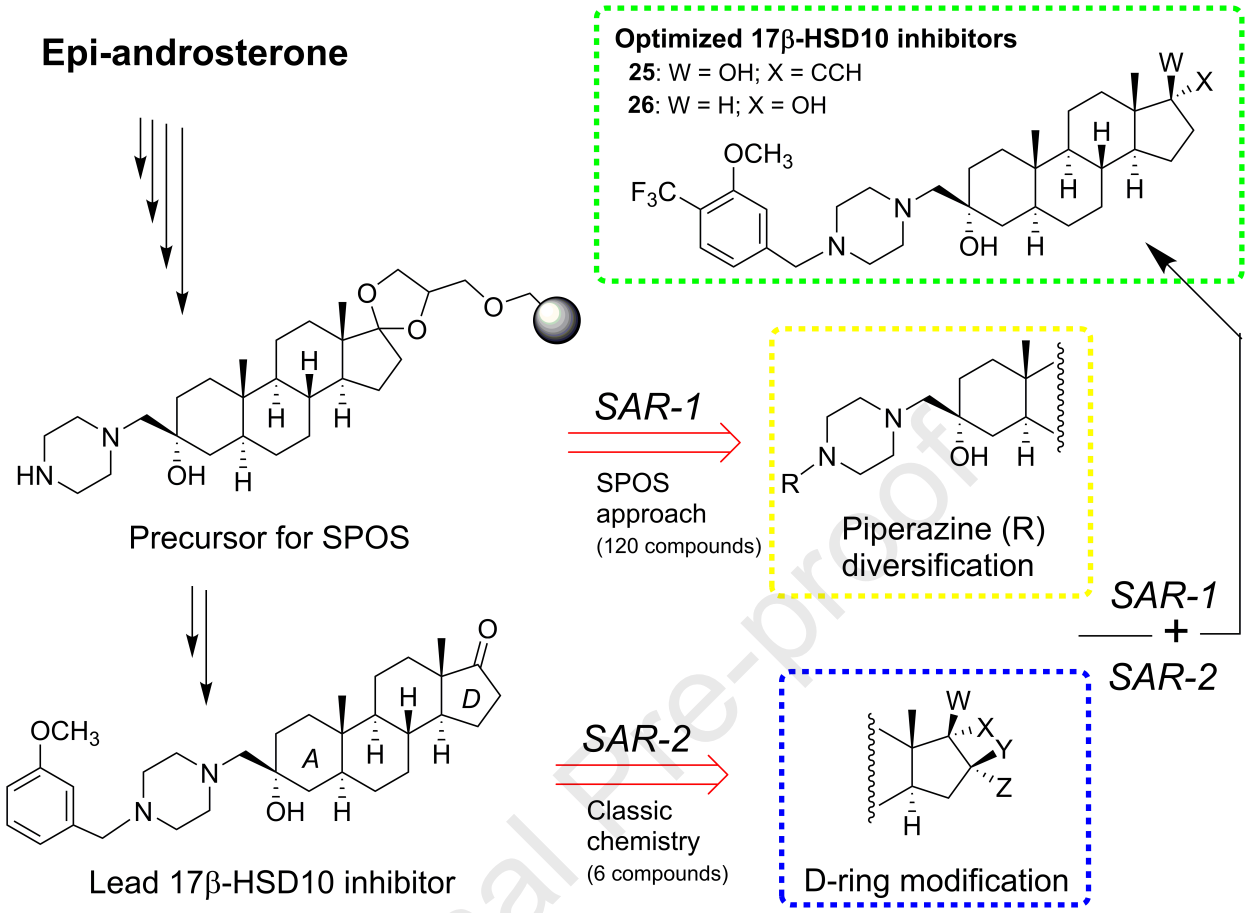
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 23 June 2020

Revised Date: 28 September 2020

Accepted Date: 3 October 2020

Please cite this article as: S. Boutin, R. Maltais, J. Roy, D. Poirier, Synthesis of 17β-hydroxysteroid dehydrogenase type 10 steroidal inhibitors: selectivity, metabolic stability and enhanced potency, *European Journal of Medicinal Chemistry*, https://doi.org/10.1016/j.ejmech.2020.112909.



Eur. J. Med. Chem. (Third revised version)

Synthesis of 17β-hydroxysteroid dehydrogenase type 10 steroidal inhibitors: selectivity, metabolic stability and enhanced potency

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Abstract:

17beta-Hydroxysteroid dehydrogenase type 10 (17β-HSD10) is the only mitochondrial member of 17β-HSD family. This enzyme can oxidize estradiol (E2) into estrone (E1), thus reducing concentration of this neuroprotective steroid. Since 17β-HSD10 possesses properties that suggest a possible role in Alzheimer's disease, its inhibition appears to be a therapeutic strategy. After we identified the androsterone (ADT) derivative **1** as a first steroidal inhibitor of 17β-HSD10, new analogs were synthesized to increase the metabolic stability, to improve the selectivity of inhibition over 17β-HSD3 and to optimize the inhibitory potency. From six D-ring derivatives of **1** (17-C=O), two (17β-H/17α-OH and 17β-OH/17α-C≡CH) were more metabolically stable and did not inhibit the 17β-HSD3. Moreover, solid phase synthesis was used to extend the molecular diversity on the 3β-piperazinylmethyl group of the steroid base core. Eight over 120 new derivatives were more potent inhibitors than **1** for the transformation of E2 to E1, with the 4-(4-trifluoromethyl-3-methoxybenzyl)piperazin-1-ylmethyl-ADT (**D-3,7**) being 16 times more potent (IC_{50} = 0.14 μM). Finally, D-ring modification of **D-3,7** provided 17β-OH/17α-C≡CH derivative **25** and 17β-H/17α-OH derivative **26**, which were more potent inhibitor than **1** (1.8 and 2.4 times, respectively).

Keywords: 17β-HSD10, Alzheimer disease, enzyme, inhibitor, steroid, solid-phase synthesis.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia [1]. About 50 million people are living with dementia worldwide and it is estimated that this prevalence will almost double every 20 years [2]. AD cause the gradual loss of important neuronal functions as language, motor functions, but especially memory. These are clinical symptoms, but neuropathologically, AD is characterized by the brain atrophy and the abundance of extracellular amyloid beta (Aβ) plaques [1]. Human 17β-hydroxysteroid dehydrogenase (17β-HSD) is a family of oxidoreductases that catalyzes the interconversions of ketones and alcohols. These enzymes transform functional groups at position 17 of the steroid backbone depending on their substrate specificity [3]. Some 17β-HSDs have thus key roles in up or down regulation of androgens, estrogens and neurosteroids, making them interesting therapeutic targets in many diseases [3-5].

Mitochondrial 17β-HSD type 10 (17β-HSD10), a member of 17β-HSD family, possesses properties that suggest a possible role in AD [6,7]. One of these properties is its capacity to transform estradiol (E2) into estrone (E1). In fact, E2 is one substrate among others of 17β-HSD10 (K_m = 14-43 μM, Fig. 1) [7-10]. The E2 concentration in brains of AD patients being reduced, the inhibition of 17β-HSD10 could help to restore E2 levels and promote its neuroprotective effects. 17β-HSD10 is also known for the formation of a high affinity neurotoxic complex with Aβ-42, the peptide responsible of the formation of Aβ plaque in the brain. In mitochondria, Aβ-42 can bind to 17β-HSD10, disrupts the oxidoreductase function of the enzyme and causes dysfunction and neuronal cell death [11]. Furthermore, several studies showed that inhibiting this neurotoxic complex formation is a promising therapeutic approach [6,7, 12-14]. It is also important to note that higher concentrations of 17β-HSD10 are found in the brain of AD patients [7,15]. Consequently, the development of 17β-HSD10 inhibitors offer the opportunity to validate the role of 17β-HSD10 in AD and provide a new therapeutic approach against this disease.

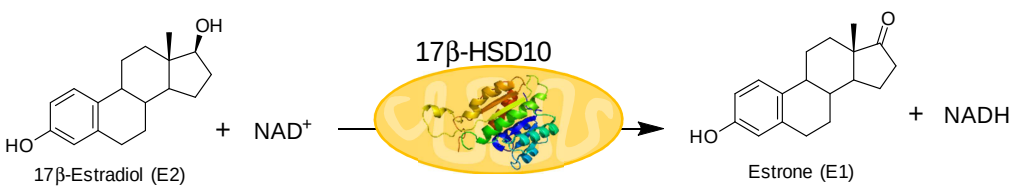


Fig. 1. Oxidation of the 17β-OH of estradiol catalysed by 17β-HSD10 and cofactor NAD^+ to form estrone, a 17-ketosteroid, and NADH.

There are few known 17β-HSD10 inhibitors [16-18]. One of them is AG18051, an irreversible inhibitor with a pyrimidine core identified by Kissinger *et al* [19]. Benzothiazole and frentizole derivatives were also synthesized and evaluated as potent inhibitors of 17β-HSD10 [20-28]. None of these inhibitor, including AG18051, were however based on a steroid scaffold. In fact, our research group reported compound **1** (Fig. 2) as the first steroidal inhibitor of 17β-HSD10 [29,30]. This 5α-androstane-3α,17β-diol derivative inhibited (IC_{50} = 0.55 μM) the transformation of E2 to E1 by HEK-293[17β-HSD10] cells, but it also inhibited 17β-HSD3, an enzyme that catalyzes the reduction of 4-androstene-3,17-dione (4-dione) into testosterone (T). Since a low level of androstane (T or 5α-dihydrotestosterone) is associated with an increased risk of AD [31], and considering the expression of 17β-HSD3 in hippocampus [32], the type 10/type 3 selectivity of compound **1** needed to be improved. Furthermore, a first round of metabolic stability assay also showed that compound **1** was weakly stable in the conditions of the assay using human liver microsomes, highlighting a potential issue toward its translation into a drug.

Three different strategies (Fig. 2) were tested to improve the biological activities of 17β-HSD10 steroidal inhibitor **1**. In the first one, D-ring derivatives of **1** (modifications at C16 or C17) were synthesized using classical chemistry in solution to increase its metabolic stability and type 10/type 3 selectivity. In the second strategy, libraries of amide, sulfonamide, urea, thiourea and amine derivatives of **1** (side chain modifications) were generated by parallel solid phase-synthesis to increase its inhibitor potency and selectivity. Finally, in the third strategy, two hybrid compounds were synthesized to combine the results of strategies 1 and 2.

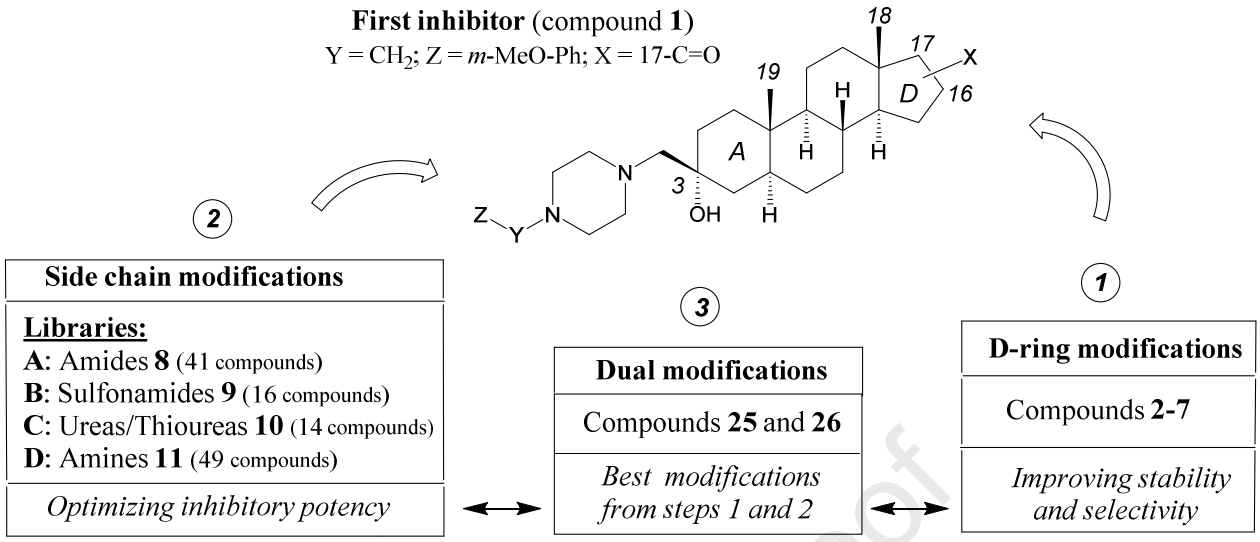
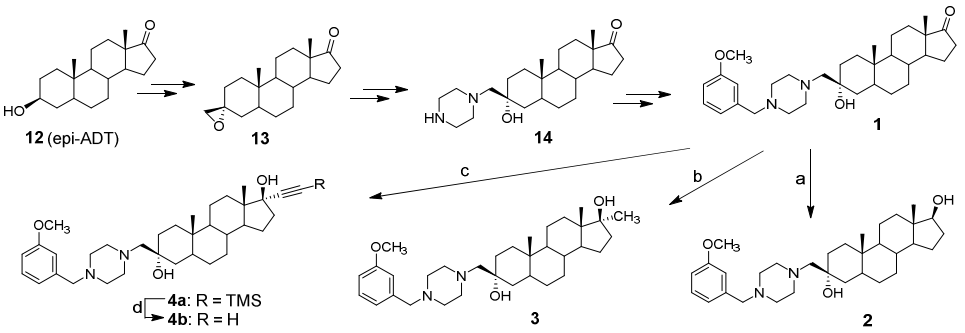


Fig. 2. Three different strategies (#1-3) behind the optimization of lead compound **1**. *1)* Derivatization of compound **1** to improve the metabolic stability and selectivity for 17β-HSD10 over 17β-HSD3 (D-ring derivatives **2-7**, Schemes 1 and 2); *2)* Side chain modification of compound **1** to optimize the inhibitory potency for 17β-HSD10 (libraries A-D members, 120 compounds represented by **8-11**, Scheme 3); and *3)* Two hybrid inhibitors, compounds **25** and **26**, combining best elements from steps 1 and 2 (Scheme 4). Selected positions 3, 16, 17, 18 and 19 as well as key A- and D-rings of steroid scaffold were represented.

2. Results

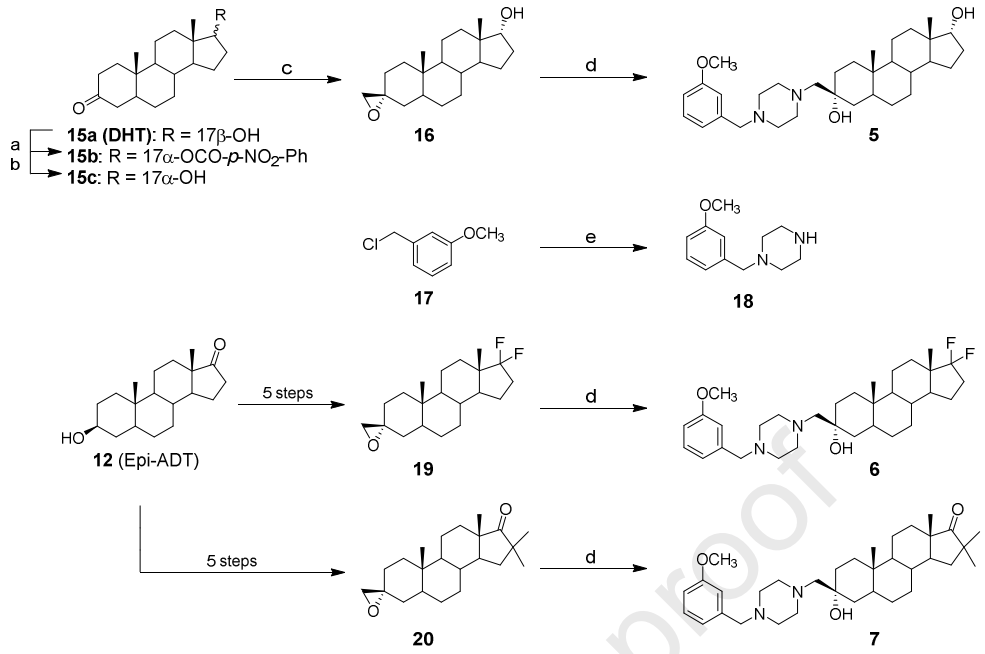
*2.1 Chemical synthesis of D-ring derivatives **2-7** (first strategy)*

The lead compound **1** was first synthesized from epi-androsterone (epi-ADT; **12**) or dihydrotestosterone (DHT; **15a**) using the conditions previously published [33], and then used as starting material for the synthesis of **2-4** (Scheme 1). The C-17 carbonyl of **1** underwent a stereoselective reduction using sodium borohydride to give 17β-OH derivative **2**. The compound **3** (17β-OH/17α-CH_3) was synthesized through a Grignard reaction with methyl magnesium iodide. Compound **4b** (17β-OH/17α-C≡CH) was synthesized in two steps by adding lithium(trimethylsilyl)ethylinide giving intermediate **4a**, which underwent hydrolysis with K_2CO_3 in methanol to afford **4b** [34]. As previously reported [35], the steric hindrance of the axial methyl-18 is responsible for the stereoselectivity of these three reactions involving the C-17 carbonyl and providing 17β-OH configuration in **2**, **3** and **4a**.



Scheme 1. D-ring steroid derivatives **2** (17β-OH), **3** (17β-OH/17α-CH_3) and **4b** (17β-OH/17α-C≡CH). Reagents and conditions: (a) $NaBH_4$, MeOH, 0°C (4 h); (b) CH_3MgI (3.0 M in Et_2O), toluene, 80°C (4 h) to rt (16 h); (c) TMS-C≡CH, CH_3Li, Et_2O, THF, 0°C to rt (16 h); (d) K_2CO_3 5%, MeOH, rt (17 h).

For the synthesis of 17α-OH derivative **5** (Scheme 2), we first attempted to start from **2** by performing a Mitsunobu reaction followed by a hydrolysis of the intermediate ester [36]. However, the first step, the inversion of the C-17β alcohol by reacting with 4-nitrobenzoic acid, triphenylphosphine and diethyl azodicarboxylate (DEAD) did not lead to the corresponding 17α-ester. One hypothesis is the presence of two tertiary amines in the piperazine derivative **2**, which could promote the formation of a salt in these conditions and thus prevent any further reaction. After this unsuccessful attempt, compound **5** was obtained in four steps from commercially available 5α-dihydrotestosterone (DHT, **15a**) (Scheme 2). The chiral center at position 17 (17β-OH) of **15a** was first inversed in two steps (Mitsunobu and hydrolysis reactions) to give the corresponding 17α-alcohol **15c**. The ketone at C-3 was then transformed into the oxirane **16** using the conditions of the Johnson-Corey-Chaykovsky reaction [37,38] and this oxirane was regioselectively opened by the secondary amine **18** to afford **5**. The building block **18**, not commercially available, was obtained by refluxing 3-methoxybenzyl chloride in ethanol (**17**) with piperazine in excess.

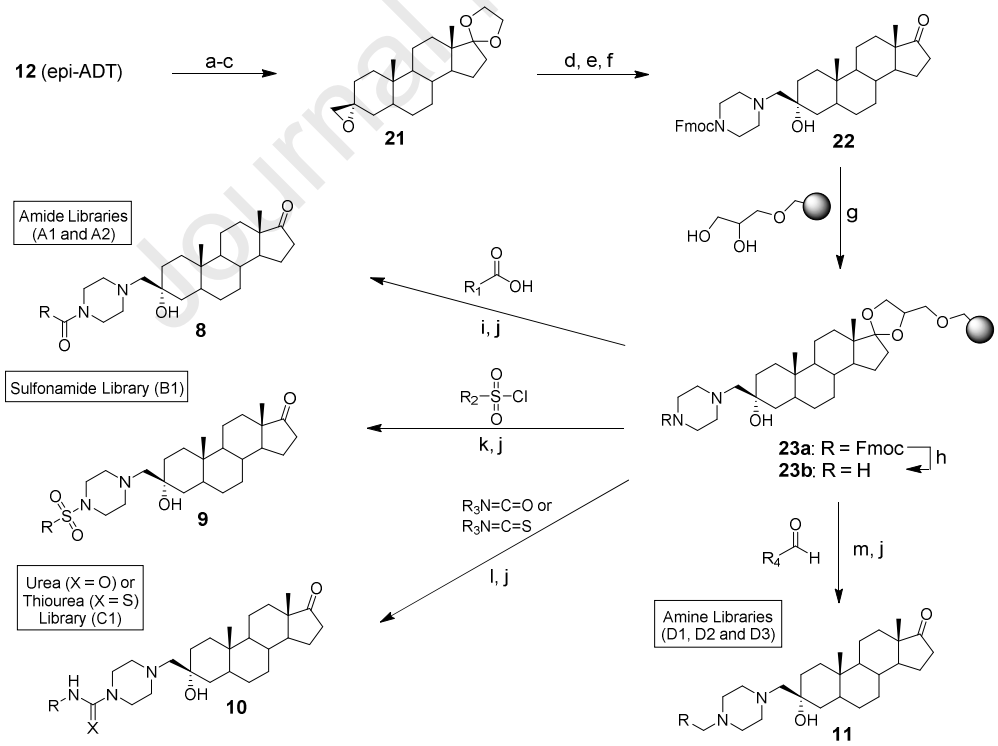


Scheme 2. Synthesis of D-ring steroid derivatives **5** (17α-OH), **6** (17-CF_2) and **7** (17-C=O/16-C(CH_3)$_2$). Reagents and conditions: (a) 4-Nitrobenzoic acid, PPh_3, DEAD, toluene, 80°C (18 h); (b) KOH 10%, MeOH, rt (6 h); (c) $(CH_3)_3SOI$, NaH, DMSO, 50°C (16 h); (d) 1-(3-Methoxybenzyl)-piperazine (**18**), EtOH, 60°C (24 h); (e) Piperazine, DCM, rt (19 h).

C-17-difluoro derivative **6** was obtained from **19** (Scheme 2), which was synthesized previously in five steps from epi-ADT (**12**) as reported in literature [39]. Briefly, the 3β-alcohol of **12** was first protected as an acetate and this intermediate reacted with DAST reagent, allowing the addition of two fluorides on the C-17 ketone. Then, the ester at C-3 was hydrolyzed, the corresponding alcohol oxidized, and the corresponding ketone transformed into the oxirane **19**. Finally, **19** reacted with piperazine derivative **18** to afford **6**. C-16 dimethyl derivative **7** was generated from **20**, which was also synthesized from epi-ADT (**12**) in five steps as reported in literature [39]. Briefly, after protecting the alcohol of **12** as an acetate derivative, the dimethylation at C-16 was afforded using sodium hydride and methyl iodide in excess. The deprotection of the alcohol and its oxidation to a ketone provided the oxirane **20**, which then reacted with **18** to afford the final compound **7**. A direct dimethylation of **1** to obtain **7** was not attempted since the methyl iodide used in the methylation step would have react with the piperazine nitrogens, producing a salt.

2.2 Chemical synthesis of libraries A, B, C and D (second strategy)

To generate libraries **A** to **D** members, represented by compounds **8**-**11**, the androstane derivative **22** must first be synthetized from epi-ADT (**12**) and, then, loaded on the glycerol polymer-bound (Scheme 3). Briefly, and as previously published [40], the ketone at position 17 of epi-ADT was protected as an acetal via a transacetalization with ethylene glycol and *p*-TSA using a Dean-Stark apparatus. The 3β-OH was then oxidized using tetrapropylammonium perruthenate (TPAP), N-methylmorpholine N-oxide (NMO) and molecular sieves, as desiccant, and this ketone was transformed into the oxirane **21** using the Johnson-Corey-Chaykovsky conditions [37, 38]. An excess of piperazine was used to open regioselectively the oxirane **21** at position 3 to give a piperazino derivative, which was protected as a Fmoc derivative using Fmoc-*O*-succinimide and $NaHCO_3$ as base. Finally, the acetal at position 17 was hydrolysed under acidic conditions to afford **22** in 72% yield for three steps. The protection of the secondary amine of the piperazino derivative was necessary since the acidic conditions needed for the acetalization could favorize the intermolecular formation of an imine between the piperazine and the 17-ketone, forming a dimer, and thus preventing the loading of the steroid on the solid support (resin).



Scheme 3. Parallel solid-phase synthesis of amide (**A-1** and **A-2**), sulfonamide (**B-1**), urea and thiourea (**C-1**) and amine (**D-1**, **D-2** and **D-3**) libraries of steroid derivatives **8-11**. Reagents and conditions: (a) Ethylene glycol, *p*-TSA, toluene, Dean-Stark apparatus, reflux (24 h); (b) TPAP, NMO,

DCM, molecular sieves, 0°C (2 h); (c) $(CH_3)_3SOI$, NaH, DMSO, 50°C (16 h); (d) Piperazine, EtOH, reflux; (e) Fmoc-*O*-succinimide, $NaHCO_3$, THF/H_2O (5:1), rt (16h); (f) HCl/MeOH (1.0 M):DCM (75:25), rt (16 h); (g) Glycerol polymer-bound, trimethyl-orthoformate, *p*-TSA (10%), toluene, rt (17 h); (h) Piperidine/DMF (2:8), rt (3.5 h); (i) Carboxylic acid, HBTU, DIPEA, DMF, rt (3 h); (j) HCl / MeOH / DCM (25:75), rt (20 h); (k) Sulfonyl chloride, TEA, DCM, rt (3 h); (l) Isocyanate or isothiocyanate, DCM, rt (5 h); (m) Aldehyde, AcOH (1%), $NaBH_3CN$, DMF, rt (5 h).

Compound **22** was added to a suspension of glycerol polymer bound (loading capacity of 1.00 mmol/g) in presence of *p*-TSA to provoke the acetalization. Tri-methyl orthoformate was added to scavenge water produced as a side product during the formation of **23a**. The Fmoc of resin **23a** was cleaved in presence of piperidine, a weak base commonly used for this type of reaction, to give resin **23b**. The global loading of **22** after Fmoc hydrolysis (resin **23b**) was established to 0.41 mmol/g from weight difference. The glycerol polymer-bound was selected since the ketal functionality generated is stable under basic conditions used to produce, from **23b** in parallel synthesis, the library members represented by compounds **8** - **11**. Advantageously, the cleavage of the steroids from resins is easily done by a hydrolysis under weak acidic conditions, thus giving the desired ketosteroids in good yields (libraries A-D; Supplementary data).

Amide derivatives **8** (libraries **A-1** and **A-2**) were synthetized by reacting equal portions of resin **23b** with diverse carboxylic acids (21 for each library), which were previously activated with HBTU. The final step of this solid-phase synthesis required an acid cleavage (HCl generated *in situ*) to provide the free androstane derivatives **8** (**A-1,1** to **A-1,21** and **A-2,1** to **A-2,21**). For the amide library **A-1**, the HPLC purity of all 21 synthetized compounds ranged from 71.4% to 99.4%, with an average purity of 95.2 ± 6.1% (Table 1). For the amide library **A-2**, 20 derivatives of 21 were selected and the HPLC purity ranged from 76.8% to 99.7%, with an average purity of 95.6 ± 5.6%.

Table 1. Assessment of solid-phase chemical synthesis of libraries A1 to D3

Library Number		Family Compound	Number of Building Blocks	Number of Selected Compounds[a]	Range of Purity (%)[b]	Average of Purity (%)[b,c]
A	1	Amides **8**	21	21	71.4 – 99.4	95.2 ± 6.1
A	2	Amides **8**	21	20	76.8 – 99.7	95.6 ± 5.6
B	1	Sulfonamides **9**	21	16	65.8 – 99.3	87.2 ± 11.7
C	1	Ureas/Thioureas **10**	21	14	68.0 – 98.5	89.6 ± 10.4
D	1	Amines **11**	21	16	60.0 – 95.7	85.2 ± 9.6
D	2	Amines **11**	21	17	65.0 – 98.3	87.8 ± 8.3
D	3	Amines **11**	21	16	72.0 – 97.2	92.3 ± 7.1

[a] Compounds with NMR and HPLC purity over 60%
[b] For selected compounds
[c] Reported as purity ± SD

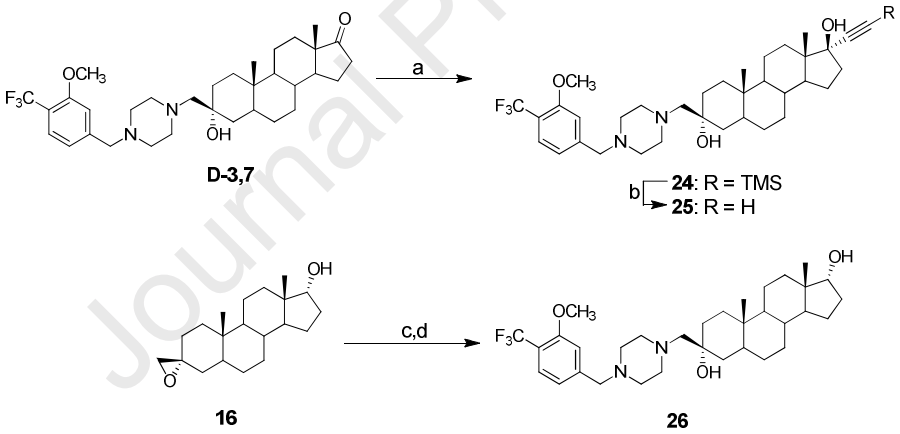
To obtain sulfonamide derivatives **9** (library **B-1**), equal portions of resin **23b** were placed in presence of a solution of each sulfonyl chloride to allow a nucleophilic substitution. The free sulfonamide derivatives **9** (**B-1,1** to **B-1,21**) were obtained after the acid cleavage. From this third library (**B-1**), five compounds were eliminated because of their low NMR or HPLC purity. Thus, 16 sulfonamide derivatives, with a range of HPLC purity between 65.8% and 99.3% and an average purity of 87.2 ± 11.7%, were selected to undergo biological assays.

To produce urea and thiourea derivatives **10** (library **C-1**), equal portions of resin **23b** were reacted with a solution of each appropriate isocyanate or isothiocyanate under basic conditions. After final cleavage, compounds **10** (**C-1,1** to **C-1,21**) were obtained. A smaller number of compounds were selected from this urea and thiourea library since the purity of some members was not high enough. From library **C-1**, 14 urea and thiourea derivatives were selected for biological assays. These compounds had a range of HPLC purity between 68.0% and 98.5% with an average purity of 89.6 ± 10.4%. The difference of reactivity of the building blocks may explain why more compounds were not selected. In fact, when the resins were cleaved, a mixture of the desired compound **10** and the non-derived amine (3β-piperazinyl-methyl-ADT) were obtained in some cases. Since we always used the same reaction conditions, indifferently of the building blocks, maybe more time would have favorized completion of the reaction.

Amine derivatives **11** (libraries **D-1**, **D-2** and **D-3**) were synthetized by a reductive amination. Equal portions of resin **23b** were treated in presence of each aldehyde under low acidic conditions to promote the formation of an iminium. Then, a solution of sodium cyanoborohydride was added to reduce the iminium into the corresponding tertiary amine. After cleavage of the acetal link, amines **11** (**D-1**,**1** to **D-1**,**21**, **D-2**,**1** to **D-2**,**21** and **D-3**,1 to **D-3**,**21**) were successfully obtained. For library **D-1**, 15 amine derivatives were selected, with a range of HPLC purity between 60.0% and 95.7%, and an average purity of 85.2 ± 9.6%. For library **D-2**, 17 amine derivatives were selected, with a range of HPLC purity between 65.0% and 98.3%, and an average purity of 87.8 ± 8.3%. Finally, for library **D-3**, 16 amine derivatives were selected, with a range of HPLC purity between 72.0% and 97.2%, and an average purity of 92.3 ± 7.1%.

*2.3 Chemical synthesis of hybrid inhibitors **25** and **26** with dual modifications (third strategy)*

The most potent inhibitor resulting from libraries A-D, compound **D-3,7**, was modified at position C17 to generate two D-ring derivatives (compounds **25** and **26**). Compound **25** was synthesized from **D-3,7** (Scheme 4) following the two steps procedure used for the synthesis of **4b** (Scheme 1). Briefly, lithium(trimethylsilyl)ethylinide was added to ketone **D-3,7** to give the intermediate **24**, which underwent hydrolysis with K_2CO_3 in methanol to afford **25** (17β-OH/17α-CCH). The second hybrid, compound **26**, was obtained from **16** also in two steps. First, the 17α-OH-oxirane **16** was opened regioselectively with piperazine to generate a secondary amine and, next, it was reacted with 4-trifluoromethyl-3-methoxybenzaldehyde and sodium cyanoborohydride to afford **26**.



Scheme 4. Synthesis of compounds **25** and **26**. Reagents and conditions: (a) TMS-C≡CH, CH_3Li, Et_2O, THF, 0°C to rt (16 h); (b) K_2CO_3 5%, MeOH, rt (17 h); (c) piperazine, EtOH, 60°C (24 h); (d) 4-trifluoromethyl-3-methoxybenzaldehyde, AcOH (1%), $NaBH_3CN$, DMF, rt (5 h).

*2.4 Biological assessment of D-ring derivatives **2-7***

After chemical synthesis of D-ring derivatives **2-7**, those resulting from the first strategy, their potency to inhibit the transformation of E2 into E1 by17β-HSD10, their selectivity of inhibition (17β-HSD10 *vs* 17β-HSD3) and their metabolic stability were assessed and compared to the lead compound **1**. The oxidation of E2 to E1 was evaluated using stably transfected HEK-293[17β-HSD10] cells in culture. For each assay, transfected cells were incubated for 40 h with

the inhibitor (0.3 μM and 3.0 μM) and the substrate E2 (1.0 μM, E2/[^{14}C]-E2 in proportions 9:1). The steroids E2 and E1 were then extracted from the culture medium with Et_2O and separated by thin layer chromatography (TLC). The radioactivity of the substrate (E2) and the metabolite (E1) was quantified to calculate the percentage of transformation and then the percentage of inhibition (Table 2).

Table 2. Biological results for D-ring derivatives **2-7**

Cpd[a]	Derivatization	Inhibition (%) of E2 to E1 by HEK-293[17β-HSD10] cells[b]		Inhibition (%) of 4-dione to T by LNCaP[17β-HSD3] cells[b]		Metabolic stability – Remaining Quantity (%)[c]
		0.3 μM	3.0 μM	0.3 μM	3.0 μM	1.0 μM
1	C17-ketone (C=O)	40.7 ± 4.4	70.4 ± 1.3	30.2 ± 8.9[d]	84.4 ± 9.3[d]	28.0 ± 12.0
2	17β-OH/17α-H	8.5 ± 3.3	42.4 ± 4.4	5.0 ± 0.6	48.7 ± 2.4	67.2 ± 5.2
3	17β-OH/17α-CH_3	20.5 ± 1.3	41.9 ± 8.4	2.0 ± 0.2	20.6 ± 0.7	21.8 ± 4.2
4b	17β-OH/17α-CCH	22.4 ± 8.4	45.8 ± 6.2	2.0 ± 1.8	4.0 ± 1.2	45.5 ± 6.8
5	17β-H/17α-OH	13.5 ± 1.0	36.9 ± 1.8	5.2 ± 2.6	3.8 ± 0.5	70.6 ± 3.6
6	17-di-F	5.5 ± 2.0	40.8 ± 1.5	1.1 ± 0.4	-0.5 ± 2.9	34.1 ± 2.1
7	16,16-di-CH_3	10.8 ± 2.0	27.9 ± 2.8	12.4 ± 1.9	51.9 ± 1.9	54.5 ± 6.0

[a] See schemes for the full chemical structure of **1-7**.
[b] Average results of one experiment performed in triplicate ± SEM.
[c] Average results of two experiments performed in duplicate ± SEM.
[d] Average results of five experiments performed in triplicate ± SEM (see table S1 in Supporting Information).

At a concentration of 3.0 μM, the secondary 17β-alcohol **2** inhibited 42.4% of E2 transformation into E1, but the corresponding ketone **1** was more potent with 70.4% of inhibition. This result confirms that a ketone at C-17 is better than a 17β-hydroxy to inhibit 17β-HSD10 and is in accord with an observation from our previous screening study [29]. Tertiary alcohols **3** and **4b** were synthesized and tested considering that the addition of a methyl or an acetylene group at position 17α is a known strategy to increase the metabolic stability of steroids [41]. Their inhibitory potencies, 41.9% and 45.8%, respectively for **3** and **4b** at a concentration of 3.0 μM, were similar to that of **2** (42.4%). With 36.9% of inhibition, the 17α-OH derivative **5** was a less potent inhibitor than ketone **1**. In fact, the similar inhibitory potencies of **2** and **5** suggest that the stereochemistry of the alcohol at C17 has no impact on the inhibitory potency. Compound **6** was synthesized since a CF_2 group is a potential bioisostere of a ketone [42] and, also, taking into account that the C-F bond is more resistant to metabolization than corresponding C-O bond [43]. This 17-CF_2 derivative inhibits E2 into E1 transformation (40.8%) but it is less potent than the ketone **1** (70.4%) when tested at 3.0 μM. The last D-ring derivative, compound **7**, is a 17-ketone derivative modified by the addition of two methyl groups at C16 to avoid the hydroxylation at this position. We also expected that steric hindrance could prevent the reduction of the ketone. In fact, derivative **7** was the less potent inhibitor, inhibiting only 27.9%

of the E2 to E1 transformation at 3.0 μM. All the above results showed that D-ring derivatives **2-7** inhibit the transformation of E2 to E1 in HEK-293[17β-HSD10] cells, but they are less potent inhibitors than lead compound **1**.

To test the selectivity of inhibition, derivatives **2-7** were assessed as 17β-HSD3 inhibitors using LNCaP cells overexpressing 17β-HSD3 (LNCaP[17β-HSD3]) as source of enzyme. After 1 h of incubation, the radiolabeled substrate 4-dione and its metabolite T were extracted with Et_2O and separated by TLC. After quantification of the radioactivity, the percentage of transformation and then the percentage of inhibition were calculated (Table 2). In this assay, the lead compound **1** inhibited 17β-HSD3 at both concentrations tested. Derivatives **2** (17β-OH/17α-H), **3** (17β-OH/17α-CH_3) and **7** (17-C=O/16,16-di-CH_3) inhibited 17β-HSD3 less than compound **1** (17-C=O), but still prevent the formation of T and therefore they are not selective inhibitors of 17β-HSD10. However, derivatives **4b** (17β-OH/17α-C≡CH), **5** (17β-H/17α-OH) and **6** (17,17-di-F) did not inhibit significantly the enzymatic activity of 17β-HSD3 at the two concentrations tested (0.3 and 3.0 μM). Therefore, these three compounds are selective inhibitors of 17β-HSD10 at the tested concentrations.

Finally, to test the metabolic stability of derivatives **2-7**, the compounds were treated 1 h with a microsomal preparation of human liver in the presence of NADPH as cofactor. In this assay, a compound of interest is submitted to metabolism related enzyme reactions, and the remaining compound is measured at the end of the incubation time, and expressed in %. Thus the more the compound tested is present after this treatment (higher % of remaining compound), the more the compound is considered stable (less degraded by the enzymes present in the liver). Under these artificial conditions, which are more severe than those existing in the liver, the remaining quantities of derivatives **3** (21.8%) and **6** (34.1%) were not significantly different to the value of **1** (28.0%), but the other four derivatives were all more metabolically stable (45.5-70.6%). Compounds **2** and **5** were the most stable, with 67.2% and 70.6% respectively, whereas **4b** and **7** were slightly less stable with 45.5% and 54.5% of remaining compound, respectively. In fact, the tertiary alcohol **4b** cannot be oxidized, thus preventing phase-I reaction. Also, the steric hindrance of **4b** (17α-ethynyl) and **7** (two methyls at C-16) seems to stabilize these androstane derivatives, by making the D-ring less available to phase-I reactions. Compounds **2** and **5**, two possible metabolites of **1** after carbonyl reduction are probably more stable than the 17-ketone of **1** in this biological assay using the cofactor NADPH (for the reduction) over NAD^+ (for the oxidation). In summary only one reaction (oxidation at C17) can transform **2** and **5**

whereas two reactions (reduction at C17 and hydroxylation at C16) can decrease the quantity of **1**.

*2.5 Biological assessment of compounds **8-11** (libraries A-D members)*

Libraries of androsterone (ADT) derivatives resulting from the second strategy (side chain modification) were synthesized to optimize the inhibitory potency of lead compound **1** for the transformation of E2 into E1 in HEK-293[17β-HSD10] cells in culture and to potentially improve the selectivity over 17β-HSD3. In our design, we kept the 3β-piperazinyl-methyl-ADT nucleus as base core, but greatly expanded the molecular diversity (aryl group) present on the piperazine nucleus in order to extend our structure-activity relationship (SAR) study. Five functional groups to link these aryl building blocks (amide, sulfonamide, urea, thiourea and amine) were also used to extend the molecular diversity. From the 150 compounds synthesized (libraries **A-1** to **D-3**, 120 were selected for a first round of screening tests at two concentrations of 0.3 μM and 3.0 μM (Supplementary data; Table S1).

The inhibitions of E2 into E1 transformation provoked by the new compounds were compared to the lead compound **1**, and only the compounds producing a similar or better inhibition were retained. For the amide library **A-1**, although compounds **A-1,5**, **A-1,10**, **A-1,20** and **A-1,21** similarly inhibited the transformation of E2 to E1 by 17β-HSD10 as **1**, none were more potent at 0.3 or 3.0 μM. From the second amide library **A-2**, however, five amide derivatives were similarly or more potent than **1** at 0.3 μM (namely **A-2,5**, **A-2,10**, **A-2,12**, **A-2,13** and **A-2,20**), but were all more potent at 3.0 μM. These compounds were thus selected for the next round of assay (to obtain their IC_{50} values). From the sulfonamide library **B-1**, two compounds **B-1,3** and **B-1,19** were more potent than **1** at 3.0 μM, but only **B-1,3** was a stronger inhibitor at 0.3 μM, and therefore selected. In library **C-1**, representing urea and thiourea derivatives, compounds **C-1,16** and **C-1,12** were more potent inhibitor than **1** at the two concentrations tested and thus selected. From the amine library **D-1**, any compound inhibited E2 into E1 transformation at sufficient level to justify their selection. For the amine library **D-2**, compound **D-2,12** was more potent at 0.3 μM than **1** and **D-2,4** produced a similar inhibitory potency. Both compounds **D-2,4** and **D-2,12** were however more potent than **1** at 3.0 μM and therefore they were retained for additional assays. Finally, the last library **D-3** was designed based on these two compounds. The first derivatives were isomers of **D-2,4**; the position of the methyl or the methoxy group being changed. Three out of four of these derivatives had similar

inhibitory potency of 17β-HSD10 as **1**, and only one (**D-3,4**) was more potent at both concentrations tested. Derivatives with a trifluoromethyl group, which increased the hydrophobicity [44], were also synthesized for comparison with the ones with methyl substituent. Interestingly, the trifluoromethyl analog of **D-2,4**, compound **D-3,7**, was the most potent 17β-HSD10 inhibitor from this library and all the others.

Compounds from libraries **A-1** to **D-3** were also screened against 17β-HSD3 to determine their selectivity of inhibition (17β-HSD10 over 17β-HSD3). LNCaP[17β-HSD3] cells were incubated to determine if the transformation of 4-dione into T was affected by each library member (Table S1, Supplementary data). From the data expressed as percentages of inhibition, almost all compounds inhibited 17β-HSD3 activity at 0.3 μM (between 10% and 70%) and more at 3.0 μM (between 60% and 100%). Thus, a selectivity of inhibition for 17β-HSD10 over 17β-HSD3 cannot be obtained by modifying the capping group added on the piperazine nucleus. In fact, the two enzymes probably have a similar binding pocket, mainly hydrophobic, where the side chain at position C-3β can produce key favorable interactions affecting their inhibitory potency, but not their selectivity of inhibition. However, as previously exemplified by D-ring derivatives (Table 1), the area surrounding D-ring seems more promising to obtain a selectivity of inhibitor action.

Selected library members from the screening study reported above are structurally closely related to lead compound **1** and they were reported in Table 3. For a better comparison they have been tested again in HEK-293[17β-HSD10] cells using a range of concentrations to obtain inhibition curves, which were used to calculate IC_{50} values. Eight of the twelve derivatives had lower IC_{50} values than **1** in their respective assay (Table 3), but the amine derivatives **D-2,4** and **D-3,7** were the best inhibitors. Thus, **D-2,4** has a 5.4 folds better inhibition than lead compound **1** (IC_{50} = 0.42 μM and 2.26 ± 0.36 μM, respectively). The 3-methoxy-4-benzyl derivative **D-2,4** differs from **1** only by the presence of a methyl group in the *para* position on the benzyl substituent. Overall, the 4-trifluoromethyl-3-methoxybenzyl derivative **D-3,7** was the most potent inhibitor of all derivatives with an IC_{50} value of 0.14 μM (16 fold better than **1**).

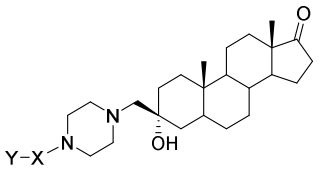
Interestingly, the addition of a hydrophobic groups on the benzyl part of the androstane derivative improves the inhibitory potency of some compounds (Table 3). As example, the difference between **1**, **D-2,4** and **D-3,7** is related to the nature of the groups on the *para* position of the phenyl, respectively being a hydrogen, a methyl and a trifluoromethyl group. The ClogP,

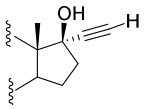
one of the criteria of Lipinski rule of 5 [45,46], allows to estimate the lipophilicity of a molecule and the estimated ClogP values of **1**, **D-2,4** and **D-3,7** are respectively 4.4, 4.7 and 5.2. These estimated values show a correlation between the hydrophobicity and potency of the three inhibitors (Supplementary data; Fig. S8A). Thus, we hypothesized that hydrophobic interactions between the enzyme and the side chain of the steroidal inhibitor enhanced inhibitory potency against 17β-HSD10. However, even if derivatives **A-2,13** and **C-1,12** were more hydrophobic than **D-3,7** they were not more potent. In fact, compound **A-2,13** possesses a voluminous rigid side chain, and the phenyl moiety of **C-1,12** is distant by a thiourea bond from the piperazine which is longer than the methylene group compound **1** (Ar-CH_2-NH-C=S-piperazine-ADT for **C-1,12** *vs* Ar-CH_2-piperazine-ADT for **1**). The rigidity and the length of the side chain seem to prevent the formation of optimal hydrophobic interactions in that enzyme region, and thus, these derivatives do not provide a marked inhibition improvement. In fact, the plot for Log P *versus* IC_{50} values for all compounds in Table 3 showed no correlation (Supplementary data; Fig. S8B). Finally, the presence of a methoxy group, a hydrogen accepting group, on the aryl ring seems to be helpful to inhibition, potentially by producing a hydrogen bonding with the enzyme. Therefore, we can hypothesize that 17β-HSD10 possesses a pocket where the 3β-side chain of the steroid inhibitors is well positioned to form beneficial hydrophobic and hydrogen bond interactions.

In addition to 17β-HSD10 inhibition, penetration across the blood brain barrier (BBB) is also an important attribute to consider. Two different prediction scores (CNS MPO and CNS ACD) were then used to estimate the BBB penetration of our inhibitors (Table 3). The CNS multiparameter optimization (MPO) score was developed by Wager et al. [47] while the ACD score was generated from ACD/Percepta 14.0.0 software [48], both scores use different physicochemical properties such as hydrogen bond donors, topological polar surface area, pKa, ClogP and ClogD. On a scale from 0-6, it was observed that marketed central nervous system (CNS) drugs displayed a CNS MPO score of ≤ 2 (1%), between 2 and 4 (25%) and ≥ 4 (74%) [47]. With CNS MPO scores ranging from 1.9 to 3.8, our best inhibitors fall into the second category. For the ACD score, which is expressed as a negative value, a molecule is considered to have a good penetrating BBB power when its score is $\geq$ -3. Most inhibitors reported in Table 3 have a CNS ACD score that is higher than -3 (-2.43 to -2.98) or close to -3 (-3.10 to -3.71). It is interesting to note that the same trend is observed between the two predictive scores. It is also important to mention that compound **1**, with a CNS ACD score of -2.54, demonstrated a very good ability to

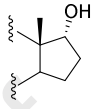
cross the BBB when assessed in mice using the *in situ* cerebral perfusion technique (see Fig. S1 in [30]). We can therefore conclude that with scores similar to or close to that of 17β-HSD10 steroid inhibitor **1**, the new inhibitors reported in Table 3 should penetrate the BBB.

Table 3. Biological results for a selection of compounds from libraries A-D

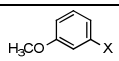
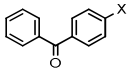
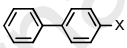
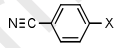
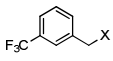
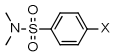
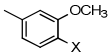
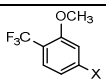




25



26

Cpd	Family	X	Y (N-Aryl Group)	IC_{50} (μM)[a] (E2 to E1)	Potency (fold)[b]	ClogP[c]	CNS MPO[d]	CNS ACD[e]
1	Amine	CH_2		**2.26 ± 0.36**[f]	---	4.43	3.1	-2.54
A-2,5	Amide	C=O		2.85	0.79	4.99	2.8	-3.53
A-2,10	Amide	C=O		2.05	1.10	4.66	3.0	-2.82
A-2,12	Amide	C=O		2.30	0.98	3.85	3.4	-2.98
A-2,13	Amide	C=O		2.60	0.87	5.30	2.8	-3.66
A-2,20	Amide	C=O		1.66	1.36	3.81	3.4	-2.55
B-1,3	Sulfonamide	SO_2		1.57	1.44	2.97	3.8	-2.43
C-1,12	Thiourea	NH-C=S-		1.41	1.60	5.54	2.5	-3.29
C-1,16	Thiourea	NH-C=S		1.07	2.11	4.25	1.9	-3.10
D-2,4	*Amine*	CH_2		***0.42***	***5.38***	***4.72***	***3.0***	***-2.60***
D-2,12	Amine	CH_2		0.97	2.33	4.58	3.0	-2.82
D-3,4	Amine	CH_2		3.00	0.75	4.72	3.0	-2.60
D-3,7	*Amine*	CH_2		***0.14***	***16.1***	***5.25***	***2.8***	***-2.95***
25	Amine	CH_2		1.26	1.8	5.79	2.5	-3.71
26	Amine	CH_2		0.95	2.4	5.54	2.5	-3.20

[a] For the transformation of E2 to E1 by HEK-293[17β-HSD10] cells.

[b] Fold = IC_{50} (lead compound **1**) / IC_{50} (compound).

[c] Values estimated by ACD/Percepta software [48].

[d] Values calculated according to Wager et al [47].

[e] Values estimated by ACD/Percepta [48].
[f] Average results of four experiments (2.69, 3.03, 1.75 and 1.57 µM) ± SEM.

*2.6 Assessment of hybrid compounds **25** and **26***

Up to this point, we obtained from the strategies 1 and 2 new steroidal inhibitors of 17β-HSD10 that were either more metabolically stable than **1**, selective for this enzyme over 17β-HSD3 (from D-ring derivatives **2-7**) or better inhibitors than **1** for the E2 into E1 transformation in HEK-293[17β-HSD10] (from libraries **A-D**). To obtain an inhibitor more metabolically stable and more potent than lead compound **1**, and with a selective action for 17β-HSD10 over 17β-HSD3, we synthetize two hybrid compounds (**25** and **26**) of the potent inhibitor **D-3,7** and D-ring derivatives **4b** or **5** in a third optimization strategy. The inhibition activities of these new derivatives, **25** (17β-OH/17α-C≡CH) and **26** (17β-H/17α-OH) (Scheme 4), were assessed in HEK-293[17β-HSD10] by measuring their inhibition potency of the E2 into E1 transformation, in the same conditions as previously described. Complete inhibition curves for **25** and **26** were obtained (Fig. 3) and their IC_{50} values were calculated as 1.26 and 0.95 µM, respectively. In fact, **25** and **26** were found 1.8 and 2.4 times more potent inhibitors of 17β-HSD10 than lead compound **1**, but in the same time, the modifications of D-ring of **D-3,7** were found to reduce their inhibitory potency by 7-9 times (Table 3). This loss of activity is however not detrimental since comparative activities to lead compound **1** were obtained, and most importantly, an enhanced metabolic stability and selectivity over 17β-HSD3 is expected based on results obtained with corresponding derivatives **4b** and **5**.

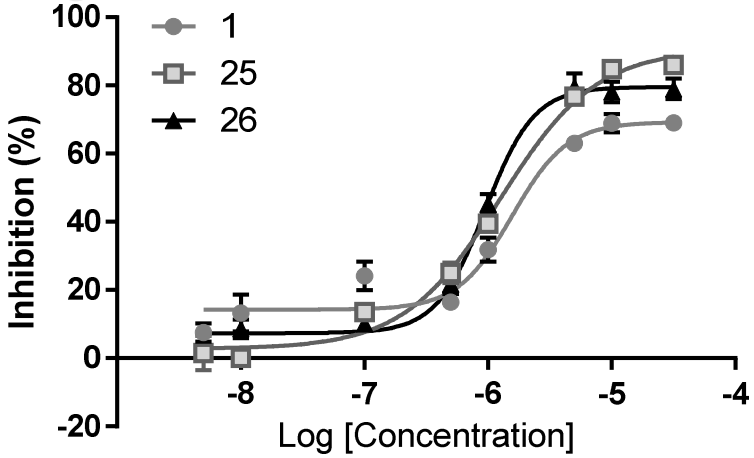


Fig. 3. Inhibition of E2 into E1 transformation by HEK-293[17β-HSD10] intact cells. The calculated IC_{50} are 1.57, 1.26 and 0.95 μM for compounds **1**, **25** and **26**, respectively. Error bars are smaller than the symbol for some concentration points.

3. Discussion

Optimization of 17β-HSD10 lead inhibitor **1** has been addressed by using three successive SAR strategies. We first performed six D-ring modifications to improve metabolic stability and/or selectivity of action over 17β-HSD3 by synthesizing compounds **2**-**7** using classic chemistry in solution. Secondly, we prepared 120 3β-piperazinyl-methyl-ADT derivatives (libraries A-D members represented by general structures **8**-**11**) by parallel solid-phase synthesis in order to increase the level of inhibition of E2 into E1 transformation by17β-HSD10. Finally, we combined the best D-ring modifications to the best inhibitors obtained from libraries A-D by synthesizing compounds **25** and **26**. Steroid derivatives generated by classic chemistry (compounds **2**-**7**) were characterized by IR, ^{1}H NMR, ^{13}C NMR and MS while library members generated by solid-phase synthesis (amides **8**, sulfonamide **9**, ureas **10** and amines **11**) were characterized by ^{1}H NMR and MS. Obtained in very small quantities and in amorphous forms because having a long hydrophobic side chain at position C3, it was not possible to determine the melting point of these 128 steroid derivatives, but their percentages of purity were however determined by HPLC and ^{1}H NMR spectra provided (Supplementary data).

Interesting observations emerged from those three optimization strategies. In the first one, six derivatives aiming D-ring modifications were synthesized and were found to inhibit E2 into E1 transformation by 17β-HSD10, but they were slightly less potent than **1**. However, three of these compounds, **4b** (17β-OH/17α-C≡CH), **5** (17β-H/17α-OH) and **6** (17,17-di-F), did not inhibit the 17β-HSD3 whereas two, **4a** and **5**, were metabolically more stable than **1**. Therefore, we had at this stage new steroidal inhibitors of 17β-HSD10, selective over 17β-HSD3 and more metabolically stable than lead compound **1**.

In the second strategy, we were interested to increase the 17β-HSD10 inhibition level of compound **1** by exploring effect of modifications at the C3 side chain. In that end, the 3β-piperazinyl-methyl-ADT nucleus as base core was kept intact but the aryl group present on the piperazine nucleus was modified to extend our SAR study. We thus synthesized new compounds by solid-phase synthesis, but only twelve compounds from the 120 shown a better 17β-HSD10 inhibition than compound **1** and their IC_{50} values were determined. Thus, from the four families

of side chain tested (amides, sulfonamides, ureas and amines), the amine derivatives **D-2,4** and **D-3,7** showed the highest inhibitions. Indeed, **D-2,4** was 5.4 folds better than lead compound **1** (IC_{50} = 0.42 μM and 2.26 μM, respectively). This molecule differs from **1** only by the addition of a methyl group at the *para* position on the benzyl substituent. Even better, when the methyl group was replaced by corresponding 4-trifluoromethyl group (**D-3,7**) an additional gain of inhibition was obtained with an IC_{50} value of 0.14 μM (16-fold better than **1**).

In a final step of optimization (third strategy), we decided to combine the two best D-ring modifications to the best inhibitor of libraries A-D (**D-3,7**) leading to hybrid compounds **25** (17β-OH/17α-C≡CH) and **26** (17β-H/17α-OH). Overall, these dual modifications provided more potent inhibitors than lead inhibitor **1** (IC_{50} of 1.26, 0.95 and 2.26 μM, respectively), but the metabolic stability and selectivity of inhibition for 17β-HSD10 over 17β-HSD3 remain to be addressed for compounds **25** and **26**.

4. Conclusion

We synthesized 128 compounds (3α-hydroxy-5α-androstane derivatives with D-ring modification or/and N-substituted 3β-piperazinylmethyl side chain) and tested their ability to inhibit the transformation (oxidation) of E2 to E1 by 17β-HSD10, a mitochondrial enzyme suspected to play a role in AD. Two D-ring modifications (17β-OH/17α-CCH and 17β-H/17α-OH) made it possible to increase the metabolic stability of lead compound **1** while making these inhibitors selective for 17β-HSD10 over the 17β-HSD3. The SAR study also made it possible to obtain trifluorinated compound **D-3,7** which is 16 times more active as an inhibitor of 17β-HSD10 (IC_{50} = 0.14 μM) than lead compound **1**. Finally, the combination of two SAR strategies generated compounds **25** and **26**, two promising candidates for future in vivo studies.

5. Experimental section

5.1 Chemistry

5.1.1 General

Chemical reagents were purchased from Sigma-Aldrich (Saint-Louis, MI, USA), Matrix Innovation (Québec, QC, Canada), Alfa Aesar (Wood Hill, MA, USA) and AstaTech (Bristol, PA, USA), Enamine Building Blocks (Cincinnati, OH, USA), Platte Valley Scientifics (Gothenburg, NE, USA), Aldlab Chemicals (Woburn, MA, USA) and LabNetwork Compounds (Cambridge, MA, USA). The glycerol polymer bound with a loading of 1.0 mmol/g was supplied

by Sigma-Aldrich. Anhydrous dichloromethane (DCM), diethyl ether (Et_2O), dimethylformamide (DMF), ethanol (EtOH), dimethylsulfoxide (DMSO), and tetrahydrofuran (THF) were obtained from Sigma-Aldrich. Ethyl acetate (EtOAc), hexanes and methanol (MeOH) were purchased from Fisher Scientific (Montréal, QC, Canada) and were used as received. The loading of steroid **22** was performed in peptide synthesis vessels with frit equipped for vaccum filtration (ChemGlass Inc.; Vineland, NJ, USA). The steps from ***i*** to ***m*** (Scheme 3) were performed with an AAPPTec Solutions automated organic synthesizer (Louisville, KY, USA) using a solid phase reaction block (96 wells). Thin layer chromatography (TLC) was performed on 0.20 mm silica gel 60 F_{254} plates (E. Merck; Darmstadt, Germany). Flash column chromatography were performed with 230-400 mesh ASTM silica gel 60 supplied by SiliCycle, (Québec, QC, Canada).

Infrared (IR) spectra were recorded with a Horizon MB 3000 ABB FTIR spectrometer (ABB, Québec, QC, Canada) and only characteristic bands were reported in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C on a Bruker Avance 400 digital spectrometer (Billerica, MA, USA). The chemical shifts (δ) are expressed in ppm and referenced to chloroform (7.26 and 77.0 ppm) for ^{1}H and ^{13}C NMR, respectively. ^{1}H NMR signals were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and broad (br). High performance liquid chromatograph (HPLC) purities of final compounds released from solid support were determined with a Shimadzu apparatus (Kyoto, Japan) using a Shimadzu SPD-M20A Photodiode array detector, a Alltima HP C18 reverse-phase column (250 mm x 4.6 mm, 5 μm) and a solvent gradient of MeOH-H_2O with 0.1% acetic acid (AcOH). The wavelength of the UV detector was selected between 190-265 nm. Low resolution mass spectra (LRMS) were recorded on a Shimadzu apparatus equipped with APCI (atmospheric pressure chemical ionization). High-resolution mass spectra (HRMS) were provided by Pierre Audet from the Department of Chemistry at Université Laval (Québec, QC, Canada).

5.1.2 Chemical synthesis of D-ring derivatives

5.1.2.1 Synthesis of 2

To a solution of compound **1** [33] (0.150 g, 0.30 mmol) in MeOH (10 mL) was added sodium borohydride (0.056 g, 1.49 mmol) at 0°C. The mixture was stirred 4 h under inert atmosphere and the resulting solution was evaporated, water added and extracted three times

with EtOAc. The organic layers were washed with water and brine, dried with $MgSO_4$, filtered and evaporated over reduced pressure. The crude compound was purified by flash column chromatography using a gradient of hexanes/EtOAc (1:1 to 8:2) to give compound **2**.

(3α,5α,17β)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-ol (**2**): Amorphous solid (151 mg, 99 %). IR (ATR) υ: 3472 (OH). ^{1}H NMR ($CDCl_3$) δ: 0.72 and 0.73 (2 s, 6H, 19-CH_3 and 18-CH_3), 0.70-1.79 (m, unassigned CH and CH_2), 1.98-2.07 (br m, 1H), 2.25 (s, 2H, NC$\underline{H}_2$-COH), 2.46 (br s, 4H, 2 x NCH_2), 2.64 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NC$\underline{H}_2$-Ar), 3.62 (t, 1H, J = 8.6Hz, 17α-CH), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.8, 1.9 Hz, CH of Ar), 6.87-6.89 (m, 2H, 2 x CH of Ar), 7.24 (t, 1H, J = 8.1 Hz, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.1, 11.2, 20.5, 23.3, 28.5, 30.5, 31.5, 32.7, 33.9, 35.5, 35.8, 36.7, 39.7, 40.8, 42.9, 51.0, 53.4 (2C), 54.2, 55.2, 55.7 (2C), 62.9, 68.9, 70.2, 81.9, 112.4, 114.6, 121.5, 129.1, 139.6, 159.5. HRMS for $C_{32}H_{51}N_2O_3$ $[M+H]^+$: calculated 511.3894, found 511.3897. HPLC purity: 98.3% (RT = 11.8 min, MeOH/H_2O/formic acid (55:45:0.1), Luna C18 column).

5.1.2.2 Synthesis of 3

To a solution of compound **1** (0.100 g, 0.20 mmol) in dry toluene (3 mL) was added methyl magnesium iodide, 3.0 M in diethyl ether (0.66 mL, 1.97 mmol) under inert atmosphere. The mixture was stirred 4 h at reflux, and then at room temperature (rt) overnight. The resulting solution was cooled at 0°C, and an aqueous solution of NH_4Cl (2.6 M) was added dropwise. The organic solvents were evaporated and the aqueous phase was extracted eight times with DCM. The organic layers were combined and washed with water, dried with $MgSO_4$, filtered and evaporated over reduced pressure. The crude compound was purified by flash column chromatography using DCM/MeOH (96:4) to give compound **3**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-methyl-17-ol (**3**): Amorphous white solid (102 mg, 98%). IR (ATR) υ: 3425 (OH). ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 1.20 (s, 3H, 17α-CH_3) 0.70-1.81 (m, unassigned CH and CH_2), 2.25 (s, 2H, N-C$\underline{H}_2$-COH), 2.46 (br s, 4H, 2 x NCH_2), 2.65 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NCH_2-Ar), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.4, 1.9 Hz, 1H, CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.21 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 14.0, 20.6, 23.2, 25.8, 28.6, 31.7 (2C), 32.7, 33.9, 35.8, 36.4, 39.0, 39.7, 40.8, 45.5, 50.7, 53.4, 54.1, 55.2, 55.7, 62.9, 68.9, 70.2, 81.7, 112.4, 114.6, 121.5, 129.1, 139.7, 159.6. HRMS for $C_{33}H_{53}N_2O_3$ $[M+H]^+$: calculated 525.4051, found 525.4060. HPLC purity: 95.9% (RT = 11.3 min, MeOH/H_2O/formic acid (55:45:0.1), Luna C18 column).

5.1.2.3 Synthesis of 4a and 4b

5.1.2.3.1 Synthesis of 4a

To a solution of trimethylsilylacetylene (0.438 g, 4.46 mmol) in anhydrous diethyl ether (25 mL) was added dropwise MeLi (1.6 M in THF, 2.23 mL, 3.57 mmol) at 0°C. The mixture was stirred at rt for 1 h and cooled again at 0°C. The compound **1** (0.150 g, 0.30 mmol) was added in anhydrous THF (25 mL) and the solution was stirred at rt for 15 h. The reaction mixture was stopped by addition of ice/water and the crude compound was extracted with EtOAc, washed with brine, dried with $MgSO_4$, filtered and evaporated. The mixture was purified by flash column chromatography using a gradient of hexanes/EtOAc (6:4 to 5:5) to give **4a**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-(trimethylsilyl)ethynyl-17-ol (**4a**): Amorphous solid (144 mg, 80%). IR (ATR) υ: 3425 (OH), 841 ($SiMe_3$). ^{1}H NMR ($CDCl_3$) δ: 0.17 (s, 9H, $Si(CH_3)_3$), 0.74 (s, 3H, 19-CH_3), 0.81 (s, 3H, 18-CH_3), 0.70-1.70 (m, unassigned CH and CH_2), 1.88-1.96 (m, 1H, 16α-CH_2), 2.19-2.24 (m, 1H), 2.26 (s, 2H, NC$\underline{H_2}$-COH), 2.47 (br s, 4H, 2 x NCH_2), 2.66 (br s, 4H, 2 x NCH_2), 3.48 (s, 2H, NC$\underline{H_2}$-Ar), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.3, 2.0 Hz, 1H, CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.24 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 0.05 (3C), 11.2, 12.9, 20.6, 23.2, 28.5, 29.7, 31.5, 32.8 (2C), 34.0, 35.7, 36.2, 38.9, 39.8, 40.8, 47.0, 50.5, 53.4, 53.8, 55.2, 55.7 (2C), 62.9, 68.9, 70.2, 80.1, 89.9, 109.5, 112.4, 114.7, 121.5, 129.1, 139.6, 159.6. LRMS for $C_{37}H_{59}N_2O_3Si$ [$M+H^+$]: 608.7.

5.1.2.3.2 Synthesis of 4b

To a solution of K_2CO_3 (5% in MeOH, 10 mL) was added **4a** (0.120 g, 0.20 mmol) and the mixture was stirred at room temperature overnight. The reaction was quenched with water, extracted three times with DCM, washed with brine and filtered and evaporated under reduce pressure. The crude solid was purified by flash column chromatography using a gradient of hexanes/EtOAc (4:6 to 2:8) to give **4b**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-ethynyl-17-ol (**4b**): Amorphous white solid (92 mg, 87%). IR (ATR) υ: 3502 and 3387 (OH), 3263 (C≡C-H). ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.75-1.71 (m, unassigned CH and CH_2), 1.91-1.99 (m, 1H, 16α-CH_2), 2.25 (s, 2H, NC$\underline{H_2}$-COH), 2.22-2.30 (m, 1H), 2.46 (br s, 4H, 2 x NCH_2), 2.57 (s, 1H, C≡CH), 2.64 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NC$\underline{H_2}$-Ar), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.7, 1.9 Hz, 1H, CH of Ar), 6.88 (m, 2H, 2 x CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.22 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ:

11.2, 12.8, 20.6, 23.1, 28.5, 31.5, 32.7 (2C), 33.9, 35.8, 36.1, 38.9, 39.7, 40.7, 46.9, 50.4, 53.4 (2C), 53.7, 55.2, 55.7 (2C), 62.9, 68.9, 70.2, 73.8, 79.9, 87.6, 112.4, 114.7, 121.5, 129.1, 139.7, 159.6. HRMS for $C_{34}H_{51}N_2O_3$ $[M+H]^+$: calculated 535.3894, found 535.3898. HPLC purity: 99.5% (RT = 11.3 min, MeOH/H_2O/formic acid (55:45:0.1), Luna C18 column).

5.1.2.4 Synthesis of 5

5.1.2.4.1 Synthesis of 15b

To a solution of 4-nitrobenzoic acid (0.920 g, 5.51 mmol) in toluene (20 mL) was added, under argon atmosphere and over ice bath, triphenylphosphine (1.370 g, 5.23 mmol) and diethyl azodicarboxylate (DEAD) (0.7 mL, 4.46 mmol). The mixture was stirred for 30 min, and 5α-dihydrotestosterone (**15a**) (0.400 g, 1.38 mmol) was added. The resulting solution was stirred at 80°C for 18 h. The solvent was evaporated and the crude compound purified by flash column chromatography using a gradient of hexanes/EtOAc (95:5 to 85:15) to give **15b**.

5α-androstan-(17α-(4-nitrobenzoate))-3-one (**15b**): Amorphous white solid (325 mg, 53 %). IR (ATR) υ: 1713 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.85 (s, 3H, 19-CH_3), 1.03 (s, 3H, 18-CH_3), 0.78-2.43 (m, unassigned CH and CH_2), 5.08 (d, J = 6.1 Hz, 1H, 17β-CH), 8.19 (d, J = 8.8 Hz, 2H, 2 x CH of Ar), 8.30 (d, J = 8.8 Hz, 2H, 2 x CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.5, 16.7, 20.8, 24.8, 28.8, 30.1, 32.0, 32.1, 35.6, 35.8, 38.1, 38.5, 44.6, 45.2, 46.6, 50.4, 53.5, 83.7, 123.5 (2C), 130.4, 130.6 (2C), 136.1, 150.4, 164.2, 211.8. LRMS for $C_{26}H_{34}NO_5$ $[M+H]^+$: 440.25.

5.1.2.4.2 Synthesis of 15c

Compound **15b** (0.315 g, 0.72 mmol) was solubilized in a solution of 10% KOH in MeOH (30 mL). The solution was stirred at rt for 6 h. The solvent was evaporated and water was added. The mixture was extracted with EtOAc (three times), washed with brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude solid was purified by flash column chromatography using hexanes/EtOAc (7:3) to give **15c**.

5α-androstan-17α-ol-3-one (15c): Amorphous white solid (146 mg, 70%). IR (ATR) υ: 3441 (OH), 1697 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.68 (s, 3H, 19-CH_3), 1.02 (s, 3H, 18-CH_3), 0.73-2.43 (m, unassigned CH and CH_2), 3.74 (d, J = 5.0 Hz, 1H, 17β-CH). ^{13}C NMR ($CDCl_3$) δ: 11.5, 17.0, 20.9, 24.6, 28.9, 31.4, 32.0, 32.3, 35.6, 35.7, 38.1, 38.6, 44.6, 45.3, 46.6, 48.5, 53.6, 79.9, 212.1. LRMS for $C_{19}H_{31}O_2$ $[M+H]^+$: 291.25.

5.1.2.4.3 Synthesis of 16

To a solution of trimethylsulfoxonium iodide (0.212 g, 0.96 mmol) in dry DMSO (7 mL) over an ice bath and under argon atmosphere was slowly added sodium hydride 60% in oil

(0.038 mg, 0.95 mmol). The solution was stirred at rt for 1 h before adding compound **15c** (0.135 g, 0.46 mmol) dissolved in dry THF (4 mL). The reaction mixture was stirred overnight, then poured in water (500 mL), and extracted with EtOAc (three times). The combined organic layers were washed with water, brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude compound was purified by flash column chromatography using hexanes/EtOAc (8:2) to give **16**.

17*α*-Hydroxy-spiro-3(*R*)-oxirane-5*α*-androstane (**16**): Amorphous white solid (124 mg, 87%). IR (ATR) υ: 3455 (OH). ^{1}H NMR ($CDCl_3$) δ: 0.66 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.78-1.78 (m, unassigned CH and CH_2), 1.87 (t, J = 13.6 Hz, 1H), 2.04 (td, J = 14.1, 4.7 Hz, 1H), 2.10-2.18 (m, 1H), 2.61 (s, 2H, CH_2(O)C), 3.72 (d, J = 5.9 Hz, 1H, 17β-CH). ^{13}C NMR ($CDCl_3$) δ: 11.3, 17.0, 20.5, 24.6, 28.5, 29.2, 31.4, 32.1, 32.3, 35.5, 35.7, 35.9, 35.8, 43.6, 45.3, 48.7, 53.5, 53.8, 58.6, 80.0. LRMS for $C_{20}H_{33}O_2$ $[M+H]^+$: 291.25.

5.1.2.4.4 Synthesis of building block 18

To a solution of piperazine (1.65 g, 19.20 mmol) in DCM (50 mL) was added 3-methoxybenzylchloride (**17**) (0.300 g, 1.92 mmol) at 0°C. The solution was next stirred at rt for 19 h. After completion, water was added, and the solution was extracted with DCM (four times), washed with water, brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The mixture was purified by flash column chromatography using DCM/MeOH/Et_3N (94:5:1) to give the building block **18**.

1-(3-methoxybenzyl)-piperazine (**18**): Gummy brownish solid (398 mg, 99%). IR (ATR) υ: 3225 (NH). ^{1}H NMR ($CDCl_3$) δ: 2.42 (br s, 4 H, 2 x NCH_2), 2.88-2.90 (br m, 5H, 2 x NCH_2 and NH), 3.45 (s, 2H, NCH_2-Ar), 3.78 (s, 3H, OCH_3), 6.78 (dd, J = 7.1, 1.5 Hz, 1H, CH of Ar), 6.88-6.91 (m, 2H, 2 x CH of Ar), 7.22 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 45.8, 54.0 (2C), 55.2, 63.5 (2C), 112.4, 114.6, 121.5, 129.1, 139.7, 159.6. LRMS for $C_{12}H_{19}N_2O$ $[M+H]^+$: 207.2.

5.1.2.4.5 Synthesis of 5

To a solution of compound **16** (0.040 g, 0.13 mmol) in EtOH (10 mL) was added the piperazine derivative **18** (0.136 g, 0.66 mmol). The resulting mixture was stirred at 60°C for 24 h and the solvent evaporated. The crude compound was purified by flash column chromatography using DCM:MeOH (97:3) to give **5**.

(3*α*,5*α*,17*α*)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-ol (5): Amorphous brownish solid (59 mg, 85%). IR (ATR) υ: 3448 (OH). ^{1}H NMR ($CDCl_3$) δ: 0.64 (s,

3H, 19-CH_3), 0.73 (s, 3H, 18-CH_3), 0.77-1.80 (m, unassigned CH and CH_2), 2.04-2.17 (m, 1H), 2.25 (s, 2H, NC<u>H</u>$_2$-COH), 2.46 (br s, 4H, 2 x NCH_2), 2.64 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NCH_2-Ar), 3.71 (d, J = 5.9 Hz, 1H, 17α-CH), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.4, 1.9 Hz, 1H, CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.22 (t, J = 8.1 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 17.1, 20.4, 24.6, 28.7, 31.5, 32.3, 32.8 (2C), 34.0, 35.7, 35.8, 39.8, 40.7, 45.3, 48.7, 53.4 (2C), 53.9, 55.2, 55.7 (2C), 62.9, 69.0, 70.2, 80.0, 112.4, 114.6, 121.5, 129.1, 139.7, 159.6. HRMS for $C_{32}H_{51}N_2O_3$ $[M+H]^+$: calculated 511.3894, found 511.3900. HPLC purity: 98.5% (RT = 15.3 min, MeOH/H_2O/formic acid (55:45:0.1), Luna C18 column).

5.1.2.5 Synthesis of 6

To a solution of compound **19** [39; compound D in Supporting Information] (0.017 g, 0.05 mmol) in EtOH (3 mL) was added the piperazine derivative **18** (0.054 g, 0.26 mmol) and the resulting mixture was stirred at 60°C for 23 h. The solvent was evaporated and the crude compound was purified by flash column chromatography using DCM:MeOH (98:2) to give **6**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}-17-difluoroandrostane (**6**): Amorphous brownish solid (18 mg, 65%). IR (ATR) υ: 3490 (OH), 1311 (C-F). ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 3H, 19-CH_3), 0.85 (s, 3H, 18-CH_3), 0.75-1.72 (m, unassigned CH and CH_2), 1.97-2.19 (m, 2H), 2.26 (s, 2H, NC<u>H</u>$_2$-COH), 2.46 (br s, 4H, 2 x NCH_2), 2.65 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NC<u>H</u>$_2$-Ar, 2H), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.5, 1.9 Hz, 1H, CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.22 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 13.4 (t, J_{CCCF} = 4.5 Hz), 19.9, 22.2 (22.3), 28.4, 28.8 (28.9), 31.0, 32.7, 32.9 (t, J_{CCF} = 25.2 Hz), 33.9, 35.4, 35.8, 39.7, 40.7, 45.3 (t, J_{CCF} = 20.1 Hz), 49.3 (49.4), 53.4, 53.7 (2C), 55.2, 55.7 (2C), 62.9, 68.9, 70.1, 112.4, 114.7, 121.5, 129.1, 132.7 (dd, J_{CF} = 259.1, 250.8 Hz, 17-CF_2), 139.7, 159.6. HRMS for $C_{32}H_{49}F_2N_2O_2$ $[M+H]^+$: calculated 531.3757, found 531.3763. HPLC purity: 94.9% (RT = 18.9 min, MeOH/H_2O/formic acid (40:60:0.1), Luna C18 column).

5.1.2.6 Synthesis of 7

To a solution of compound **20** [39] (0.007 g, 0.02 mmol) in EtOH (10 mL) was added piperazine derivative **18** (0.022 g, 0.11 mmol) and the resulting mixture was stirred at 60°C for 23 h. The solvent was evaporated and the crude compound was purified by flash column chromatography using a gradient of DCM:MeOH (98:2 to 96:4) and to give **7**.

(3α,5α)-3-Hydroxy-3-{[4-(3-methoxybenzyl)piperazin-1-yl]methyl}-16,16-dimethyl-androstan-17-one (**7**): Amorphous brownish solid (9 mg, 81%). IR (ATR) υ: 3450 (OH), 1728 (C=O). ^{1}H NMR ($CDCl_3$) δ: 0.75 (s, 3H, 19-CH_3), 0.87 (s, 3H, 18-CH_3), 1.02 (s, 3H, 16-CH_3), 1.16 (s, 3H, 16-CH_3), 0.80-1.82 (m, unassigned CH and CH_2), 2.26 (s, 2H, NC$\underline{H_2}$-COH), 2.46 (br s, 4H, 2 x NCH_2), 2.65 (br s, 4H, 2 x NCH_2), 3.47 (s, 2H, NC$\underline{H_2}$-Ar), 3.80 (s, 3H, OCH_3), 6.79 (dd, J = 7.4, 1.9 Hz, 1H, CH of Ar), 6.87-6.90 (m, 2H, 2 x CH of Ar), 7.22 (t, J = 8.0 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 14.4, 20.1, 25.9, 27.3, 28.4, 31.0, 32.3, 32.7, 33.8, 34.6, 35.9, 37.9, 39.7, 40.8, 45.1, 48.1, 48.9, 53.4 (2C), 54.4, 55.2, 55.8 (2C), 62.9, 68.9, 70.1, 112.4, 114.7, 121.5, 129.1, 139.7, 159.6, 225.7. HRMS for $C_{34}H_{53}N_2O_3$ $[M+H]^+$: calculated 537.537.4051, found 537.4055. HPLC purity: 93.7% (RT = 15.7 min, MeOH/H_2O/formic acid (70:30:0.1), Luna C18 column).

5.1.3 Solid phase synthesis of androstane derivatives

5.1.3.1 Synthesis of 22 (Fmoc protection of piperazine and deprotection of 17-ketone)

To a solution of oxirane **21** [40] (7.5 g, 21 mmol) in EtOH (500 mL) was added piperazine (7.4 g, 85 mmol) and the resulting mixture was stirred at 60°C for 6 h. The solvent was evaporated and water was added. The mixture was extracted with EtOAc and the organic phase washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure to give 8.7 g of the piperazino derivative. To a solution of this crude compound in THF (650 mL) was added $NaHCO_3$ (1.0 M, 350 mL) and Fmoc-O-succinimide (8.39 g, 24.9 mmol). The resulting solution was stirred at rt for 16 h and then quenched with water and extracted with EtOAc. The organic phase was washed with brine, dried with Na_2SO_4 and evaporated to give 12.3 g of crude compound. To a solution of this crude compound in DCM was added HCl 1.0 M in MeOH (25:75). The reaction was stirred at rt for 16 h. The crude solution was washed with saturated aqueous $NaHCO_3$, filtered on a phase separator (Biotage, Uppsala, Sweden) and washed with DCM. The crude solid was purified by flash chromatography using a gradient of hexanes/EtOAc (7:3 to 1:1) to give **22**.

(3α,5α)-3-Hydroxy-3-{[4-((9H-fluoren-9-yl)methoxy)carbonyl)piperazin-1-yl]methyl}-16,16-dimethyl-androstan-17-one (**22**): Amorphous white solid (9.0 g, 72%). IR (ATR) υ: 3455 (OH), 1736 (C=O of Fmoc), 1705 (17-C=O). ^{1}H NMR ($CDCl_3$) δ: 0.77 (s, 3H, 19-CH_3), 0.86 (s, 3H, 18-CH_3), 0.80-1.97 (m, unassigned CH and CH_2), 2.02-2.12 (m, 1H, 16α-CH), 2.29 (s, 2H, NC$\underline{H_2}$-COH), 2.43 (dd, J = 19.2, 8.7 Hz, 1H, 16β-CH), 2.56 (br s, 4H, 2 x NCH_2), 3.47 (br s, 4H, 2 x NCH_2), 4.24 (t, J = 6.8 Hz, 1H, C$\underline{H}CH_2O$), 4.44 (d, J = 6.8 Hz, 2H, CHC$\underline{H_2}$O), 7.37 (t, J

= 7.4 Hz, 2H, 2 x CH of Ar), 7.40 (t, J = 7.5 Hz, 2H, 2 x CH of Ar), 7.56 (d, J = 7.4 Hz, 2H, 2 x CH of Ar), 7.76 (d, J = 7.5 Hz, 2H, 2 x CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 13.8, 20.2, 21.8, 28.4, 30.8, 31.6, 32.5, 33.8, 35.1, 35.8, 35.9, 39.4, 40.7, 44.1, 47.4, 47.8, 51.5, 54.2, 55.4, 67.2, 69.2, 70.5, 120.0 (2C), 124.9 (2C), 127.0 (2C), 127.7 (2C), 141.3 (2C), 144.0 (2C), 155.1, 221.3.

5.1.3.2 Coupling of steroid (synthesis of resin 23a)

To a suspension of glycerol polymer bound resin (10.0 g; loading of 1.0 mmol/g) in dry toluene was added ketone **22** in dry toluene (9.0 g, 14.7 mmol), trimethyl orthoformate (16.1 mL, 147.2 mmol) and *p*-TSA (3.8 g, 20.2 mmol). The solution was stirred at rt for 17 h, washed three times successively by DCM, MeOH and DCM. The resin was dried overnight under vacuum to give 14.8 g of **23a**.

5.1.3.3 Fmoc deprotection of piperazine (synthesis of resin 23b)

To a suspension of resin **23a** (14.3 g) in DMF and in a peptide flask was added a solution of piperidine in DMF (20%, 250 mL) and the resulting mixture was stirred at rt for 3.5 h. The resin was filtered, washed five times successively with DCM, MeOH and DMF, and dried overnight under vacuum to afford 11.3 g of resin **23b** (loading of 0.41 mmol/g by weight difference).

5.1.3.4 Synthesis of amide derivatives 8 (Libraries A1 and A2)

5.1.3.4.1 Amide bound formation

Portions of resin **23b** (50 mg; loading 0.41 mmol/g) were placed in 4 mL reactor wells of an automated synthesizer reaction block (96 well format, AAPPTec). A solution of HBTU (0.5 M, 18.9 g in 100 mL DMF) was added (3 mL) to the well and the suspension was shaken for 2 min. Then, a solution of DIPEA (1.0 M, 17.4 mL in 82.6 mL DMF) was added (1 mL) to each well followed by the addition of a solution of the appropriate carboxylic acid (0.5 M), solubilized in DMF (2 mL). The suspension was vortexed at 600 rpm for 3 h. The well was then filtered using the vacuum system and resin washed with DCM (3 mL), then EtOH (3 mL) three times.

5.1.3.4.2 Cleavage of amide derivatives

To the coupled amide resin in 4 mL reactor wells was added a solution of acetyl chloride-MeOH-DCM (1:9:30) (2.5 mL) and the resulting suspension was vortexed at 600 rpm for a total of 20 h. The resin was then filtered, washed with MeOH:DCM (1:1) (1.5 mL) and the filtrate neutralized with a saturated aqueous solution of $NaHCO_3$ (2 mL). The biphasic solutions were filtered with a phase separator syringe (Biotage) and each organic solution was evaporated and dried under reduced pressure. The amide derivatives **8** (two libraries, **A-1** and **A-2**, generated in parallel) were weighted and characterized by ^{1}H NMR (Table 3). Only compounds with

appropriate NMR signals and NMR purities (> 60%) were next analyzed by mass spectrometry and purity determined by HPLC (41 compounds, Supplementary data for quantity, purity, ^{1}H NMR and MS).

5.1.3.5 Synthesis of sulfonamide derivatives 9 (Library B)

Portions of resin **23b** (50 mg; loading 0.41 mmol/g) were placed in 4 mL reactor wells of an automated synthetizer reaction block (96 well format, AAPPTec). Triethylamine (Et_3N) was added (1 mL) to the well and a solution of appropriate sulfonyl chloride (between 0.15 M to 0.3 M) in DCM (2 mL) was added. The suspension was vortexed at 600 rpm for 3 h. The well was then filtered using the vacuum system and resin washed successively with DCM (1.5 mL) and EtOH (1.5 mL) two times. The resin was submitted to conditions of cleavage mentioned above. The sulfonamide derivatives **9** (one library, **B-1**, generated in parallel) were weighted and characterized by ^{1}H NMR (Table 3). Only compounds with appropriate NMR signals and NMR purities (> 60%) were next analyzed by mass spectrometry and purity determined by HPLC (19 compounds, Supplementary data for quantity, purity, ^{1}H NMR and MS).

5.1.3.6 Synthesis of urea and thiourea derivatives 10 (Library C)

Portions of resin **23b** (50 mg; loading 0.41 mmol/g) were placed in 4 mL reactor wells of an automated synthesizer reaction block (96 well format, AAPPTec). A solution of Et_3N (2.5%) in DCM was added (1 mL) to the well and a solution of appropriate isocyanate or isothiocyanate (0.5 M) was added (1.5 mL). The suspension was vortexed at 600 rpm for 5 h. The well was then filtered using the vacuum system and resin was washed with DCM (1.5 mL), then EtOH (1.5 mL) and DCM (1.5 mL) two times. The resin was submitted to conditions of cleavage mentioned above. Thiourea derivatives **10** (one library, **C-1**, generated in parallel) were weighted and characterized by ^{1}H NMR (Table 3). Only compounds with appropriate NMR signals and NMR purities (> 60%) were next analyzed by mass spectrometry and purity determined by HPLC (21 compounds, Supplementary data for quantity, purity, ^{1}H NMR and MS).

5.1.3.7 Synthesis of amine derivatives 11 (Libraries D1, D2 and D3)

Portions of resin **23b** (50 mg; loading 0.41 mmol/g) were placed in 4 mL reactor wells of an automated synthesizer reaction block (96 well format, AAPPTec). A solution of the appropriate aldehyde (1.0 M), solubilized in DMF/AcOH (99:1) was added (1 mL) to the well. The suspensions were vortexed at 600 rpm for 1 h. Then, a solution of $NaBH_3CN$ (1.0 M), in DCM/MeOH/AcOH (75:24:1) was added (1 mL), the suspension was vortexed at 600 rpm for 5 h. The well was filtered using the vacuum system and resin was washed with DCM (3 mL), then EtOH (3 mL) three times. The resin was submitted to conditions of cleavage mentioned above.

Amine derivatives **11** (three libraries, **D-1**, **D-2** and **D-3**, generated in parallel) were weighted and characterized by ^{1}H NMR (Table 3). Only compounds with appropriate NMR signals and NMR purities (> 60%) were next analyzed by mass spectrometry and their purity determined by HPLC (55 compounds, Supplementary data for quantity, purity, ^{1}H NMR and MS).

5.1.4 Synthesis of hybrid inhibitors 25 and 26

5.1.4.1 Synthesis of intermediate compound 24

Following the procedure reported in section **5.1.2.3.1**, compound **D3-7** (50 mg, 0.087 mmol) was transformed to **24**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(4-trifluoromethyl-3-methoxy-benzyl)piperazin-1-yl]methyl}androstan-17-(trimethylsilyl)ethynyl-17-ol (24): Amorphous white solid (40 mg, 68%). IR (ATR) υ: 3430 (OH), 1126 (C-F), 841 ($Si(CH_3)_3$). ^{1}H NMR ($CDCl_3$) δ: 0.17 (s, 9H, $Si(CH_3)_3$), 0.74 (s, 3H, 19-CH_3), 0.81 (s, 3H, 18-CH_3), 0.80-2.25 (m, unassigned CH and CH_2), 2.27 (s, 2H, N-C$\underline{H}_2$-COH), 2.47 (br s, 4H, 2 x NCH_2), 2.66 (br s, 4H, 2 x NCH_2), 3.51 (s, 2H, NCH_2-Ar), 3.90 (s, 3H, OCH_3), 6.93 (d, J = 7.9 Hz, 1H, CH of Ar), 7.01 (s, 1H, CH of Ar), 7.48 (d, J = 7.9 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 0.1 ($Si(CH_3)_3$, 11.2, 12.9, 20.7, 23.2, 28.6, 31.5, 32.8 (2C), 34.0, 35.8, 36.2, 39.0, 39.8, 40.8, 47.0, 50.6, 53.5, 53.9, 55.7, 55.9, 62.6, 69.0, 70.3, 80.1, 89.9, 109.6, 112.3, 117.5 (q, J = 30.8 Hz), 120.4, 123.7 (q, J = 271.8 Hz), 126.9 (q, J = 5.3 Hz), 144.5, 157.5. LRMS for $C_{38}H_{58}F_3N_2O_3Si$ $[M+H^+]$: 675.5.

5.1.4.2 Synthesis of 25

Following the procedure reported in section **5.1.2.3.2**, a solution of K_2CO_3 (5% in MeOH, 2 mL) was added to compound **24** (25 mg, 0.04 mmol) to give **25**.

(3α,5α,17α,17β)-3-Hydroxy-3-{[4-(4-trifluoromethyl-3-methoxybenzyl)piperazin-1-yl]methyl} androstan-17-ethynyl-17-ol (25): Amorphous white solid, 21 mg, 95%. IR (ATR) υ: 3302 (OH), 1126 (C-F). ^{1}H RMN δ: 0.75 (s, 3H, 19-CH_3), 0.83 (s, 3H, 18-CH_3), 0.80-2.10 (m, unassigned CH and CH_2), 2.23-2.30 (m, 3H, N-C$\underline{H}_2$-COH and 16-CH), 2.46 (br s, 4H, 2 x NCH_2), 2.57 (s, 1H, C≡CH), 2.66 (br s, 4H, 2 x NCH_2), 3.51 (s, 2H, NC$\underline{H}_2$-Ar), 3.90 (s, 3H, OCH_3), 6.93 (d, J = 7.9 Hz, 1H, CH of Ar), 7.01 (s, 1H, CH of Ar), 7.48 (d, J = 7.9 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 12.8, 20.6, 23.1, 28.5, 30.8, 31.5, 32.7, 33.9, 35.8, 36.2, 38.9, 39.7, 40.7, 46.9, 50.5, 53.5, 53.7, 55.7, 55.9, 62.6, 69.0, 70.3, 73.8, 79.9, 87.6, 112.3, 117.5 (q, J = 30.5 Hz), 120.3, 123.7 (q, J = 272.0 Hz), 126.9 (q, J = 5.1 Hz), 144.5, 157.6. HRMS for $C_{35}H_{50}F_3N_2O_3$ $[M+H]^+$: calculated 603.3768, found 603.3764. HPLC purity of 86.6% (RT = 19.0 min, MeOH/H_2O (40:60 to 100:0),Luna C18 column).

5.1.2.3 Synthesis of 26

To a solution of compound **16** (66 mg, 0.22 mmol) in EtOH (10 mL) was added piperazine (93 mg, 1.08 mmol). The resulting mixture was stirred at 60 °C for 20 h and the reaction was quenched by addition of water. The solution was extracted three times with EtOAc, the organic phases combined and washed with $NaHCO_3$, dried over $MgSO_4$, filtered and evaporated under reduce pressure. This crude white solid (72 mg) was then dissolved in MeOH:AcOH (99:1, 10 mL) and 4-trifluoromethyl-3-methoxybenzaldehyde (15 mg, 0.74 mmol) was added. The solution was stirred 2.5 h at rt. Then, $NaBH_3CN$ (35 mg, 0.55 mmol) was added and the mixture stirred for 18 h. The solvent was removed under reduced pressure and $NaHCO_3$ (aq. saturated) and DCM were added. The aqueous phase was extracted three times with DCM and the organic phases combined, washed with brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude compound was purified by flash column chromatography using DCM:MeOH (97:3) to afford **26**.

(3α,5α,17α)-3-Hydroxy-3-{[4-(4-trifluoromethyl-3-methoxybenzyl)piperazin-1-yl]methyl}androstan-17-ol (26): amorphous white solid (65 mg, 52%). IR (ATR) υ: 3418 (OH), 1126 (C-F). ^{1}H RMN δ: 0.65 (s, 3H, 19-CH_3), 0.74 (s, 3H, 18-CH_3), 0.80-2.20 (m, unassigned CH and CH_2), 2.27 (s, 2H, N-C$\underline{H}_2$-COH), 2.47 (br s, 4H, 2 x NCH_2), 2.66 (br s, 4H, 2 x NCH_2), 3.51 (s, 2H, NC$\underline{H}_2$-Ar), 3.71 (d, J = 5.8 Hz, 17β-CH), 3.90 (s, 3H, OCH_3), 6.93 (d, J = 7.9 Hz, 1H, CH of Ar), 7.01 (s, 1H, CH of Ar), 7.48 (d, J = 7.9 Hz, 1H, CH of Ar). ^{13}C NMR ($CDCl_3$) δ: 11.2, 17.1, 20.5, 24.6, 28.7, 31.5, 32.3, 32.8, 34.0, 35.8, 39.8, 40.7, 45.3, 48.7, 53.5, 53.9, 55.7, 55.9, 62.6, 69.0, 70.3, 80.0, 112.3, 117.5 (q, J = 30.7 Hz), 120.4, 123.7 (q, J = 272.1 Hz), 126.9 (q, J = 5.1 Hz), 144.5, 157.6. HRMS for $C_{33}H_{50}F_3N_2O_3$ $[M+H]^+$: calculated 579.3768, found 579.3768. HPLC purity of 90.1% (RT = 20.7 min, MeOH/H_2O (40:60 to 100:0) Luna C18 column).

5.2 Biological assays

5.2.1 Inhibition of 17β-HSD10 activity

5.2.1.1 Generation of stably transfected human embryonic kidney (HEK)-293 cells expressing 17β-HSD10

Cells were cultured in six-well falcon flasks to approximately 3 x 10^5 cells/wells in Dubelcco's Modified Eagle's Medium (DMEM) (Life Technologies, Burlington, ON, Canada) supplemented with 10% of foetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT, USA) at 37°C under a 95% air-5% CO_2 humidified atmosphere. Five micrograms of pCMVneo-

h17βHSD10 plasmids were transfected using a lipofectin transfection kit (Life Technologies, Burlington, ON, Canada). After 6 h of incubation, at 37°C, the transfection medium was removed, and 2 mL DMEM were added. Cells were further cultured for 48 h and then transferred into 10 cm petri dishes and cultured in DMEM containing 700 μg/mL of Geneticin (G418; Wisent, Montréal, QC, Canada) to inhibit the growth of non-transfected cells. Medium containing G418 was changed every 2 days until resistant colonies were observed.

5.2.1.2 Cell culture

Stably transfected HEK-293 cells were cultured in minimum essential medium (MEM) containing non-essential amino acids (0.1 nM), glutamine (2 mM), sodium pyruvate (1 mM), 10% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL) and G418 (700 μg/mL).

5.2.1.3 Inhibition of E2 to E1 transformation by HEK-293 overexpressing 17β-HSD10

HEK-293 cells stably transfected with 17β-HSD10 were seeded at 2.5 x 10^5 cells in a 24-well plate at 37°C under a 95% air-5% CO_2 humidified atmosphere in 990 μL of culture medium. Inhibitor stock solution were prepared in DMSO (10 mM) and diluted with culture medium. After 24 h, 5 μL of these solutions were added to the cells to obtain a final inhibition concentration of 0.3 and 3.0 μM. For the most active inhibitors, concentrations of 0.01 μM to 5 μM were tested to determine their IC_{50} value. The final DMSO concentration in each well, including the wells for the blank control without any compound, was adjusted to 0.5%. Additionally, 5 μL of a solution containing [^{14}C]-17β-estradiol (55 mCi/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) and 17β-estradiol (Sigma-Aldrich) (1:9) was added to obtain a final concentration of 1 μM and cells were incubated for 40 h. Each inhibitor was assessed in triplicate. After incubation, the culture medium was removed, and steroids (E1 and E2) were extracted with diethyl ether. The organic phase was evaporated to dryness under nitrogen. Residues were dissolved in DCM and dropped on silica gel thin layer chromatography plates (EMD Chemicals Inc, Gibbstown, NJ, USA) and eluted with a mixture toluene/acetone (4:1). Substrate [^{14}C]-E2 and metabolite [^{14}C]-E1 were identified by comparing them with reference steroids (E2 and E1) and quantified using the Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA). After subtracting the value corresponding to the blank control, percentages of transformation and next the percentages of inhibition were calculated as follow: % of transformation = 100 x [^{14}C]-E1/([^{14}C]-E2 + [^{14}C]-E1) and % of inhibition = 100 x (% transformation without inhibitor - % transformation with inhibitor/ % transformation without inhibitor). The specific activity for the transformation of E2 into E1 was estimated to be 1.3 x 10^{-13} μmol min^{-1} $cell^{-1}$.

5.2.2 Inhibition of 4-dione to T transformation by LNCaP cells overexpressing 17β-HSD3

LNCaP transfected cells (LNCaP[17β-HSD3]) kindly provided by IPSEN INNOVATION (France) were maintained at 37°C under 5% CO_2 humidified atmosphere. Cells were grown in RPMI-1640 medium supplemented (v/v) with 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin, 2 mM L-glutamine, 4.5 g/L D-glucose, 10 mM Hepes, 1 mM sodium pyruvate and 250 µg/mL hygromycin. For enzymatic assays, the protocol medium had the same composition, but hygromycin, used to maintain the clone selection, was not included. LNCaP[17β-HSD3] cells were plated in a 24-well culture at 10^4 cells per well, in protocol medium. After 2 days of incubation, 15 nM of [^{14}C]-4-androstene-3,17-dione (53.6 mCi/mmol; Perkin Elmer Life Sciences Inc., Boston, MA, USA) and 10 µL of a solution of inhibitor dissolved in DMSO and culture medium was added. The final DMSO concentration in each well, including the wells for the blank control without any compound, was adjusted to 0.05%. After 1 h of incubation, the culture medium was removed from wells and steroids (4-dione and testosterone) were extracted with diethyl ether. After evaporating the organic phase to dryness with nitrogen stream, residue was dissolved in DCM, dropped on silica gel 60 F254 thin layer chromatography plates (EMD Chemicals Inc, Gibbstown, NJ, USA), and eluted with a mixture of toluene/acetone (4:1). [^{14}C]-4-dione and [^{14}C]-T were identified by comparison with reference steroids and quantified using the Storm 860 System (Molecular Dynamics). After subtracting the value corresponding to the blank control, percentages of transformation and next the percentages of inhibition were calculated using the equation reported above. The specific activity for the transformation of 4-dione into T was estimated to be 4.3 x 10^{-12} µmol min^{-1} $cell^{-1}$.

5.2.3 Metabolic stability assays

Assays were performed for 1 h at 37 °C, with or without 10 mM NADPH in the presence of 40 µg of human liver microsomes from Corning (Melrose, MA, USA) and 10 µM of substrate in a final 100 µL volume of 50 mM Tris buffer supplemented with 10 mM $MgCl_2$. Assays were ended by adding 100 µL of MeOH and the solution centrifuged at 13,000 g for 10 min to obtain a pellet of proteins. The surpernatants of 2 assays were pooled, filtered and 100 µL submitted to HPLC-MS analysis (Shimadzu LCMS-2020 APCI, Alltima HP C18 (250 mm × 4.6 mm, 5 µm) column, MeOH:water gradient). The solvent gradient started with a mixture of MeOH:water (70:30, 50:50, 30:70 or 20:80 according to the compound) and finished with MeOH (100%). The wavelength of the UV detector was selected at 190 nm. Remaining compound (expressed in %)

was calculated by dividing the area under the curve of the substrate for the assays with NADPH by the one without NADPH and multiplied by 100.

Acknowledgments

This work was supported by a seed grant from Merck Sharpe & Dome – Faculté de médecine (Université Laval) and a financial support from Mitacs Inc (Montréal, QC, Canada). Sophie Boutin would like to thank the foundation of CHU de Québec (Endocrinology and Nephrology Unit) and the Faculty of Medicine of Université Laval for two fellowships. The authors would like to thank IPSEN INNOVATION (France) for providing LNCaP[17β-HSD3] cells. Our thanks also to Dr. Martin Perreault, for its participation to the metabolic stability assay as well as discussion, to Ms. Marie-Claude Trottier for NMR data and to Ms. Micheline Harvey for the careful reading of this manuscript.

Declaration of competing interest

RM, JR and DP have ownership interests on patent applications and patents related to 17β-HSD inhibitors. SB declares no conflict of interest.

Supplementary data

Structures and screening results (inhibition of 17β-HSD10 and 17β-HSD3) for all members of libraries A, B, C and D (Table S1). Structures (Figures S1-S7), chemical data (^{1}H NMR, MS and HPLC purity) and spectra (^{1}H NMR) of 120 library members (compounds **8**-**11**). Spectra (^{1}H NMR and ^{13}C NMR) for D-ring derivatives **2**, **3**, **4b**, **5**, **6**, **7**, **25** and **26**. ClogP *versus* IC_{50} values (Figure S8) for compounds from Table 3.

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Highlights

- 128 *N*-substituted 3β-piperazinylmethyl-3α-OH-5α-androstane derivatives were prepared.
- They inhibited the oxidation of estradiol to estrone by 17β-HSD10.
- Compound D-3,7 is the best inhibitor with IC_{50} value of 0.14 μM.
- D-ring modifications increased metabolic stability.
- D-ring modifications increased selectivity of inhibition (17β-HSD10 *vs* 17β-HSD3).

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

RM, JR and DP have ownership interests on patent applications and patents related to 17β-HSD inhibitors. SB declares no conflict of interest.