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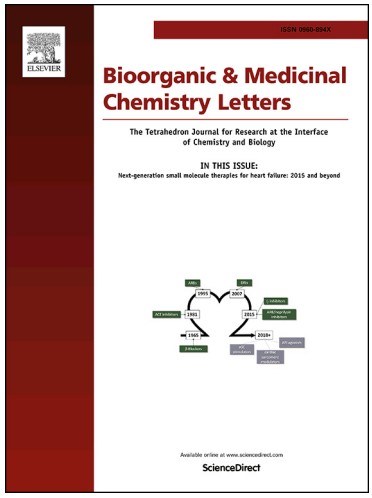

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Identification of steroidal derivatives inhibiting the transformations of allopregnanolone and estradiol by 17β-hydroxysteroid dehydrogenase type 10

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Abstract

17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) is a mitochondrial enzyme known for its potential role in Alzheimer's Disease (AD). 17β-HSD10, by its oxidative activity, could decrease the concentration of two important neurosteroids, allopregnanolone (ALLOP) and 17β-estradiol (E2), respectively preventing their neurogenesis and neuroprotective effects. Since the inhibition of 17β-HSD10 could lead to a new treatment for AD, we developed two biological assays using labeled ALLOP or E2 as substrates to measure the inhibitory activity of compounds against pure 17β-HSD10 protein. After the optimization of different parameters (time, concentration of enzyme, substrate and cofactor), analogs of the first reported steroidal inhibitor of 17β-HSD10 in intact cells were screened to determine their inhibitory potency for the ALLOP or the E2 oxidation. One compound, androstane derivative **5**, possesses the best dual inhibition against both transformations (ALLOP, IC_{50} = 235 μM and E2, IC_{50} = 610 μM). Some compounds are dual inhibitors to a lesser extent, and others seem selective for one of the transformations in particular. By developing two reliable assays and by identifying a first generation of steroidal inhibitors of pure 17β-HSD10, this preliminary study opens the door to new and more potent inhibitors.

Keywords: neurosteroids, allopregnanolone, estradiol, 17β-HSD10, Alzheimer

Alzheimer's Disease (AD) is a neurodegenerative disorder and the most common form of dementia. AD is characterized by the loss of cognitive function including memory, behavioral function, and reasoning, among others.[1,2] In recent years, the focus was on understanding the underlying mechanisms of this disease to find therapeutic targets.[3-5] The implication of neuroactive steroids in AD has therefore been explored and studied.[6] Neurosteroids can be produced in the brain from cholesterol following the steroidogenesis pathway, or can be metabolized locally from circulating steroids synthesized by peripheral steroidogenic organs. They could also have positive effects on various diseases of the central nervous system.[7,8] More specifically, the neuronal steroids allopregnanolone (ALLOP) and 17β-estradiol (E2) possess properties possibly linked to the prevention and the treatment of AD.[9-13] Allopregnanolone is a positive allosteric modulator of the $GABA_A$ receptors[9], and by its action in the regulation of ion-exchange dynamics, this neurosteroid would promote neurogenesis.[10] Thus, increasing ALLOP concentration in the brain should promote the regeneration of neuronal cells.[11] On the other hand, E2 exerts neuroprotective effects by its regulation of amyloid-β (Aβ) protein

precursor trafficking and metabolism.[12] It was also reported that E2 treatment reduces the formation of Aβ in both *in vivo* and *in vitro* experiments.[13]

The mitochondrial enzyme 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) possesses properties suggesting a possible role in the development of AD.[14] First, higher concentrations of 17β-HSD10 are found in the brain of people affected by AD, compared to those who do not have the disease.[15,16] Secondly, 17β-HSD10 is known for its formation of a high affinity neurotoxic complex with amyloid beta 42 (Aβ-42)[17], the peptide responsible for the formation of Aβ plaque in the brain. Neuropathologically, AD is characterized by the abundance of these extracellular Aβ plaques.[18] Furthermore, several studies suggested that inhibiting the neurotoxic complex 17β-HSD10 and Aβ-42 formation is a promising therapeutic approach.[19-20] Finally, 17β-HSD10 catalyzes the oxidation of several substrates, including ALLOP and E2, using the nicotinamide adenine dinucleotide (NAD^+) as cofactor (Fig. 1A).[9,21,22] In an AD affected brain, 17β-HSD10 activity could lower the levels of ALLOP and E2, thus promoting the development of the disease. Therefore, by preventing the oxidation of ALLOP and E2, inhibitors of 17β-HSD10 could increase the concentration of these neurosteroids and promote their neuroprotective and neuroregenerative effects. Thus, inhibition of 17β-HSD10's enzymatic activity could provide a new approach for the treatment of AD, as well as a new tool to study the mechanisms involved in the disease.

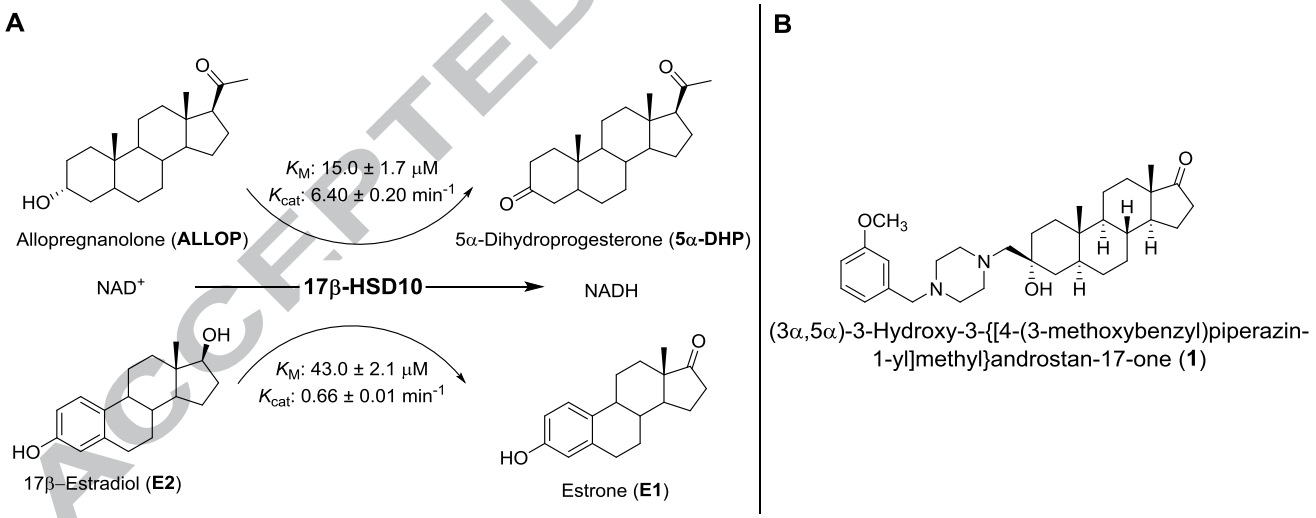


Fig. 1. **A.** Transformation of two neurosteroids catalyzed by 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) in the presence of NAD^+ as cofactor. The K_M and K_{cat} values reported are taken from *Yang et al.*[15] **B.** Chemical structure of lead compound **1**, a first steroidal inhibitor of 17β-HSD10 identified previously in intact cells.

Until now, the published biological assessments of potential 17β-HSD10 inhibitors mostly report the use of S-acetoacetyl CoA as a substrate. In fact, this assay requires measuring the decrease in absorbance (oxidation) of the cofactor NADH. This implies that 17β-HSD10 reduces, and does not

oxidize, the S-acetoacetyl CoA, a non-steroidal substrate.[23-26] Therefore, in an inhibition assay, the use of steroidal substrates that would be oxidized by 17β-HSD10, such as ALLOP and E2, should be promoted.

There are few known inhibitors of 17β-HSD10,[23-26] and until now, none were based on a steroid scaffold. Recently, however, the androstane derivative **1** (Fig. 1B) was reported to inhibit the transformation of E2 into E1 in HEK-293[17β-HSD10] intact cells.[27] Analogs of this androstane-based inhibitor had however been synthesized to optimize its inhibitory potency, metabolic stability and selectivity for 17β-HSD10 over the 17β-HSD3,[28] another member of 17β-HSD family.[29] In a fundamental investigation effort regarding 17β-HSD10 inhibition, we herein report the development of two biological assays to measure the inhibition of steroidal derivatives against pure 17β-HSD10 using labeled ALLOP or E2 as steroidal substrates. Different parameters were optimized and a series of androstane derivatives, all analogs of **1**, was screened to determine their inhibitory potency for the oxidative activity of pure 17β-HSD10.

Inhibition of 17β-HSD10 transformation of allopregnanolone to 5α-dihydroprogesterone

Before the screening of potential inhibitors against the ALLOP transformation into 5α-DHP by 17β-HSD10, the assay conditions needed to be determined. In a first assay, the incubation time (1, 2 or 5 h), the enzyme concentration (5, 10, 50 or 100 ng/mL) and the substrate concentration (0.5, 1 or 2 μM) were modified to obtain the optimal transformation, while the NAD^+ cofactor concentration was fixed at 1 mM. However, neither of the combinations of conditions cause an important transformation of ALLOP into 5α-DHP. In fact, the maximum transformation obtained in this assay, 2.8% after 5 h of incubation using 100 ng/mL of enzyme and 1 μM of substrate, was found inadequate. In a second assay, we kept the concentration of substrate (1 μM) and cofactor NAD^+ (1 mM), but the time of incubation was increased to either 24 or 48 h and the concentration of 17β-HSD10 was either 25, 50 or 100 ng/mL. The best percentage of transformation (ALLOP to DHP) was 6.6% obtained after 48 h using the higher concentration of enzyme (100 ng/mL) (Fig. 2A). Still, the transformation was not high enough to perform sensitive and reproducible inhibition assays compatible with the radioactivity detection method. In a third assay, the incubation time was fixed at 24 h and the concentrations of ALLOP (0.1, 1 or 10 μM) and 17β-HSD10 (100 or 200 ng/mL) were modified. The highest transformations (6.6% and 6.1%) were obtained at 0.1 and 1 μM of substrate, respectively, using 200 ng/mL of 17β-HSD10 (Fig. 2B). Worthy of mention, a higher concentration of ALLOP (10 μM)

inhibited the transformation instead of promoting it, seemingly showing a substrate inhibition effect, a phenomenon that was observed for other steroidogenic enzymes.[30,31] The transformation of 3.7%, gained in the assay with 100 ng/mL of enzymes (24 h), was similar to the one previously obtained (4.0%, Fig. 2A) showing the reproducibility of the test. Finally, the transformation values when using 0.1 µM or 1 µM of substrate being not significantly different, it was therefore decided to fix for further assays the concentration of ALLOP at 1 µM, which is not so far from the K_m value of the enzyme (15 µM).

In a fourth assay, to promote an optimal transformation of ALLOP into 5α-DHP, the enzyme concentration and the incubation time were increased to 400 ng/mL and 48 h, respectively, whereas the concentration of the cofactor NAD^+ was modified (0.001, 0.01, 0.1, 0.5 or 1 mM). The results clearly showed that the transformation of ALLOP into 5α-DHP is directly dependent of the quantity of cofactor, since a dose-response (activity of the enzyme) is observed when the concentration of cofactor is increased (Fig. 2C). The highest transformation (20.1%) was obtained when using a concentration of 1 mM of NAD^+. Following these results, we fixed the incubation time at 48 h, the enzyme concentration at 400 ng/mL and the NAD^+ concentration at 1 mM.

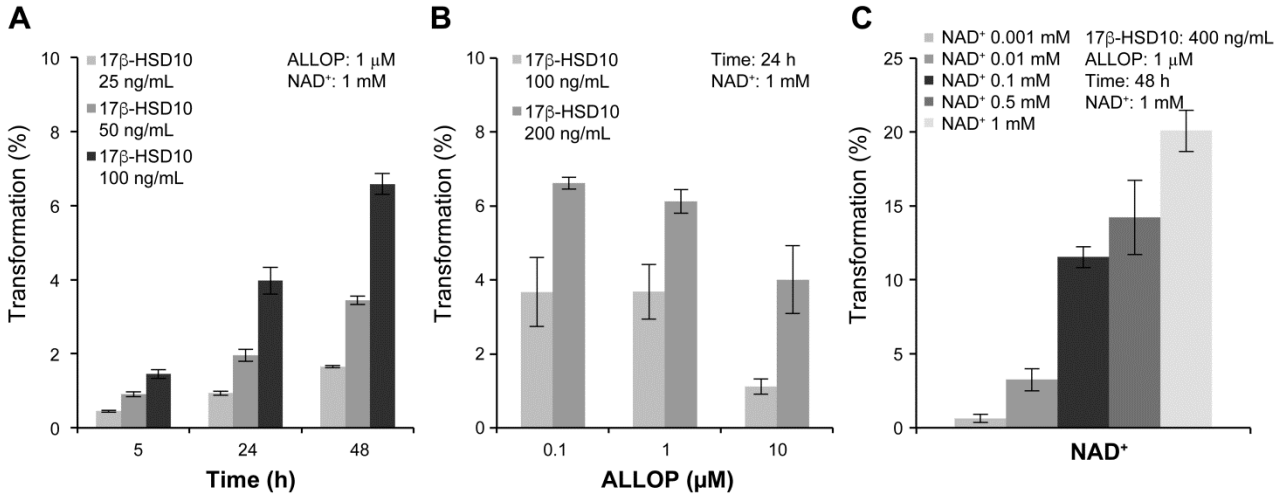


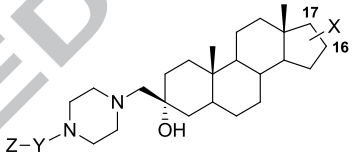
Fig. 2. Transformation of ALLOP into 5α-DHP by purified 17β-HSD10 protein. **A.** Modifying the time of incubation and the enzyme concentrations, with fixed concentrations of NAD^+ (1 mM) and substrate (1 µM (1:99) of [^{3}H]-ALLOP/ALLOP) (2nd assay). **B.** Modifying the concentration of [^{3}H]-ALLOP and ALLOP (proportion 1:99) and enzyme, with fixed time (24 h) and concentration of NAD^+ (1 mM) (3rd assay). **C.** Modifying NAD^+ concentration with fixed time (48 h), concentration of [^{3}H]-ALLOP/ALLOP (1 µM, 1:99) and concentration of enzymes (400 ng/mL) (4th assay).

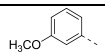
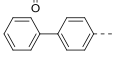
As part of our project to develop more stable and selective steroidal inhibitors of 17β-HSD10, we selected 15 androstane derivatives (compounds **2**-**16**) that are closely related to compound **1** and possessing either of these characteristics.[28] The latter has demonstrated a very good ability to cross the blood brain barrier (BBB) in mice (Supplementary data, Fig. S1), matching with a predicted CNS Score of -2.63 (Table 1).

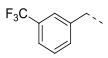
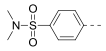
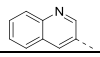
Compounds **2-16**, on the other hand, are all able to penetrate the brain based on the predicted CNS Score > -3.00, except **8** and **11**. Some relevant complementary physicochemical properties were also reported in Table 1 including hydrophobicity (Log*P*), hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and topological polar surface area (TPSA).

Compounds **1-16** were all previously tested using HEK-293[17β-HSD10] intact cells and only for the transformation of E2 into E1, but have never been tested in pure enzyme.[28] Using the optimized conditions developed above for the transformation of ALLOP to 5α-DHP by 17β-HSD10 recombinant protein, the inhibitory potency of this series of compound **1** analogs (compounds **2** to **16)** was assessed (see Table 1). At 100 μM, these compounds were either not, or moderately potent inhibitors with inhibition ranging from 0 to 41.7%. More precisely, D-ring derivative **2**, amide derivatives **8**, **9** and **10**, and sulfonamide derivative **12** did not significantly inhibit the ALLOP transformation (0 to 8.0%). Lead compound **1**, D-ring derivatives **3**, **4b** and **7**, amide derivative **11**, thiourea derivative **14** and amine derivatives **15** and **16** slightly inhibited (11.1-21.9%) the 5α-DHP formation in the assay. The most potent inhibitors were compounds **5** (17β-H/17α-OH derivative of **1**), **6** (17,17-difluoro derivative of **1**) and **13** (aryl thiourea derivative of **1**), having an inhibitory potency of 41.7, 38.6 and 34.2%, respectively.

Table 1. Inhibition of pure 17β-HSD10 protein by a series of steroidal derivatives (compounds **1** to **16**)



#	X	Y	Z	CNS Score[a]	Log*P*[b]	TPSA[b]	HBA/ HBD[b]	Inhibition (%) of ALLOP to DHP[c]	Inhibition (%) of E2 to E1[d]
1	17-ketone (C=O)	CH_2		-2.54	4.43	53.01	5/1	14.7 ± 5.3	30.0 ± 0.9
2	17β-OH/17α-H	CH_2		-2.61	4.67	56.17	5/2	0.0 ± 2.4	13.7 ± 2.9
3	17β-OH/17α-CH_3	CH_2		-2.69	5.06	56.17	5/2	21.9 ± 4.1	22.6 ± 0.8
4b	17β-OH/17α-C≡CH	CH_2		-2.96	4.93	56.17	5/2	20.0 ± 1.2	44.5 ± 0.6
5	17β-H/17α-OH	CH_2		-2.61	4.67	56.17	5/2	41.7 ± 2.4	31.7 ± 5.4
6	17,17-di-F	CH_2		-2.81	5.57	35.94	4/1	38.6 ± 3.0	13.3 ± 2.7
7	17-ketone (C=O) /16,16-di-CH_3	CH_2		-2.60	5.57	53.01	5/1	11.1 ± 3.1	24.6 ± 0.8
8	17-ketone (C=O)	C=O		-3.53	4.99	77.92	6/1	8.0 ± 1.9	58.8 ± 2.8
9	17-ketone (C=O)	C=O		-2.82	4.86	60.85	5/1	1.5 ± 2.1	----
10	17-ketone (C=O)	C=O		-2.98	3.85	77.92	6/1	5.0 ± 2.7	----
11	17-ketone (C=O)	C=O		-3.66	5.30	60.85	5/1	19.3 ± 9.9	29.4 ± 1.2

12	17-ketone (C=O)	SO_2		-2.43	2.97	89.54	7/1	0.0 ± 2.5	10.2 ± 1.5
13	17-ketone (C=O)	NH-C=S		-2.98	3.85	87.90	5/2	34.2 ± 0.1	8.5 ± 0.3
14	17-ketone (C=O)	NH-C=S		-3.46	3.32	156.44	8/4	13.3 ± 6.8	0.3 ± 1.7
15	17-ketone (C=O)	CH_2		-2.60	4.72	53.01	5/1	12.5 ± 2.6	31.0 ± 1.6
16	17-ketone (C=O)	CH_2		-2.83	4.58	56.67	5/1	15.2 ± 2.1	36.1 ± 0.4

[a] CNS Score is a BBB permeation score (S_{BBB}) defined as a logarithm of the product of brain/plasma equilibration rate and the extent of penetration when this equilibrium is achieved (calculated from ACD/Percepta 14.0.0 software (Toronto, ON, Canada). Value ≤ -3.50 (Non-penetrant); -3.50 to -3.00 (Weak penetrant); Value ≥ -3.00 (Penetrant).
[b] Log*P*: partition coefficient octanol/water; HBA (hydrogen bond acceptor), HBD (hydrogen bond donor) and TPSA (topological polar surface area) were calculated from ACD/Percepta software 14.0.0 (Toronto, ON, Canada).
[c] Inhibition (%) at 100 μM of inhibitor, after 48 h of incubation (400 ng/mL of enzymes, 1 mM NAD^+, 1 μM [^{3}H]-ALLOP and ALLOP [1:99]). Results are reported as mean ± SEM. 20.1% of transformation without inhibitor.
[d] Inhibition (%) at 300 μM of inhibitor after 48 h of incubation (400 ng/mL of enzymes, 1 mM NAD^+, 1 μM [^{14}C]-E2 and E2 [1:9]). Results are reported as mean ± SEM. 35.1% of transformation without inhibitor.

Dose-response inhibition results were obtained for the most potent inhibitors, compounds **5** and **6**. Their inhibitory potencies were also compared to lead compound **1**, to ALLOP and to E2. As seen in Fig. 3A, the substrate ALLOP completely inhibits 17β-HSD10 activity at all the concentrations tested, so the IC_{50} would be lower than 50 μM. The second substrate, E2, inhibited the transformation of ALLOP, with an estimated IC_{50} of 770 μM (Fig. 3B). Compound **1** slightly inhibited the 17β-HSD10 (IC_{50} = 985 μM) while compound **5** is the most potent inhibitor (after ALLOP), with an estimated IC_{50} of 235 μM. Finally, compound **6** is slightly more potent than **5** at 50, 100 and 300 μM but is less potent at 600 μM. At higher concentrations, this compound was less soluble and its IC_{50} value could not be determined for this reason. As one of the criteria of Lipinski rule of 5,[32,33] the estimated Log*P* values of **5** and **6** (4.67 and 5.57, respectively, Table 1) support that **6** is more hydrophobic than **5** and should be less soluble in the buffer solution. The estimated solubility of **5** and **6** (0.84 and 0.07 mg/mL, respectively; ACD/Percepta 14.0.0) is also consistent with this conclusion. Thus, the inhibition of 17β-HSD10 activity measured for **6** is underestimated at high concentrations of 600 and 1000 μM. In summary, we have established a new assay using ALLOP as a steroidal enzyme substrate and identified the first steroidal derivatives inhibiting 5α-DHP formation by 17β-HSD10.

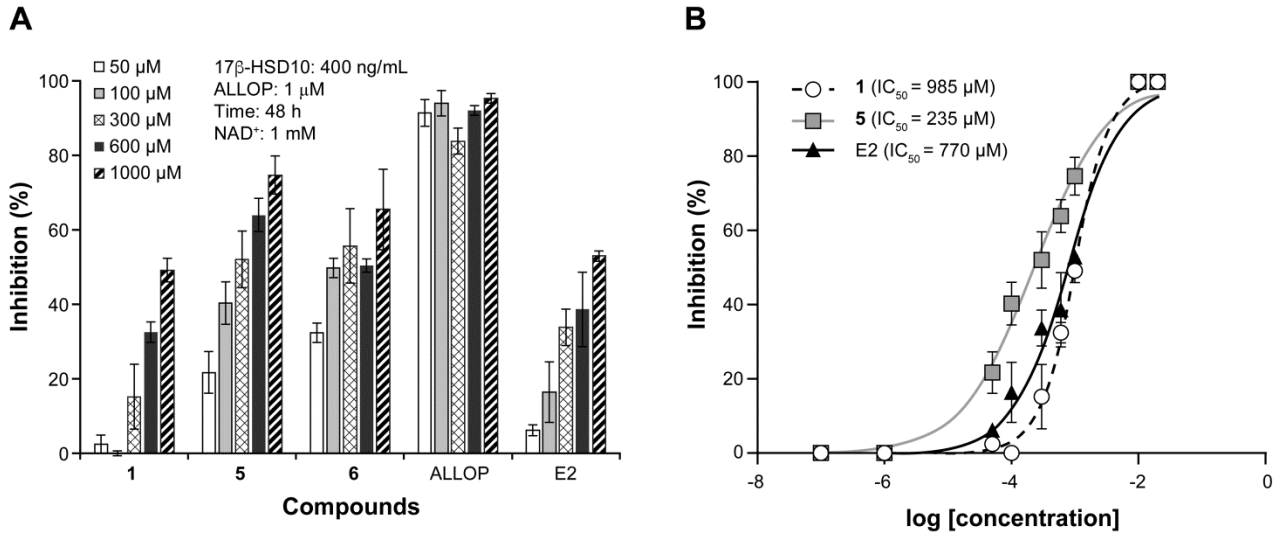


Fig. 3. Inhibition of 17β-HSD10 protein by steroidal derivatives **1**, **5**, **6** and substrates ALLOP and E2. **A.** Inhibition of the transformation of labeled ALLOP to 5α-DHP (400 ng/mL of enzyme, NAD^+ 1 mM, [^{3}H]-ALLOP and ALLOP 1 µM [1:99], 48 h) (5^{th} assay). Results are reported as mean ± SEM. **B.** Inhibition curves for **1**, **5** and **E2** (IC_{50} of ALLOP << 50 µM).

2.2 Inhibition of 17β-HSD10 transformation of 17β-estradiol to estrone

The same conditions as in the assay with ALLOP (400 ng/mL of enzyme, NAD^+ 1 mM, 48 h) were used to verify the capacity of the pure 17β-HSD10 to transform E2 to E1. A mixture of [^{14}C]-E2 and E2 (1 µM, 1:9) was however used as substrate and the percentage of E2 to E1 transformation gained (~30%) was found acceptable for further assays. Also, the first assay showed that 100 µM of the compound tested was not sufficient to significantly inhibit the 17β-HSD10 oxidation of E2. Therefore, the screening round was performed using a concentration of 300 µM (Table 1). Compounds **14** and **13** did not significantly inhibit the E2 transformation to E1 (0.3-8.5%), while **2**, **3**, **6**, **7** and **12** slightly inhibited the E1 formation (10.2-24.6%). Better inhibitions were obtained with compounds **1**, **5**, **11**, **15** and **16** (29.4 and 36.1%), and the most potent inhibitors in the screening assay were compounds **4b** and **8**, inhibiting 44.5 and 58.8%, respectively, of the transformation of E2 to E1 by 17β-HSD10.

For the dose-response inhibition (E2 to E1), compounds **1**, **5**, **6** and natural substrates ALLOP and E2 were next selected. Thus, their inhibitory potency could be directly compared to the ones obtained when ALLOP was previously used as the substrate. In this assay, neither of the compounds tested was more potent inhibitors than E2, inhibiting almost 50% of the enzymatic activity at 100 µM (Fig. 4A). From the inhibition curves (Fig. 4B), the IC_{50} values of **1**, **5** and E2 were estimated at 710, 610 and 155 µM, respectively. Surprisingly, ALLOP did not significantly inhibit the E2 transformation into E1. Since E2 inhibited the ALLOP transformation to 5α-DHP, the contrary should had been an

expected result, if 17β-HSD10 possesses only one active site. Therefore, from our results using both steroidal substrates, ALLOP and E2, we can hypothesize that this enzyme has at least two active sites. Thus, E2 can bind to both, but ALLOP can only bind to one. However, further assays need to be performed to confirm this preliminary conclusion.

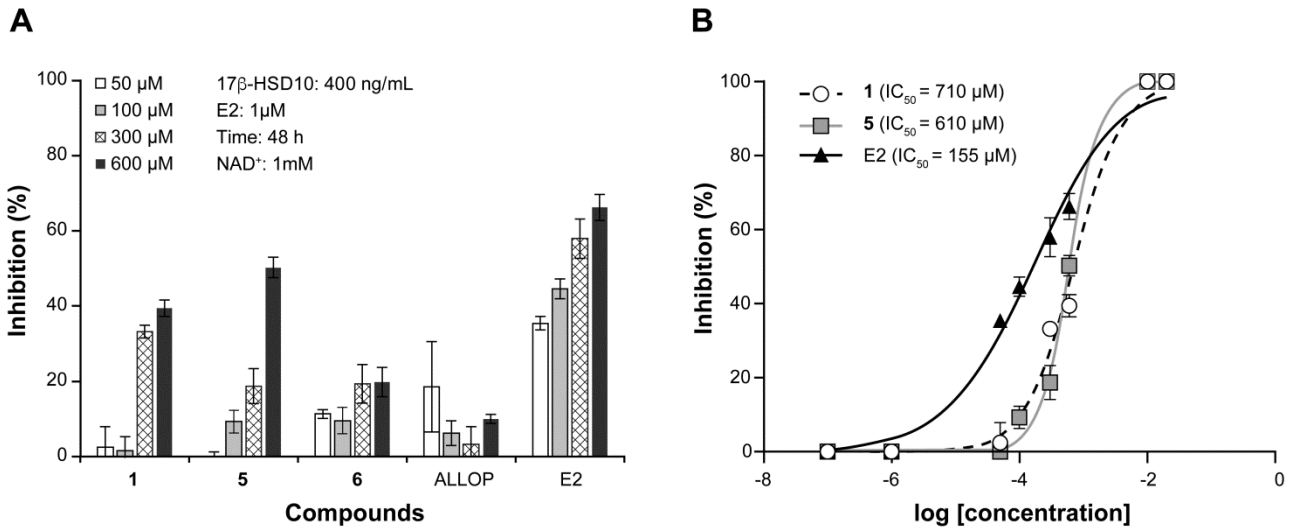


Fig. 4. Inhibition of 17β-HSD10 protein by steroidal derivatives **1**, **5** and **6**, and substrate ALLOP and E2. **A.** Inhibition of the transformation of labeled E2 to E1 (400 ng/mL of enzyme, NAD^+ 1 mM, [^{14}C]-E2 and E2 1 µM [1:9], 48 h). Results are reported as mean ± SEM. **B.** Inhibition curves for **1**, **5** and **E2**.

In conclusion, biological assays were developed to measure the inhibition potency of diverse compounds against pure 17β-HSD10, using two natural steroidal substrates, ALLOP and E2. When ALLOP was used as a substrate, the 17α-OH alcohol **5** was the most potent inhibitor of the steroidal derivatives screened (41.7% of inhibition at 100 µM; estimated IC_{50} = 235 µM). Compound **5** also inhibited the E2 to E1 transformation by 17β-HSD10 (31.7% of inhibition at 300 µM; estimated IC_{50} = 610 µM), being one of the most potent inhibitors of all the assays. This inhibitor therefore possessed the best dual inhibition activity against both 17β-HSD10 steroidal substrates and the ability to cross BBB as assessed by the CNS Score (-2.61). Other compounds were also dual inhibitors to a lesser extent than **5**, while others seem more selective for one particular transformation. Compound **8** was the most potent inhibitor for the E2 to E1 transformation (58.8% of inhibition at 300 µM), but it slightly inhibited the ALLOP to 5α-DHP transformation (8.0%) and it should not cross BBB (-3.53). On the other hand, compound **13** seems more selective for the ALLOP to 5α-DHP transformation (34.2% of inhibition vs 8.5% for E2 into E1) and it should not be able to cross BBB (-2.98). No correlation was observed between the inhibition of 17β-HSD10 by compounds **1**-**16** and calculated physicochemical properties, such as Log*P*, HBA, HBD and TPSA. In summary, we report the first

steroidal derivatives inhibiting pure 17β-HSD10 and active against one or two steroidal substrates, ALLOP and E2.

Acknowledgments

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A. Supplementary data

These data include 1) the enzymatic assays, 2) quantification of the brain uptake coefficient of compound **1**, and 3) the chemical synthesis and characterization (data and spectra) of representative androstane derivative **5**.

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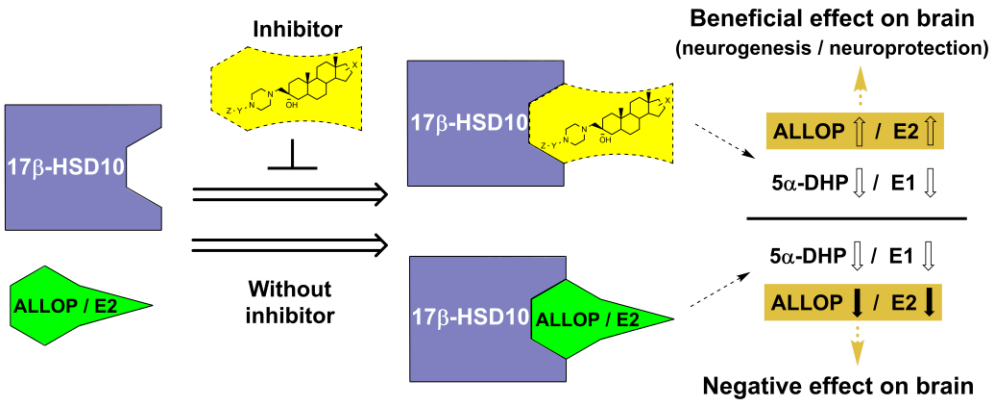

Highlights

- Two assays were developed to assess the 17β-HSD10 inhibitory potency of compounds
- Two labeled natural steroid substrates, ALLOP and E2, were tested
- Androstane derivatives were screened and their 17β-HSD10 inhibitory activity measured
- Some compounds are dual inhibitors, and others are selective inhibitors
- A first generation of steroidal inhibitors of pure 17β-HSD10 were identified