

3 β -Alkyl-androsterones as inhibitors of type 3 17 β -hydroxysteroid dehydrogenase: Inhibitory potency in intact cells, selectivity towards isoforms 1, 2, 5 and 7, binding affinity for steroid receptors, and proliferative/antiproliferative activities on AR⁺ and ER⁺ cell lines

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Abstract

Type 3 17 β -hydroxysteroid dehydrogenase (17 β -HSD) is involved in the biosynthesis of the potent androgen testosterone (T), which plays an important role in androgen-sensitive diseases. In an attempt to design compounds to lower the level of T, we designed androsterone (ADT) derivatives substituted at the position 3 β as inhibitors of type 3 17 β -HSD, and then selected the eight most potent ones (compounds 1–8) for additional studies. In an intact cell assay, they inhibited efficiently the conversion of natural substrate 4-androstene-3,17-dione into T, although they were less active in intact cells (IC₅₀ ~ 1 μ M) than in homogenated cells (IC₅₀ = 57–100 nM). A study of the inhibitory potency with four other 17 β -HSDs revealed they were selective, since they do not inhibit reductive types 1, 5 and 7, nor oxidative type 2. Interestingly, they did not show any binding affinity for steroid receptors (androgen, estrogen, glucocorticoid and progestin). Only two inhibitors, 3 β -phenyl-ADT (5) and 3 β -phenylmethyl-ADT (6) showed some proliferative activities on an AR⁺ cell line and on an ER⁺ cell line, but their effects were not mediated through the androgen or estrogen receptors. This study identified selective inhibitors of type 3 17 β -HSD acting through a mixed-type inhibition, and devoid of non-suitable androgenic and estrogenic proliferative activities. The more potent inhibitors were 3 β -hexyl-ADT (2), 3 β -cyclohexylethyl-ADT (4) and 3 β -phenylethyl-ADT (7).

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1. Introduction

The synthesis of androgens and estrogens from the abundant steroid cholesterol involves a series of reactions catalyzed by steroidogenic enzymes. Among these enzymes, the 17 β -hydroxysteroid dehydrogenases (17 β -HSDs) are responsible for the last step, which is the reduction of the C17-carbonyl group into the corresponding C17 β -hydroxyl group, or the reverse oxidative reaction. In the 90s, several new types of 17 β -HSD were reported, indicating that a fine regulation is carried out (Peltoke et al., 1999; Adamski and Jakob, 2001; Mindnich et al., 2004). More importantly, each 17 β -HSD isoform has a specific tissue distribution and displays selective substrate affinity. Moreover, in intact cells its activity is unidirectional (reductive or oxidative) (Luu-The et al., 1995; Luu-The, 2001a). The

crucial role played by the 17 β -HSD family in steroid biology probably explains the existence of a large series of isoforms having individual cell-specific expression, substrate specificity and regulatory mechanisms (Vihko et al., 2004; Mindnich et al., 2004; Labrie et al., 1997, 2000; Moghrabi and Andersson, 1998). Moreover, it makes this group of enzymes a unique target for the control of the intracellular concentration of active sex steroids by using selective inhibitors (Poirier, 2003; Smith et al., 2001; Penning, 1996).

We are especially interested in the third member of this ubiquitous enzyme family. In fact, type 3 17 β -HSD, also referred to as testicular 17 β -HSD, is mainly found in the microsomal fraction of Leydig cells of the testis, where it reduces 4-androstene-3,17-dione (Δ^4 -dione) into potent androgen testosterone (T) using nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor (Fig. 1) (Le Lain et al., 2001; Andersson et al., 1996). Deficiencies in testicular 17 β -HSD (type 3) have been associated with pseudohermaphroditism (Geissler et al., 1994), thus showing the importance of this enzyme in the production

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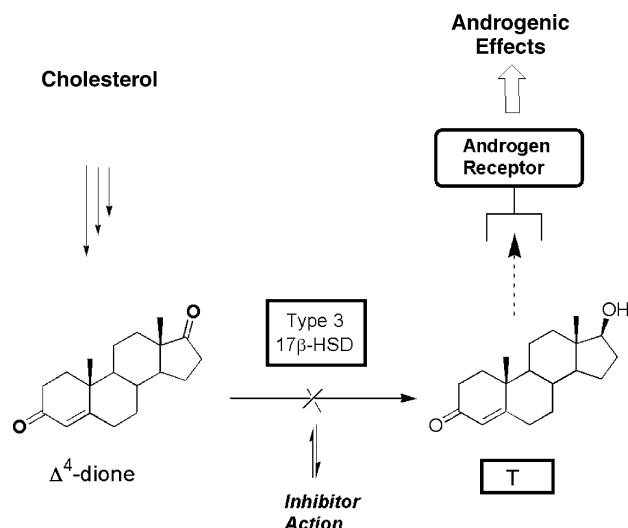


Fig. 1. Role of type 3 17 β -HSD inhibitors.

of T from Δ^4 -dione. It follows that inhibition of this enzyme could block the biosynthesis and, consequently, the action of androgens originating from the testis. On this basis, selective inhibitors of type 3 17 β -HSD have the potential for being used in preventing the development of androgen-sensitive diseases like benign hyperplasia and prostate cancer. These inhibitors could also be used as adjuvants, to enhance the efficacy of androgen receptor antagonists. Considering the crucial role of T in spermatogenesis (Verhoeven, 1992), it would also be interesting to study the potential of such inhibitors as contraceptive agents.

Although several inhibitors of reductive type 1 17 β -HSD, which is involved in the synthesis of potent estrogen estradiol, have been developed in the past (review in Poirier, 2003) and also more recently (Lawrence et al., 2005; Poirier et al., 2006; Gobec et al., 2004; Bérubé and Poirier, 2004), little effort was made to synthesize inhibitors of type 3. Previous screening studies (Pittaway, 1983; Poirier et al., 1995) led us to target the steroid androsterone (ADT) and its derivatives as potential inhibitors of this reductive isoform. Based on results obtained in our group for inhibitors of type 1 17 β -HSD (Tremblay and Poirier, 1998), we carried out the synthesis of ADT derivatives substituted at position 16, but they turned out to be weak inhibitors of type 3 (Tchédam Ngatcha et al., 2002). On the other hand, the exploration of position 3 β of the ADT nucleus led to potent inhibitors. Indeed, IC_{50} values of 50–100 nM were obtained for some ADT derivatives substituted at position 3 with an alkyl or a phenylalkyl group (Table 1) (Tchédam Ngatcha et al., 2000, 2005). Following these interesting results, we undertook two studies. In the first one, we were interested in determining the characteristics of this new family of inhibitors, whereas the second study focussed on the optimization of their inhibitory potency. Indeed, using solid-phase and liquid-phase parallel synthesis, we prepared libraries of 3 β -ADT derivative analogues of the first series of inhibitors (Maltais et al., 2001, 2002). In this paper, we report the results obtained from the former study.

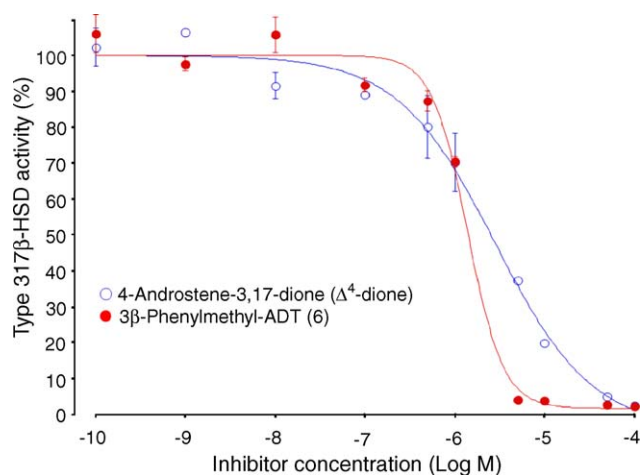


Fig. 2. Inhibition of type 3 17 β -HSD by compound 6 (IC_{50} = 1.40 μ M) and unlabeled Δ^4 -dione (IC_{50} = 2.82 μ M) in transfected HEK-293 cells in culture (intact cells).

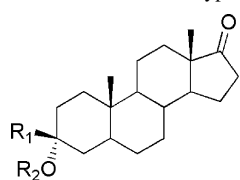
Eight ADT derivatives (compounds 1–8) known as potent inhibitors of type 3 17 β -HSD in homogenated transfected cells were selected for determining some of their biological characteristics. Thus, we examined their type 3 17 β -HSD inhibitory activity in intact transfected cells, their selectivity towards other isoforms of 17 β -HSD (types 1, 2, 5 and 7), their binding affinities on four steroid receptors (androgen, estrogen, glucocorticoid and progesterin), as well as their proliferative and antiproliferative activities on two cell lines: an androgen receptor-positive one (Shionogi), and an estrogen receptor-positive one (T-47D).

2. Material and methods

2.1. Assessment of the inhibition of type 3 17 β -HSD in intact cells

Human embryonic kidney (HEK)-293 cells were plated at 200,000 cells/well in a 12-well Falcon flask at 37 $^{\circ}$ C under 95% air 5% CO_2 humidified atmosphere in minimum essential medium (MEM) containing non-essential amino acids (0.1 mM), glutamine (2 mM), sodium pyruvate (1 mM), 10% foetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μ g/ml). The expression vector encoding for type 3 17 β -HSD (Luu-The et al., 1995) was transfected using the Exgen 500 procedure (Fermentas, Burlington, Ont., Canada) with 2 μ g of recombinant plasmid per well. For the inhibitory activity assay, 50 nM of [^{14}C]-4-androstene-3,17-dione (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) and an ethanolic solution of inhibitor (0.5%, v/v) at concentrations of 0.1, 1 or 10 μ M (Table 1) or of 0.1 nM to 0.1 mM (Fig. 2), were added to freshly changed culture medium and the mixture was incubated for 20 h. Each inhibitor was assessed in duplicate. After incubation, the culture medium was removed and steroids (Δ^4 -dione and T) were extracted twice with 1 ml of diethyl ether. The organic phases were pooled and evaporated to dryness with nitrogen. Residues were dissolved in CH_2Cl_2 and dropped on silica gel 60 F $_{254}$ thin layer chromatography plates (EMD Chemicals Inc., Gibbstown, NJ, USA) and eluted with a toluene/acetone (4:1) solvent system. The substrate ([^{14}C]- Δ^4 -dione) and metabolite ([^{14}C]-T) were identified by comparison with reference steroids and quantified using the Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA). The percentage of transformation and the percentage of inhibition were calculated as follows: % transformation = $100 \times \frac{[^{14}C]-T}{([^{14}C]-\Delta^4-dione + [^{14}C]-T)}$ and % inhibition = $100 \times \frac{(\% \text{ transformation without inhibitor} - \% \text{ transformation with inhibitor})}{\% \text{ transformation without inhibitor}}$. The IC_{50} values, the concentrations of inhibitor, which cause 50% of enzymatic inhibition, were calculated using the DE $_{50}$ program (CHUL Research Center, Que., Canada).

Table 1
Selected inhibitors of type 3 17 β -HSD (compounds 1–8) and their inhibitory potency in homogenated and intact cells^a



Inhibitors # (name)	R ₁	R ₂	Homogenated cells ^b IC ₅₀ (nM)	Intact cells inhibition (%) at 0.1 μ M	Intact cells inhibition (%) at 1.0 μ M	Intact cells inhibition (%) at 10 μ M
ADT (Androsterone) ^c	H	H	330 \pm 60	1 \pm 0.1	7 \pm 4	86 \pm 1
1 (3 β - <i>n</i> -propyl-ADT)	CH ₃ (CH ₂) ₂	H	67 \pm 6	0 \pm 0.2	10 \pm 3	98 \pm 0.1
2 (3 β - <i>n</i> -hexyl-ADT)	CH ₃ (CH ₂) ₅	H	100 \pm 10	2 \pm 0.7	58 \pm 12	98 \pm 0.1
3 (3 β - <i>s</i> -butyl-ADT)	CH ₃ CH ₂ (CH ₃)CH	H	73 \pm 5	0 \pm 0.1	14 \pm 4	97 \pm 0.1
4 (3 β -cyclohexylethyl-ADT)	CyclohexylCH ₂ CH ₂	H	60 \pm 16	3 \pm 2	62 \pm 3	98 \pm 0.1
5 (3 β -phenyl-ADT)	Phenyl	H	81 \pm 6	0	8 \pm 4	96 \pm 1
6 (3 β -phenylmethyl-ADT)	PhenylCH ₂	H	57 \pm 5	1 \pm 0.1	14 \pm 3	98 \pm 0.3
7 (3 β -phenylethyl-ADT)	PhenylCH ₂ CH ₂	H	99 \pm 1	0 \pm 0.1	43 \pm 7	89 \pm 1
8 (3 β -phenylethyl-3 α -methyl- <i>O</i> -ADT)	PhenylCH ₂ CH ₂	CH ₃	73 \pm 11	1 \pm 0.3	17 \pm 2	97 \pm 1

^a For the transformation of [¹⁴C]- Δ^4 -dione (100 or 50 nM) into [¹⁴C]-T by homogenated or intact HEK-293 cells over-expressing type 3 17 β -HSD, respectively.

^b Data from Tchédam Ngatcha et al. (2000, 2005).

^c 3 α -Hydroxy-5 α -androstane-17-one.

2.2. Assessment of the inhibition of isoforms 1, 2, 5 and 7 of 17 β -HSD

2.2.1. Type 1 17 β -HSD

Compounds 1–8 were evaluated for their ability to inhibit the reductive transformation of [¹⁴C]-estrone (0.1 μ M) into [¹⁴C]-estradiol by type 1 17 β -HSD (transfected HEK-293 sonicated cells) in the presence of cofactor NADH according to an established procedure (Tremblay et al., 2005).

2.2.2. Type 2 17 β -HSD

Compounds 1–8 were evaluated for their ability to inhibit the oxidative transformation of [¹⁴C]-testosterone (0.1 μ M) into [¹⁴C]-4-androstene-3,17-dione by type 2 17 β -HSD (transfected HEK-293 sonicated cells) in the presence of cofactor NAD⁺ according to an established procedure (Poirier et al., 2001).

2.2.3. Type 5 17 β -HSD

Compounds 1–8 were evaluated for their ability to inhibit the reductive transformation of [¹⁴C]-4-androstene-3,17-dione (0.1 μ M) into [¹⁴C]-testosterone by type 5 17 β -HSD (transfected HEK-293 intact cells) according to an established procedure (Luu-The et al., 2001b).

2.2.4. Type 7 17 β -HSD

Compounds 1–8 were evaluated for their ability to inhibit the reductive transformation of [¹⁴C]-estrone (0.15 μ M) into [¹⁴C]-estradiol by type 7 17 β -HSD (transfected HEK-293 intact cells) according to an established procedure (Bellavance et al., 2004).

2.3. Assays for binding affinity to steroid receptors

The binding affinity assays on estrogen and progestin receptors from rat uterus were carried out under the standard procedure established in our laboratory (Luo et al., 1997). The assay with the androgen receptor from the rat ventral prostate was performed according to the procedure described by Luo et al. (1996). In the case of the glucocorticoid receptor from rat liver, the affinity binding assay was done using a slightly modified version of the procedure described by Asselin et al. (1980). Herein, a dextran-coated charcoal adsorption, instead of a protamine sulfate precipitation, was used to achieve the separation of bound and free steroids.

2.4. Assessment of proliferative/antiproliferative activities on Shionogi (AR⁺) cells

The assay for determining the proliferation of Shionogi mammary carcinoma cells as well as the inhibition of 0.3 nM DHT-induced proliferation was carried out according to the procedure described by Luo et al. (1996).

2.5. Assessment of proliferative/antiproliferative activities on T-47D (ER⁺) cells

The assay for determining the effects of the inhibitors on the proliferation of T-47D human breast cancer cells, as well as the inhibition of 0.1 nM estradiol-induced proliferation, was carried out according to the procedure described by Simard et al. (1997).

3. Results and discussion

3.1. Inhibitors of type 3 17 β -HSD

During our work on the development of inhibitors of type 3 17 β -HSD, the exploration of position 3 of the ADT nucleus led us to synthesize 3 α -ether derivatives of ADT, and to evaluate their ability to inhibit the transformation of Δ^4 -dione into T (Tchédam Ngatcha et al., 2000, 2005). Among the various substituents we tried (methyl, *n*-alkyls, allyl, hydroxypropyl, bromopropyl), the 3 α -methoxy derivative gave the highest percentage of inhibition at 0.3 μ M. On the other hand, for the various 3 β -substituted derivatives of ADT synthesized and tested, the highest percentages of inhibition at 0.3 μ M were obtained for *n*-propyl (1), *n*-hexyl (2), *s*-butyl (3), cyclohexylethyl (4), phenyl (5), phenylmethyl (6) and phenylethyl (7) ADT derivatives (Table 1). In order to see the effect that the combination of these two elements, 3 α -ether and 3 β -substitution, could have on a single molecule, we carried out the synthesis of a series of disubstituted ADT derivatives at position 3. However, for a given 3 β -substituent, the 3 α -ether analogue showed a

weaker inhibitory activity on type 3 17 β -HSD, except for 3 β -phenylethyl-3 α -methyl-*O*-ADT (**8**), which was a better inhibitor than the corresponding 3 β -phenylethyl-ADT (**7**). Based on our SAR study, we then selected compounds **1–8** for additional experiments, because they were found to be the most potent inhibitors of type 3 17 β -HSD in our routine assay using a crude preparation of enzyme overexpressed in HEK-293 cells (sonicated cells). The chemical structure of each inhibitor, as well as their inhibitory potency (IC₅₀ values = 57–100 nM) are reported in Table 1.

3.2. Inhibitory potency in intact cells

Selected inhibitors were tested for their ability to inhibit the reductive transformation of labeled substrate Δ^4 -dione (50 nM) into T catalyzed by type 3 17 β -HSD in transfected HEK-293 cells in culture (20 h). We first performed the test with the unlabeled natural substrate Δ^4 -dione and with inhibitor **6** in order to try out the intact cell system. The IC₅₀ values obtained from inhibition curves (Fig. 2) clearly demonstrated a much lower inhibitory activity in intact cells, as compared to homogenated cells; from 0.33 to 2.82 μ M for unlabeled Δ^4 -dione and from 0.057 to 1.4 μ M for inhibitor **6**. In intact cells, the inhibitor must cross the cell membrane as well as resist metabolization; this could explain, at least in part, the significant drop of inhibitory potency observed. In fact, intact cells in culture constitute a more sophisticated model than homogenated cells and allow a better evaluation of our inhibitors. All selected inhibitors were next tested at three concentrations with the same protocol using transfected HEK-293 intact cells (Table 1). All compounds showed maximal inhibition at 10 μ M, whereas no inhibition was obtained at 0.1 μ M. The relative potency of each inhibitor could, however, be discriminated at the intermediate concentration of 1 μ M. Thus, compounds **2**, **4** and **7** emerged as better inhibitors than the other ADT derivatives. The IC₅₀ values of inhibitors **2** (3 β -hexyl-ADT) and **4** (3 β -cyclohexylethyl-ADT) were not formally determined, but they can be estimated to be less than 1 μ M. These results were found satisfactory considering that the K_m for the same enzymatic transformation in a microsomal

preparation of human testis was found to be 0.77 μ M (Le Lain et al., 2001).

3.3. Selectivity towards other isoforms of 17 β -HSD

We studied the selectivity of type 3 17 β -HSD inhibitors over other isoforms of 17 β -HSD using a series of four enzymatic assays available in our laboratory for types 1, 2, 5 and 7. All assays used transfected HEK-293, cells either homogenated (types 1 and 2) or intact (types 5 and 7). Thus, the ability of ADT derivatives **1–8** to inhibit the reductive isoforms 1 and 7 was evaluated for the transformation of estrone into estradiol, their ability to inhibit the oxidative type 2 was measured for the transformation of T into Δ^4 -dione, whereas their ability to inhibit the reductive type 5 was measured for the transformation of Δ^4 -dione into T. Our results, given as percentage of inhibition at two concentrations, are summarized in Table 2.

Compounds **1–8** did not show any inhibitory activity on type 1 17 β -HSD, neither at the lower concentration (0.3 μ M), nor at the higher one (3 μ M). This result was expected given the fact that the chemical structure of such ADT derivatives (C19 steroids) is very different from that of the natural substrate of type 1, the C18 steroid estrone. Furthermore, no inhibitor of isoform 1 having a C19 steroid scaffold is known (Poirier, 2003), although C19 steroids, such as DHT and DHEA have been reported as enzyme substrates with high K_m values of 26 and 24 μ M, respectively (Gangloff et al., 2001). For purposes of comparison, the K_m of estrone is 0.07 μ M (Gangloff et al., 2001).

Type 2 17 β -HSD was not inhibited by compounds **1–8** tested at concentrations of 0.3 and 3 μ M, although this enzyme is well known to accept a C19 steroid nucleus as substrate or inhibitor (Poirier et al., 2001). The main role of isoform 2 being to promote the formation of less active androgens and estrogens (by oxidation of 17 β -OH into ketone), its inhibition is not warranted in the treatment of hormone-sensitive diseases, and so the selectivity of type 3 17 β -HSD inhibitors is a valuable characteristic from a therapeutic point of view.

Compounds **1–8** did not inhibit type 5 17 β -HSD either. Such a result was to be expected considering the very different

Table 2
Percentages of inhibition of various 17 β -HSD isoforms (types 1, 2, 5 and 7)^a

Compounds	Type 1 ^b		Type 2 ^b		Type 5 ^c		Type 7 ^c	
	0.3 μ M	3 μ M	0.3 μ M	3 μ M	0.3 μ M	3 μ M	1 μ M	10 μ M
ADT	0	20	0	0	N/D	N/D	0	0
1	N/D	N/D	2	0	N/D	N/D	6	25
2	0	22	1	0	0	0	3	29
3	0	0	0	0	13	17	12	20
4	0	0	0	0	6	6	10	38
5	0	0	5	12	0	0	12	23
6	0	0	0	9	3	16	6	21
7	0	0	4	0	0	0	3	13
8	N/D	N/D	5	0	6	6	0	22

^a Error \pm 10%. N/D: not determined.

^b Homogenated cells.

^c Intact cells.

Table 3
Percentages of binding affinities of compounds 1–8 for four steroid receptors

Compounds	Androgen		Estrogen		Glucocorticoid		Progesterin	
	10 nM	1 μ M	10 nM	1 μ M	10 nM	1 μ M	10 nM	1 μ M
ADT	0 \pm 2	2 \pm 2	0 \pm 1	0 \pm 2	1 \pm 1	4 \pm 1	0 \pm 3	0 \pm 4
1	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
2	0 \pm 3	0 \pm 3	0 \pm 3	1 \pm 2	3 \pm 2	4 \pm 2	1 \pm 2	0 \pm 3
3	1 \pm 2	3 \pm 3	N/D	N/D	N/D	N/D	N/D	N/D
4	0 \pm 1	0 \pm 1	0 \pm 2	0 \pm 4	3 \pm 4	0 \pm 2	0 \pm 1	0 \pm 2
5	0 \pm 1	1 \pm 2	0 \pm 2	3 \pm 2	7 \pm 2	10 \pm 2	0 \pm 3	0 \pm 3
6	1 \pm 1	1 \pm 1	0 \pm 1	0 \pm 2	0 \pm 1	0 \pm 1	3 \pm 1	3 \pm 1
7	0 \pm 2	0 \pm 3	0 \pm 2	0 \pm 2	1 \pm 3	0 \pm 4	6 \pm 1	2 \pm 3
8	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
DHT	70 \pm 1	100 \pm 1	2 \pm 2	4 \pm 1	2 \pm 2	6 \pm 2	3 \pm 2	40 \pm 2
E ₂	0 \pm 2	34 \pm 1	75 \pm 1	100 \pm 1	5 \pm 2	12 \pm 2	6 \pm 3	25 \pm 2
DEX	0 \pm 1	2 \pm 1	0 \pm 3	0 \pm 1	66 \pm 2	99 \pm 1	0 \pm 3	1 \pm 2
R5050	1 \pm 4	28 \pm 2	5 \pm 2	4 \pm 1	9 \pm 2	85 \pm 2	65 \pm 2	99 \pm 2

N/D: not determined. DHT: dihydrotestosterone (androgen); E₂: estradiol (estrogen); DEX: dexamethasone (synthetic glucocorticoid); R5050: synthetic progesterin.

nature of types 3 and 5 enzymes. Indeed, whereas type 3 is a membrane-bound enzyme belonging to the short-chain dehydrogenase/reductase family and is located mainly in the testis, type 5 is a cytosolic enzyme of the aldo–keto reductase family and it is located mainly in peripheral tissues.

Compounds 1–8 were all poor inhibitors of type 7 17 β -HSD, no inhibition being detected at 1 μ M, while only weak inhibitions (13–38%) were obtained at 10 μ M. In summary, the ADT derivatives 1–8 are selective inhibitors of type 3 17 β -HSD.

3.4. Binding affinity for steroid receptors

The binding affinities to steroid receptors of the selected inhibitors 1–8 were evaluated at two concentrations: 10 nM and 1 μ M (Table 3). These compounds were expected to show some affinity for the androgen receptor, because they are C19 steroids like the natural substrates of this receptor. Interestingly, none was observed at the concentrations tested. This might be due in part to the presence of a keto group at position 17 of the molecules, instead of a 17 β -hydroxy group, and to the absence of a carbonyl group at position 3. Indeed, known steroidal androgens, such as DHT and T bear a hydroxyl group at position 17 β and a C3-ketone, suggesting these are required for a good binding affinity to the androgen receptor. As expected for estrogen, glucocorticoid and progesterin receptors, inhibitors 1–8 did not bind to them at all. This was to be expected since their chemical structure (C19 steroid derivatives) is very different from that of the natural receptor substrates estrogens (C18 steroids, such as estradiol), glucocorticoids (C21 steroids, such as cortisol) and progesterins (C21 steroids, such as progesterone).

3.5. Proliferative/antiproliferative activities on cells expressing the androgen receptor

In order to assess any residual hormonal activity, we next performed assays on proliferation of AR-expressing cells, as well as on the inhibition of DHT-induced proliferation. These activities were evaluated on Shionogi mammary carcinoma cells, known

to be androgen receptor-positive (AR⁺), at two concentrations (0.1 and 1 μ M) of inhibitors 1–8 (Table 4).

No cell proliferation was observed for compounds 1–4, but a proliferative activity appeared for the phenyl derivative 5 and a more important one for phenylmethyl derivative 6. Indeed, 4 and 58% of proliferative activities at 0.1 μ M were, respectively, obtained for these two compounds (100% set as the activity induced by 0.3 nM DHT). At higher concentration (1 μ M), they both exhibited strong proliferative activities (72 and 82%, respectively). However, switching from a phenylmethyl group (compound 6) to a phenylethyl group (compound 7) led to an inhibitor devoid of proliferative activity on Shionogi cells, and this property remained after the 3 α -methylation of compound 7 to generate 8.

ADT derivatives 1–8 showed some antiproliferative activity on Shionogi cells at 1 μ M, except for 6 and 8. However, the antiproliferative activities observed are lower than that obtained

Table 4
Proliferative and antiproliferative activities of compounds 1–8 on Shionogi (AR⁺) cells^a

Compounds	Proliferative activities (%) ^b		Antiproliferative activities (%) ^c	
	0.1 μ M	1 μ M	0.1 μ M	1 μ M
ADT	14	0	0	20
1	0	0	1	14
2	0	0	11	53
3	0	0	0	23
4	0	0	13	51
5	4	72	11	53
6	58	82	0	0
7	0	0	14	73
8	0	0	0	4
OH-Flu ^d	0	0	56	100

^a Error \pm 10%.

^b 100% was set as the cell proliferation induced by 0.3 nM of androgen DHT.

^c The cells were stimulated by 0.3 nM of DHT.

^d Hydroxyflutamide, an antiandrogen (Neri et al., 1972; Poyet and Labrie, 1985).

Table 5
Proliferative and antiproliferative activities of compounds **1–8** on T-47D (ER⁺) cells^a

Compounds	Proliferative activities (%) ^b		Antiproliferative activities (%) ^c	
	0.1 μM	1 μM	0.1 μM	1 μM
ADT	52	54	0	0
1	1	37	0	3
2	8	2	0	10
3	3	0	0	9
4	6	15	14	18
5	0	92	12	11
6	0	32	4	12
7	10	0	0	32
8	0	0	6	0
EM-139 ^d	10	16	95	98

^a Error ±10%.

^b 100% was set as the cell proliferation induced by 0.1 nM of estrogen estradiol.

^c The cells were stimulated by 0.1 nM of estradiol.

^d *N-n*-Butyl,*N*-methyl-11-(16′ α -chloro-3′,17 β -dihydroxy-estra-1′,3′,5′(10′)-trien-7 α -yl)undecanamide, a potent antiestrogen (Lévesque et al., 1991).

with hydroxyflutamide, a known potent antiandrogen (Neri et al., 1972; Poyet and Labrie, 1985). Since none of the selected inhibitors showed affinity to the androgen receptor (Table 4), it can be inferred that the proliferative or antiproliferative activities observed on Shionogi cells are not mediated through the androgen receptor.

3.6. Proliferative/antiproliferative activities on cells expressing the estrogen receptor

Assays on the proliferation and on the inhibition of E₂-induced proliferation of ER-expressing cells were carried out on T-47D cells, which are known to be estrogen receptor positive (ER⁺). These activities were evaluated at two concentrations (0.1 and 1 μM) of inhibitors **1–8** (Table 5). Surprisingly, ADT was found to exert some proliferative activity on T-47D cells (52 and 54%). The compounds bearing a substituent at position 3 β of the ADT nucleus exhibited, however, a much reduced proliferative activity, and even none at all in the cases of **2**, **3**, **4**, **7** and **8**. Nevertheless, with 92% of proliferation, 3 β -phenylmethyl-ADT (**5**) exhibited stronger proliferative activity than ADT, but only at 1 μM. Moderate proliferative activities were also observed for compounds **1** and **6** (37 and 32%, respectively).

On the other hand, ADT and its 3 β -derivatives did not show any significant antiproliferative activity at 0.1 μM, while no or very weak activity (up to 32%) was detected at 1 μM on the ER⁺ T47-D cells. As for the Shionogi cell line assay, the effect on the T-47D cell line does not appear to be mediated through the estrogen receptor since no binding was detected.

3.7. Mechanism of inhibition of type 3 17 β -HSD

Considering the chemical structure of compounds **1–8**, we expected them to be reversible inhibitors. Indeed, no strong inter-

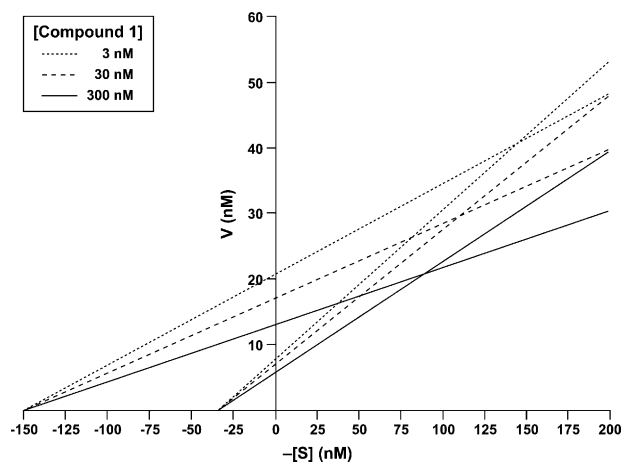


Fig. 3. Mechanism of inhibition by 3 β -propyl-ADT (**1**), of type 3 17 β -HSD for the transformation of [¹⁴C]- Δ^4 -dione (30 and 150 nM) into [¹⁴C]-T. The enzymatic assay was done using homogenated transfected HEK-293 cells as previously reported (Tchédam Ngatcha et al., 2000, 2005).

actions, such as a covalent bond, can be generated between the inhibitor and an enzyme residue, which would inactivate the enzyme. Compound **1**, 3 β -propyl-ADT, was then selected as a representative inhibitor for an enzymatic kinetic study. As illustrated in direct linear (Cornish–Bowden) plot analysis (Fig. 3), increasing amount of compound **1** affected both K_m and V_{max} values suggesting a mixed-type inhibitor. Thus, compound **1** binds to both the free enzyme and the enzyme substrate complex with different affinity ($V = V_{max}S/K_m(1 + I/K_{is}) + S(1 + I/K_{iu})$) with K_{iu} and K_{is} values of 1.4 and 0.4 μM.

Inhibitors **1–8** share a common C19 steroid nucleus and a ketone at position 17 with the natural enzyme substrate Δ^4 -dione. Our previous SAR study (Tchédam Ngatcha et al., 2005), clearly established the key role of the 3 α -OH (active compound), as compared to 3 β -OH (inactive compound), as well as the requirement for the chemical group at position 3 β to be hydrophobic. It is thus possible that the ketone at position 17 of the inhibitor mimics the ketone at position 17 of Δ^4 -dione, and that the rest of the molecule, especially the 3 β group, interacts somewhere in the catalytic site, probably in a hydrophobic pocket. It is also possible that the 3 α -OH group could mimic the 17 β -OH of natural product T, the 3 β -substituent after rotation by 180° is now in the region of the position 17 α and may interact with a hypothetical hydrophobic pocket. Considering the mixed type inhibition of ADT derivatives **1–8**, this hydrophobic pocket would be located outside the area occupied by the enzyme substrate, but may be in the cofactor-binding area or another area in proximity.

Although the hydrophobicity of the chemical group at position 3 β seems to be an important requirement for inhibition of type 3 17 β -HSD, this is not the only parameter to consider, as indicated by the drop of inhibitory potency observed when the 3 β -alkyl side chain is too long. Considering the series of 3 β -substituted ADT derivatives tested in our previous SAR studies (Tchédam Ngatcha et al., 2000, 2005; Maltais et al., 2001, 2002), it is possible to estimate the size of the hydrophobic pocket that the 3 β -oriented group occupies. The results agree with the

presence of a medium-size hydrophobic pocket, although its exact topography is not known. It could be better defined by extending our SAR study to additional diversified analogues of our best inhibitors. Such an SAR study focussing on medium-sized alkyl groups could also lead to the design of a more potent inhibitor. It is, however, not possible to determine where this hypothetical hydrophobic pocket is located in the enzyme. Crystallizing a typical inhibitor with the enzyme would of course yield precious information about the shape of this hydrophobic pocket; unfortunately, the crystallization of a membrane enzyme like type 3 17 β -HSD remains a great challenge. Modeling the three-dimensional structure of type 3 17 β -HSD from its amino acid sequence and by analogy with the known coordinates of type 1 17 β -HSD (Breton et al., 1996; Ghosh et al., 1995) would be an alternative. Indeed, types 1 and 3 17 β -HSDs are both soluble enzymes that belong to the same short-chain dehydrogenase/reductase family of enzymes. Thus, some homology modeling and docking studies could be planned to potentially bring some structural basis to explain the inhibitor potency and selectivity.

4. Conclusion

Eight type 3 17 β -HSD inhibitors originating from a previous SAR study (IC_{50} = 57–100 nM) were selected for additional experimentations. They appear to be selective inhibitors of type 3, because they do not significantly inhibit the activities of three reductive 17 β -HSDs, namely types 1, 5 and 7, nor that of the key oxidative type 2. None of them showed any significant binding affinity for the four steroid receptors involved in this study at the higher concentration tested, suggesting no residual hormonal (estrogenic, androgenic, glucocorticoid and progestative) activities for the inhibitors. Although we have no explanation for the proliferative effects induced by compounds **5** and **6** on Shionogi (AR⁺) cells and on T-47D (ER⁺), these effects are not achieved through the androgen and estrogen receptors, respectively. When tested in intact cells overexpressing type 3 17 β -HSD, the inhibitory potency of compounds **1–8** decreased to the micromolar level (estimated IC_{50} values), the best inhibitory activity being observed for inhibitors **2**, **4** and **7**. The two former have an estimated IC_{50} values lower than 1 μ M. Compound **1** was tested as a representative inhibitor and was found to be a mixed-type inhibitor. Although we have no more information on the mechanism of action of this new family of type 3 17 β -HSD inhibitors, our study identified selective inhibitors devoid of non-suitable androgenic and estrogenic proliferative activities. Three more interesting inhibitors, 3 β -hexyl-ADT (**2**), 3 β -cyclohexylethyl-ADT (**4**) and 3 β -phenylethyl-ADT (**7**), thus emerged as interesting candidates for additional studies.

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