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Impact of androstane A- and D-ring inversion on 17β-hydroxysteroid dehydrogenase type 3 inhibitory activity, androgenic effect and metabolic stability

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Abstract

17β-Hydroxysteroid dehydrogenase type 3 (17β-HSD3) is a major player in human endocrinology, being one of the most important enzymes involved in testosterone production. To capitalize on the discovery of RM-532-105, a steroidal 17β-HSD3 inhibitor, we explored the effect of its backbone configuration on inhibitory activity, androgenic profile, and metabolic stability. Two modifications that greatly alter the natural shape of steroids, i.e. inversion of the methyl on carbon 13 (13α-CH_3 instead of 13β-CH_3) and inversion of the hydrogen on carbon 5 (5β-H instead of 5α-H), were tested after the syntheses in 6 steps of 2 isomeric forms (5α/13α-RM-532-105 (**6a**) and 5β/13β-RM-532-105 (**6b**), respectively) of the 17β-HSD3 inhibitor RM-532-105 (5α/13β-configurations). For compound **6b**, a *cis/trans* junction of the A/B rings did not significantly alter the inhibitory activity on 17β-HSD3 (IC_{50} = 0.15 μM) as well as the liver microsomal stability (16.6% of **6b** remaining after 1 h incubation) compared to RM-532-105 (IC_{50} = 0.11 μM and 14.1% remaining). In contrast, a *trans/cis* junction of C/D rings reduced the inhibitory activity on 17β-HSD3 (IC_{50} = 1.09 μM) but increased the metabolic stability with 29.4% of compound **6a** remaining after incubation. The structural modifications represented by compounds **6a** and **6b** did not change the non-androgenicity profile of an androsterone derivative such as RM-532-105, but slightly increased its cytotoxic activity.

Keywords: Steroid, Androsterone derivatives, Hydroxysteroid dehydrogenase, LNCaP cells, LAPC-4 cells, Prostate cancer

1. Introduction

Prostate cancer (PCa) is the second most common cancer in men, the fourth most common cancer in both sexes combined, and its incidence is highest in developed areas such as North America, Australia, as well as Western and Northern Europe.[1] For instance, in the United States, there will be an estimated 180,890 new cases and 26,120 deaths in 2016.[2] Thus, PCa is a major disease, and there are currently several therapies to improve health or help to prolong life expectancy. Hormonal therapy is one of the first choices to treat PCa.[3] Unfortunately, this cancer often evolves toward a castration-resistant form.[4] Therefore, hormonal therapy must be improved by discovering new selective and potent drug candidates to take the relay to these first-line therapies.

The initial growth of prostate carcinomas depends on testosterone (T) and its most androgenic metabolite 5α-dihydrotestosterone (5α-DHT), which both stimulate the growth of hormone-dependent prostate cancer tumors through interaction with the androgen receptor (AR).[5,6] Therefore, suppressing T and 5α-DHT action in prostate cancer cells through the inhibition of the key enzymes involved in their synthesis is an essential approach to decrease the survival and progression of PCa tumors. In this context, 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) is a key steroidogenic macromolecule involved in the synthesis of androgens (Fig. 1).[7-10] This enzyme is well-known to catalyze the reduction of 4-androstene-3,17-dione (4-dione) to potent androgen T using NADPH as a cofactor,[11] but it could be also involved in the reduction of other steroids (5α-androstane-3,17-dione, androsterone (ADT) and dehydroepiandrosterone), thus contributing to alternative pathways for the synthesis of 5α-DHT.[12-15] Although 17β-HSD3 is expressed almost exclusively in testis,[16] there have been some reports of its up-regulation in prostate tumors. For example, Pfeiffer *et al* have reported that in clinical Castrate-Resistant Prostate Cancer (CRPC) samples, the expression of 17β-HSD3 was significantly increased, even in long-term hormone depletion.[17] Thus, 17β-HSD3 is an interesting target for hormonal therapy, and its inhibition should be an effective strategy for the treatment of androgen-dependent PCa.

In previous studies,[18-20] our research group reported the synthesis and pharmacological activity of RM-532-105, which is a 3β-substituted-androsterone derivative (Fig. 1). This steroid was identified as a potent 17β-HSD3 inhibitor on transfected HEK-293[17β-HSD3] as well as in

LNCaP[17β-HSD3] cells.[18, 21] Moreover, RM-532-105 has been shown to decrease the level of T and 5α-DHT in plasma at 2 h in rats, after subcutaneous injection.[20]

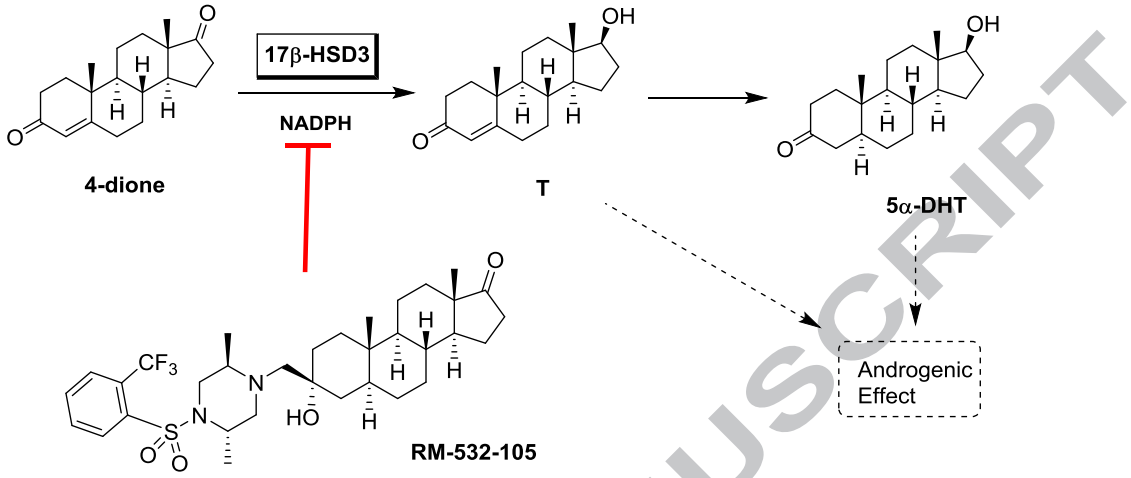


Figure 1. Contribution of 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) to the biosynthesis of the androgens testosterone (T) and 5α-dihydrotestosterone (5α-DHT) from 4-androstene-3,17-dione (4-dione). RM-532-105 is a 3β-androsterone derivative that inhibits the steroidogenic enzyme 17β-HSD3.

However, it is also known that spatial modifications of the steroidal backbone, such as inversion of the A-ring or D-ring (Fig. 2), exert a substantial effect on its biological properties.[22-26] For example, changing the 18-methyl (CH_3) group of estradiol (E2) from the β-face to α-face modifies the D-ring shape[22] and this 13α-E2 (or 18-epi-E2) was reported to be significantly less binding to the estrogen receptor alpha (ERα) than E2 with a relative binding affinity (RBA) of 1.2 and 100%, respectively.[23] Moreover, this unnatural steroid did not exert a significant proliferative effect on estrogen-sensitive MCF-7 cells (ER^+) as well as on estrogenic activity in mice.[23,24] Similarly, the *cis-trans* A/B fused rings of 5β-DHT (or etiocholan-17β-ol-3-one) exhibit 173-fold less RBA to AR than the *trans-trans* conformation of 5α-DHT.[26]

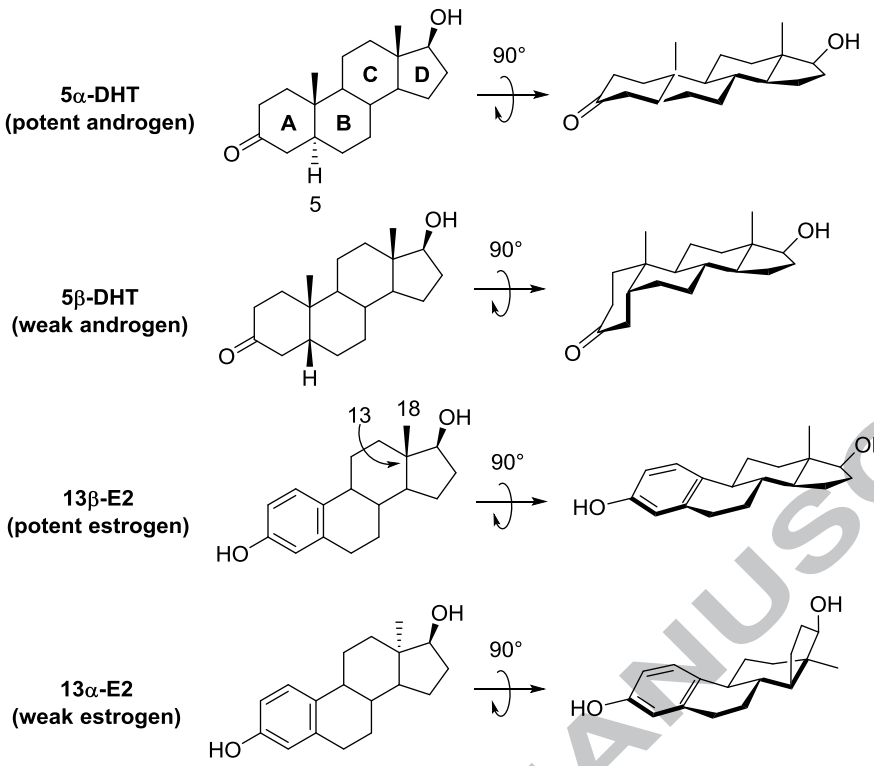


Figure 2. Conformational representations of 5α-DHT (potent androgen), 5β-DHT (weak androgen), 13β-E2 (potent estrogen) and 13α-E2 (weak estrogen).

Based on the facts reported above, we were interested to test 2 major modifications of the androstane backbone of RM-532-105 (13α-CH_3 *vs* 13β-CH_3 and 5β-H *vs* 5α-H). Because 17β-HSD3 is a membrane enzyme, its crystal structure has not yet been resolved and molecular modelling studies have been limited to homology model constructions.[27-29] Therefore, a classical structure-activity-relationship (SAR) study remains an efficient strategy to explore the tolerance of an enzyme for structural modification of an inhibitor backbone. Herein, we describe the chemical synthesis and characterization of compounds **6a** (13α-CH_3) and **6b** (5β-H). We also address the impact of the C13 and C5 stereochemistry on 17β-HSD3 inhibitory activity, the androgenic effect and metabolic stability.

2. Material and methods

2.1. Chemical Synthesis

2.1.1. General information

Compounds **1a** and **1c** were prepared by the procedure described in reference,[30] whereas compound **1b** was purchased from Steraloids (Wilton, NH, USA). The reagents for chemical synthesis were purchased from Sigma–Aldrich Canada Ltd (Oakville, ON, Canada). The usual solvents were obtained from Fisher Scientific (Montreal, QC, Canada) and were used as received. Anhydrous tetrahydrofuran (THF), dichloromethane (DCM), dimethylsulfoxyde (DMSO) and toluene were from Sigma–Aldrich. Thin-layer chromatography (TLC) and flash-column chromatography were performed on 0.20-mm Silica Gel 60 F254 plates and with Silicycle R10030B 230–400 mesh silica gel (Québec, QC, Canada). Infrared spectra (IR) were recorded with a Horizon MB 3000 ABB FTIR spectrometer (Quebec, QC, Canada), and only the significant bands reported in cm^{-1}. Samples were prepared as KBr pellets. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C with a Bruker Avance 400 digital spectrometer (Billerica, MA, USA). The chemical shifts (δ) are expressed in ppm and referenced to chloroform (7.26 ppm, ^{1}H and 77.0 ppm, ^{13}C). ^{1}H NMR signals were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and broad (br). Low-resolution mass spectra (LRMS) were recorded on a Perkin-Elmer Sciex API-150ex apparatus (Foster City, CA, USA) equipped with a turbo ion-spray source and expressed in m/z. High-resolution mass spectra (HRMS) were provided by Pierre Audet at the Laval University Chemistry Department (Quebec, QC, Canada). High-performance liquid chromatography (HPLC) analyses were carried out using a Waters system (Milford, MA, USA) equipped with a UV detector (207 nm), a reverse-phase column (Luna C18(2) 100A, 100 X 4.6 mm, 3 μm) from Phenomenex (Torrance, CA, USA) and using an appropriate system of solvents (methanol and water). HPLC purity was determined for the final compounds. The names of new compounds were obtained using ACD/Labs (Chemist's version) software (Toronto, ON, Canada). The numbering reported in Figure 3 was used for the assignment of ^{1}H and ^{13}C NMR signals.

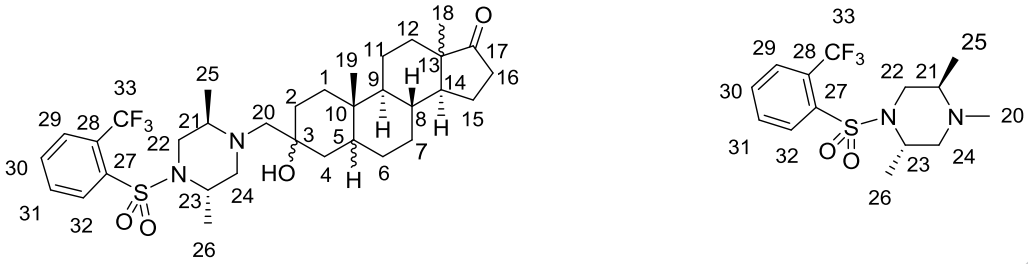


Figure 3. Carbon numbering used for the assignment of ^{1}H NMR and ^{13}C NMR signals.

2.1.2. Synthesis of 2a and 2b

To a solution of (3β,5α,13α)-3-hydroxyandrostan-17-one (**1a**, 100 mg, 0.34 mmol) or (3β,5β)-3-hydroxyandrostan-17-one (**1b**, 150 mg, 0.52 mmol) in anhydrous toluene (5 mL) was added ethylene glycol (10.0 eq) and *p*-toluenesulfonic acid (0.1 eq). The reaction was refluxed under a Dean-Stark trap for 12 h. The solution was poured into cold water (100 mL) and extracted with EtOAc (3 X 20 mL). The organic phase was washed with a 20% solution of sodium acetate (3 X 20 mL) and brine (2 X 20 mL), dried over Na_2SO_4 and filtered. The resulting solution was evaporated and purified by flash chromatography column using hexanes/EtOAc (95:5 to 80:20) to give **2a** (90 mg, 79%) or **2b** (130 mg, 74%), respectively.

(3β,5α,13α)-17-(1,3-dioxolan-2-yl)-3-hydroxyandrostane **(2a).** IR (KBr) ν_{max}: 3348 (OH), 2924, 2854 (C-H, aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.62 (m. 1H), 0.74 (s, 19-CH_3), 0.94 (s, 18-CH_3), 0.60-1.95 (m, unassigned CH and CH_2), 3.59 (m, 3α-H), 3.88 (m, OCH_2CH_2O). LRMS for $C_{21}H_{35}O_3$ $[M+H]^+$: 335.25.

(3β,5β)-17-(1,3-dioxolan-2-yl)-3-hydroxyandrostane **(2b).** IR (KBr) ν_{max}: 3286 (OH), 2932 (C-H, aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.83 (s, 19-CH_3), 0.96 (s, 18-CH_3), 1.00-1.99 (m, unassigned CH and CH_2), 3.88 (m, OCH_2CH_2O), 4.09 (m, 3α-H). LRMS for $C_{21}H_{35}O_3$ $[M+H]^+$: 335.15.

2.1.3. Synthesis of 3-keto derivatives 3a and 3b

To a solution of compound **2a** (90 mg, 0.27 mmol) or **2b** (130 mg, 0.39 mmol) in DCM (5 mL) was added Dess-Martin periodinane (1.3 eq). The reaction was stirred at room temperature for 1 h, the resulting white suspension was evaporated. The residue was diluted with EtOAc (20 mL) and washed with a saturated solution of sodium bicarbonate (3 X 20 mL) and brine (2 X 20 mL). The organic phase was dried with Na_2SO_4 and evaporated. The crude product was purified by flash chromatography column, using hexanes/EtOAc (95:5 to 80:20) to give **3a** (40 mg, 44%) or **3b** (110 mg, 84%), respectively.

(5α,13α)-17-(1,3-dioxolan-2-yl)-androstan-3-one **(3a).** IR (KBr) ν_{max}: 2924, 2870 (C-H, aliphatic), 1705 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.94 (s, 19-CH_3), 0.95 (s, 18-CH_3), 0.65-2.42 (m, unassigned CH and CH_2), 3.89 (m, OCH_2CH_2O). LRMS for $C_{21}H_{33}O_3$ $[M+H]^+$: 333.15.

(5β)-17-(1,3-dioxolan-2-yl)-androstan-3-one **(3b).** IR (KBr) ν_{max}: 2932, 2862 (C-H, aliphatic), 1713 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (s, 19-CH_3), 1.02 (s, 18-CH_3), 1.05-2.19 (m, unassigned CH and CH_2), 2.33 (td, J_1 = 14.6 Hz, J_2 = 5.3 Hz, 2-CH), 2.68 (t, J = 14.6 Hz, 4-CH), 3.91 (m, OCH_2CH_2O). LRMS for $C_{21}H_{33}O_3$ $[M+H]^+$: 333.25.

2.1.4. Synthesis of the oxirane derivatives 4a and 4b

To a solution of trimethylsulfoxonium iodide (2.0 eq), in dry DMSO (3 mL) was carefully added sodium hydride 60% in oil (2.0 eq). The solution was stirred at room temperature under argon atmosphere for 1 h before adding **3a** (40 mg, 0.12 mmol) or **3b** (100 mg, 0.30 mmol) dissolved in THF (1 mL). The reaction mixture was stirred at room temperature for 5 h and poured in ice/water (100 mL) and extracted with EtOAc. The combined organic layer was washed with brine and dried with Na_2SO_4. The resulting solution was filtered, evaporated and the crude product purified by flash chromatography column using hexanes/EtOAc (85:15) to give **4a** (30 mg, 72%) or **4b** (82 mg, 79%), respectively.

(5α,13α)-17-(1,3-dioxolan-2-yl)-(3R)-oxiran-2-yl-androstane **(4a).** IR (KBr) ν_{max}: 2939, 2862 (C-H, aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.77 (s, 19-CH_3), 0.95 (s, 18-CH_3), 0.75-2.08 (m, unassigned CH and CH_2), 2.61 (s, 20-CH_2), 3.88 (m, OCH_2CH_2O). LRMS for Chemical Formula: $C_{22}H_{35}O_3$ $[M+H]^+$ = 347.20.

(5β)-17-(1,3-dioxolan-2-yl)-(3S)-oxiran-2-yl-androstane **((4b).** IR (KBr) ν_{max}: 2932, 2862 (C-H, aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (s, 19-CH_3), 0.99 (s, 18-CH_3), 1.03-2.01 (m, unassigned CH and CH_2), 2.32 (t, *J* = 13.8 Hz, 4-CH), 2.62 (s, 20-CH_2), 3.88 (m, OCH_2CH_2O). LRMS for $C_{22}H_{35}O_6$ $[M+H]^+$: 347.25.

2.1.5. Synthesis of intermediates 5a and 5b

To a solution of **4a** (28 mg, 0.08 mmol) or **4b** (80 mg, 0.23 mmol) in anhydrous ethanol (4 mL) was added *trans*-2,5-dimethylpiperazine (5.0 eq). The solution was stirred 12 h at 70 °C. The resulting solution was poured in ice/water (50 mL). The white precipitate was separated by vacuum filtration, and the solid was purified by flash chromatography column using DCM/MeOH/TEA (96:2:2) to give **5a** (20 mg, 54%) or **5b** (52 mg, 50%), respectively.

(3α,5α,13α)-17-(1,3-dioxolan-2-yl)-3-[(trans-2,5-dimethylpiperazin-1-yl)methyl]-3-hydroxyandrostane **(5a).** IR (KBr) ν_{max}: 3425 (NH and OH), 2939 (C-H, aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.67 (s, 19-CH_3), 0.94 (s, 18-CH_3), 0.98-1.01 (m, 2 X $C\underline{H_3}CH$) 1.10-2.08 (m, unassigned CH and CH_2), 2.30 (m, 1H), 2.51-2.62 (m, 1H), 2.82-2.87 (m, 1H), 2.88-2.93 (m, 1H), 3.88 (m, OCH_2CH_2O). LRMS for $C_{28}H_{49}N_2O_3$ $[M+H]^+$: 461.30.

(3β,5β)-17-(1,3-dioxolan-2-yl)-3-[(trans-2,5-dimethylpiperazin-1-yl)methyl]-3-hydroxyandrostane **(5b).** IR (KBr) ν_{max}: 3433, 3317 (NH and OH), 2932, 2862 (C-H, aliphatic).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.84 (s, 19-CH_3), 0.96 (s, 18-CH_3), 0.98-1.01 (m, 2 X C$\underline{H_3}$CH) 1.02-2.10 (m, unassigned CH and CH_2), 2.30 (m, 1H), 2.51-2.56 (m, 2 X CH_3C$\underline{H}$), 2.72 (m, 1H) 2.82-2.87 (m, 2H), 3.84-3.96 (m, OCH_2CH_2O). LRMS for $C_{28}H_{49}N_2O_3$ $[M+H]^+$: 461.45.

2.1.6. Synthesis of sulfonamides 6a and 6b

To a solution of **5a** (20 mg, 0.04 mmol) or **5b** (44 mg, 0.09 mmol) in anhydrous DCM (3 mL) was added triethylamine (4.0 eq) and 2-(trifluoromethyl)benzenesulfonyl chloride (2.0 eq). The reaction mixture was stirred at room temperature for 4 h, then evaporated, and the remaining oily residue was diluted with acetone (5 mL) and concentrated hydrochloric acid (0.1 mL). The reaction mixture was stirred into an ice bath for 30 min. The resulting solution was evaporated and diluted with EtOAc (50 mL), washed with water, dried with Na_2SO_4 and evaporated. After filtration, the residue was purified by flash chromatography column using hexanes/EtOAc (90:10 to 60:40) to give **6a** (11 mg, 44% for 2 steps) or **6b** (29 mg, 52% for 2 steps), respectively.

(3*α*,5*α*,13*α*)-3-[(*trans*-2,5-dimethyl-4-{[2-(trifluoromethyl)phenyl]sulfonyl}piperazin-1-yl)methyl]-3-hydroxyandrostan-17-one (6a). IR (KBr) ν_{max}: 3448 (OH), 2924, 2854 (C-H, aliphatic), 1736 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.56 (s, 19β-CH_3), 0.60-0.75 (m, 7-CH and 9α-H), 0.85-0.90 (m, 8β-H), 0.86 and 0.88 (2d, J = 6.5 Hz, 25-CH_3), 0.96 (s, 18α-CH_3), 1.16 and 1.19 (2d, J = 5.4 Hz, 26-CH_3), 1.15-1.45 (m, 1-CH, 2-CH, 15-CH, 6-CH and 12-CH), 1.45-1.70 (m, 1-CH, 7-CH and 14α-H) 1.80 (m, 1H), 1.95-2.05 (m, 2H). 2.10-2.23 (m, 12-CH, 16-CH and 20-CH), 2.32 (m, 16-CH, 20-CH and 24-CH), 2.80 (br, OH), 2.89 (m, 21-H), 3.08 (br d, J = 10.2 Hz, 24-CH), 3.35 (m, 22-CH), 3.49 (dd, J_1 = 12.9 Hz, J_2 = 2.9 Hz, 22-CH), 4.05 (m, 23-CH), 7.68 (m, 30-CH and 31-CH), 7.87 (m, 29-CH), 8.17 (m, 32-CH). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ: 8.6 (C25), 10.9 (C19), 15.7 (C26), 21.2 (C11), 22.4 (C15), 25.2 (C18), 28.6 (C6), 29.7 (C7), 32.2 (C12), 32.3 (C2), 33.6 (C1), 33.8 (C16), 35.9 (C10), 37.8 (C8), 39.4 (C4), 40.1 (C5), 46.0 (C22), 49.5 (C23), 50.1 (C13), 50.8 (C14), 51.4 (C9), 52.4 (C24), 54.7 (C21), 65.7 (C20), 70.9 (C3), 122.6 (q, J_{C-F} = 274.4 Hz, C33), 127.5 (q, J_{C-C-F} = 43.8 Hz, C28), 128.5 (q, $J_{C-C-C-F}$ = 6.4 Hz, C29), 131.9 (C32), 132.1 (C31), 132.5 (C30), 139.3 (C27), 221.4 (C17). HRMS for

$C_{33}H_{48}F_3N_2O_4S$ $[M+H]^+$: 625.3281 (calculated), 625.3271 (found). HPLC purity of 95.5% (retention time = 17.6 min, 70:30 MeOH/H_2O-isocratic, Luna C18 column).

(3β,5β)-3-[(*trans*-2,5-dimethyl-4-{[2-(trifluoromethyl)phenyl]sulfonyl}piperazin-1-yl)methyl]-3-hydroxyandrostan-17-one (6b). IR (KBr) ν_{max}: 3502 (OH), 2932, 2862 (C-H, aliphatic), 1736 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.85 (s, 18β-CH_3), 0.88 and 0.89 (2d, *J* = 6.5 Hz, 25-CH_3), 0.98 (s, 19β-CH_3), 1.05-1.15 (m, 4β-CH and 7-CH), 1.17 and 1.18 (2d, *J* = 6.7 Hz, 26-CH_3), 1.20-1.37 (m, 2-CH_2, 6α-CH, 9α-H, 11α-CH and 14α-H), 1.40-1.64 (m, 1-CH, 7-CH, 8β-CH, 11β-CH, 12-CH and 15β-CH), 1.72-1.80 (5β-H and 12β-CH), 1.88-1.95 (m, 6β-CH and 15α-CH), 2.04 (m, 16α-CH), 2.12 (d, *J* = 13.9 Hz, 20-CH), 2.30 (d, *J* = 14.1 Hz, 20-CH), 2.38 (m, 24-CH), 2.44 (dd, J_1 = 19.1 Hz, J_2 = 8.7 Hz, 16β-CH), 2.80 (br, OH), 2.89 (m, 21-CH), 3.08 (dd, J_1 = 12.0 Hz, J_2 = 3.7 Hz, 24-CH), 3.35 (m, 22-CH), 3.49 (dd, J_1 = 12.9, J_2 = 2.8 Hz, 22-CH), 4.05 (m, 23-CH), 7.68 (m, 30-CH and 31-CH), 7.87 (m, 29-CH), 8.17 (m, 32-CH). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ: 8.6 (C25), 13.8 (C18), 15.7 (C26), 20.3 (C11), 21.8 (C15), 23.5 (C19), 25.2 (C7), 26.4 (C6), 34.4 (C2), 31.6 (C1), 31.7 (C12), 34.9 (C10), 35.2 (C8), 35.9 (C16), 36.9 (C4), 38.0 (C5), 40.0 (C9), 46.0 (C22), 47.9 (C13), 49.5 (C23), 51.5 (C14), 52.3 (C24), 54.6 (C21), 65.7 (C20), 71.4 (C3), 122.6 (q, J_{C-F} = 274.2 Hz, C33), 127.5 (q, J_{C-C-F} = 33.1 Hz, C28), 128.5 (q, $J_{C-C-C-F}$ = 6.4 Hz, C29), 131.9 (C32), 132.1 (C31), 132.5 (C30), 221.4 (C17). HRMS for $C_{33}H_{48}F_3N_2O_4S$ $[M+H]^+$: 625.3281 (calculated), 625.3250 (found). HPLC purity of 99.6% (retention time = 16.8 min, 70:30 MeOH/H_2O-isocratic, Luna C18 column).

2.1.7. Synthesis of *trans*-2,5-dimethyl-1-{[2-(trifluoromethyl)phenyl]sulfonyl}piperazine (7)

A solution of *trans*-2,5-dimethylpiperazine (3.0 g, 26.3 mmol) in DCM (20 mL) was cooled to -5 °C in an ice-salt bath. The stirrer was started, and 2-(trifluoromethyl)benzenesulfonyl chloride (2.1 mL, 13.6 mmol) was slowly added to the solution. After the addition, the reaction was stirred for 1 h at room temperature. The resulting solution was evaporated and purified by flash chromatography column using DCM/MeOH/TEA

(from 98:1:1 to 90:8:2) to give 2.9 g (66%) of compound **7** as a yellow solid. IR (KBr) ν_{max}: 3348 (NH), 3086 (CH aromatic), 2970, 2932, 2878 (CH aliphatic). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.06 (d, J = 6.6 Hz, 25-CH_3), 1.07 (d, J = 6.7 Hz, 26-CH_3), 1.51 (br, 1H), 2.58 (dd, J_1 = 12.7 Hz, J_2 = 5.6 Hz, 1H of 24-CH_2), 2.96 (dd, J_1 = 12.7 Hz, J_2 = 5.5 Hz, 1H of 24-CH_2), 3.04 (m, 21-CH), 3.11 (dd, J_1 = 12.7 Hz, J_2 = 3.6 Hz, 1H of 22-CH_2), 3.63-3.68 (m, 1H of 22-CH_2 and 23-CH), 7.68 (m, 30-CH and 31-CH), 7.87 (m, 29-CH), 8.17 (m, 32-CH). LRMS for $C_{13}H_{18}F_3N_2O_2S$ $[M+H]^+$: 323.10.

2.1.8. Synthesis of (*trans*-2,5)-1,2,5-trimethyl-1-{[2-(trifluoromethyl)phenyl]sulfonyl} piperazine (8)

To a solution of compound **7** (150 mg, 0.46 mmol) in DCM and cooled into an ice bath was added diisopropylamine (0.1 mL). To this solution was added methyl iodide (84 µL, 1.3 mmol) and the reaction mixture was stirred at 0 °C for 3 h and then evaporated. The crude product was purified by chromatography column using DCM/MeOH/TEA (from 99:0.5:0.5 to 92:7:1) to give 75 mg (48%) of **8** as a white solid. IR (KBr) ν_{max}: 3086 (CH aromatic), 2978, 2932, 2854 (CH aliphatic), 1165 (CF_3). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (d, J = 2.6 Hz, 25-CH_3), 1.11 (d, J = 2.6 Hz, 26-CH_3), 2.17 (dd, J_1 = 12.7 Hz, J_2 = 5.6 Hz, 1H of 24-CH_2), 2.25 (s, 3H, N-CH_3), 2.66 (m, 21-CH), 2.72 (dd, J_1 = 12.7 Hz, J_2 = 5.6 Hz, 1H of 24-CH_2), 3.15 (dd, J_1 = 12.7 Hz, J_2 = 3.6 Hz, 1H of 22-CH_2), 3.59 (dd, J_1 = 12.8 Hz, J_2 = 3.1 Hz, 1H of 22-CH_2), 3.88 (m, 23-CH), 7.68 (m, 30-CH and 31-CH), 7.87 (m, 29-CH), 8.17 (m, 32-CH). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ: 9.3 (C25), 16.3 (C26), 42.5 (C20), 48.4 (C22), 50.9 (C23), 54.6 (C21), 55.1 (C24), 122.6 (q, J_{C-F} = 274.4 Hz, C33), 126.7 (q, J_{C-C-F} = 43.8 Hz, C28), 128.5 (q, $J_{C-C-C-F}$ = 6.4 Hz, C29), 131.9 (C32), 132.1 (C29), 132.5 (C30), 140.1 (C27). HRMS for $C_{14}H_{20}F_3N_2O_2S$ $[M+H]^+$: 337.1192 (calculated), 337.1189 (found). HPLC purity of 99.9% (retention time = 18.6 min, 40:60 MeOH/H_2O-isocratic with 0.1% of formic acid, Luna C18 column).

2.2. Inhibition of 17β-HSD3 (intact 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 10,000 cells per well, in protocol medium. After 2 days of incubation, 15 nM of [14C]-4-androstene-3,17-dione and 10 µL of a solution of inhibitor dissolved in DMSO and culture medium were added. The final DMSO concentration in each well was adjusted to 0.05%. After 1 h of incubation, the culture medium was removed from wells and steroids (4-androstene-3,17-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-androstene-3,17-dione and [^{14}C]-testosterone were identified by comparison with reference steroids and quantified using the Storm 860 System (Molecular Dynamics, Sunnyvale, CA, USA). Percentages of transformation and inhibition were then calculated.

2.3. Proliferative activities on LAPC-4 (AR^+) cells

Androgen-sensitive human prostate cancer LAPC-4 cells were kindly provided by Robert E. Reiter from the University of California (Los Angeles, CA, USA). Cells were grown at 37 °C under 5% CO_2 humidified atmosphere in RPMI-1640 medium supplemented (v/v) with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% insulin and 1% penicillin/streptomycin. To determine the effect of novel compounds on cell proliferation, LAPC-4 cells were suspended with the medium supplemented with 5% dextran-coated charcoal treated FBS rather than 10% FBS, to remove the remaining hormones. Triplicate cultures of 10,000 cells in a total of 100 µL medium in 96-well microtiter plates (Becton–Dickinson Company, Lincoln Park, NJ, USA) were pre-incubated for 24 h at 37 °C under 5% CO_2 humidified atmosphere. Tested compounds were dissolved in DMSO to prepare the stock solution of 10^{-2} M. The compounds were then diluted at several concentrations with culture medium, added to corresponding wells, and incubated for 3

days. Control wells were treated with vehicle DMSO. MTS method was used for the quantification of cell growth, using CellTitter 96® AQueous Solution Cell Proliferation Assay (Promega, Nepean, ON, Canada) and following the manufacturer's instructions. The proliferative (androgenic) activity was expressed as difference between the cell proliferation (in %) caused by a given compound and the basal cell proliferation fixed at 100%.

2.4. Metabolic stability assays

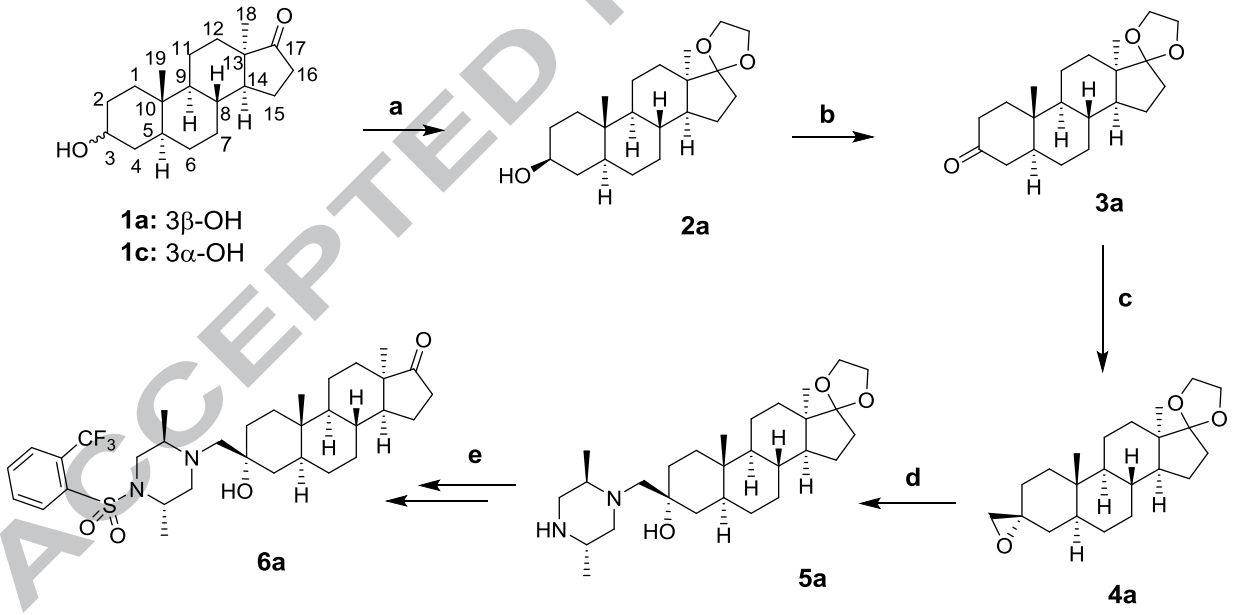
Assays were performed for 1 h at 37 °C, with or without 10 mM NADPH, in the presence of 40 µg of human liver microsomes from Corning (Melrose, MA, USA) and 10 µM of substrate in a final 100 µL volume of 50 mM Tris buffer supplemented with 10 mM $MgCl_2$. Assays were ended by adding 100 µL of MeOH, centrifuged at 13,000 g for 10 min to obtain a pellet of proteins. The supernatant of 2 assays were pooled, filtered and 100 µL submitted to HPLC-MS analysis (Shimadzu LCMS-2020 APCI, Altima HP C18 (250 mm x 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. Values represent the average of 4 independent experiments.

3. Results and discussion

3.1. Chemistry

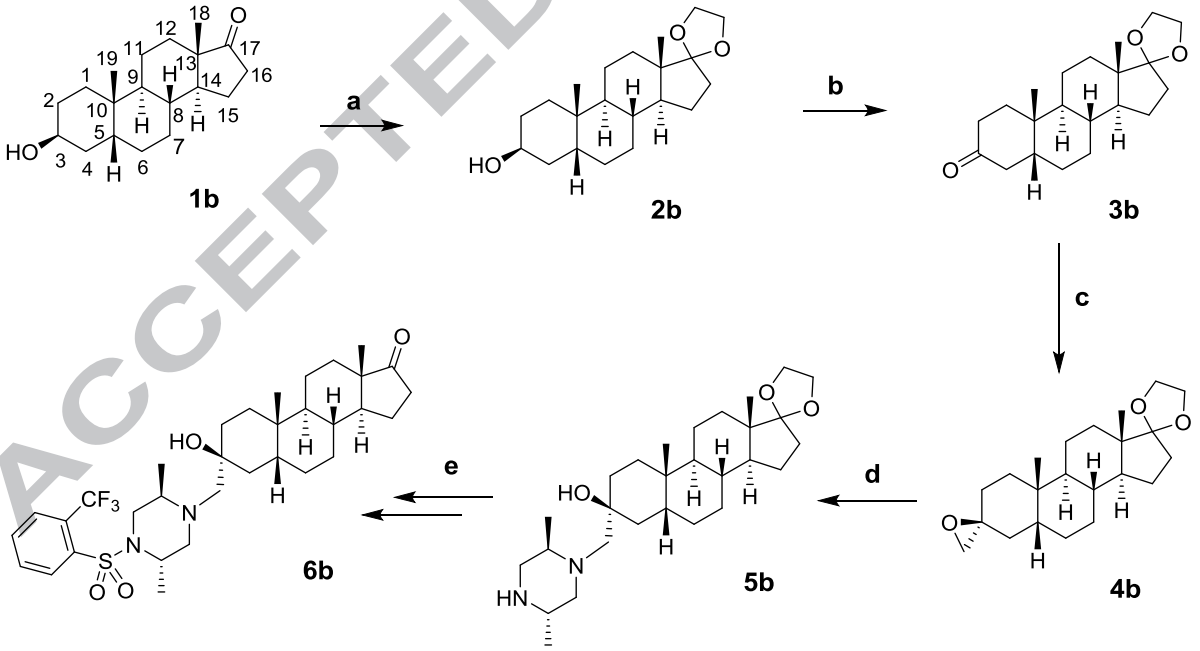
The chemical synthesis of the first RM-532-105 isomer, compound **6a**, is disclosed in Scheme 1. This compound, with an 18-methyl (CH_3) group in α-steroid face (18-epi or 13α-RM-532-105, **6a**) was prepared from **1a**, which was previously obtained from commercially available

epiandrosterone. Thus, the epimerization of the 13β-CH_3 group of epiandrosterone to give the 13α-isomer **1a** (13α-CH_3) was carried out following the method described by Yaremenko and Khvat,[30] which uses 1,2-phenylenediamine in boiling acetic acid. Treatment of **1a** with ethylene glycol and *p*-toluenesulfonic (*p*-TSA) acid in refluxing toluene yielded the protected C17 ketone as dioxolane **2a**. The secondary alcohol at C3 was oxidized in mild conditions by using Dess-Martin periodinane to give **3a**. This steroidal ketone was efficiently reacted under the Corey-Chaykovsky epoxidation conditions,[31] using trimethylsulfoxonium iodide and sodium hydride, to obtain **4a**. Under these conditions, it is known that a 5α-H-androstane-3-ketone give an oxirane with the *R*-configuration as the major compound.[18, 32-36] The treatment of **4a** with *trans*-2,5-dimethylpiperazine in refluxing ethanol opened the oxirane group and provided the tertiary alcohol **5a** with the *R*-configuration at C3, which cannot be modified during the last two steps of the synthesis. Finally, the free NH of **5a** was submitted to a reaction with 2-(trifluoromethyl)benzenesulfonyl chloride and trimethylamine (TEA) and the dioxolane protecting group was subsequently removed in acidic conditions to yield the desired product **6a** in an overall yield of 6% (6 steps from **1a**).



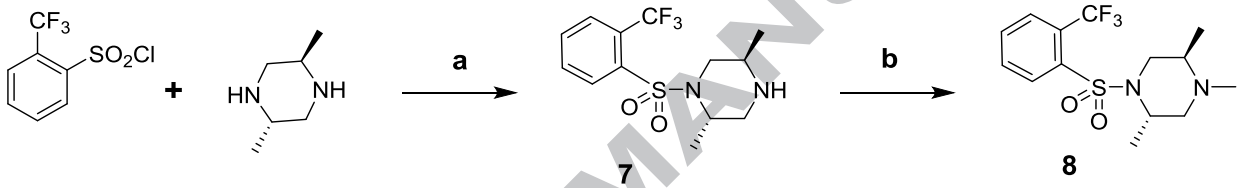
Scheme 1. Reagents and conditions: a) Ethylene glycol, *p*-TSA, toluene, reflux, 12 h; b) Dess-Martin reagent, DCM, rt, 1 h; c) $(CH_3)_3SOI$, NaH, DMSO, THF, rt, 5 h; d) *trans*-2,5-dimethylpiperazine, ethanol, 70 °C, 12 h; e) *1.* 2-(trifluoromethyl)benzenesulfonyl chloride, TEA, DCM, rt, 4 h, *2.* HCl 36%, acetone, 0 °C, 30 min.

The chemical synthesis of the RM-532-105 isomer with the *cis-trans* conformation to the A/B fused rings (5β-RM-532-105, **6b**) is disclosed in Scheme 2. Starting from 5β-epi-androsterone (**1b**), the synthetic pathway uses the same sequence of reactions reported for **6a**: 1) protection of C17 ketone as dioxolane **2b**, 2) oxidation of the secondary alcohol at C3 to the ketone **3b**, 3) epoxidation to obtain the oxirane **4b**, 4) opening of the oxirane to provide **5b**, 5) formation of the sulfonamide and 6) hydrolysis of the dioxolane protecting group to yield the desired compound **6b** in an overall yield of 12% (6 steps from **1b**). The only difference between the sequences of reactions providing **6a** and **6b** (Schemes 1 and 2) is the configuration of the oxirane generated at C3. In fact, contrary to the 3*R*-oxirane obtained from the 5α-androstan-3-one (compound **3a**), a 3*S*-oxirane was obtained from the 5β-androstan-3-one (compound **3b**). Aher *et al*[37] unambiguously confirmed by X-ray analysis the *S*-oxirane configuration generated from a 5β-steroidal 3-ketone. Consequently, the configuration at C3 is expected to be *S* for the tertiary alcohol **4b**. Since the last two steps toward **6b** can not modified the stereochemistry at C3, **5b** and **6b** will be of 3*S*-configuration. The C-3 stereochemistry of **6b** will be however addressed in an upcoming section.



Scheme 2. Reagents and conditions: a) Ethylene glycol, *p*-TSA, toluene, reflux, 12 h; b) Dess-Martin reagent, DCM, rt, 1 h; c) $(CH_3)_3SOI$, NaH, DMSO, THF, rt, 5 h; d) *trans*-2,5-dimethylpiperazine, ethanol, 70 °C, 12 h; e) *1.* 2-(trifluoromethyl)benzenesulfonyl chloride, TEA, DCM, rt, 4 h, *2.* HCl 36%, acetone, 0 °C, 30 min.

Compound **8**, which represents the side chain introduced at position 3β of the steroid scaffold of **6a** and **6b**, was prepared for the purposes of comparison (Scheme 3). Briefly, an excess of *trans*-2,5-dimethylpiperazine was treated with 2-(trifluoromethyl) benzenesulfonyl chloride to give **7**, and the free NH was next reacted with methyl iodide to yield the desired compound **8** in a moderate 34% yield for 2 steps.



Scheme 3. Reagents and conditions: a) DCM, 0 °C, 1 h; b) CH_3I, DIPEA, 0 °C, 3 h.

The structures of final compounds **6a**, **6b** and **8** were confirmed based on their spectral data (IR, ^{1}H NMR, ^{13}C NMR and MS) and their purity, determined by HPLC and found to be >95%. Additionally, 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) were carried out to obtain a full assignation for H and C atoms, and to find evidence for the conformational geometry (13α-CH_3 and 5β-H) of the2 steroid C3-derivatives (see supporting information). For compound **6a**, the chemical shift data of 13α-CH_3 group is in agreement with previous studies for 13α-ADT.[30] Compared to RM-532-105, this methyl group has shifted downfield from 0.85 ppm (13β-CH_3) to 0.96 ppm (13α-CH_3) in the ^{1}H NMR spectra and from 13.8 (13β-CH_3) to 25.2 ppm (13α-CH_3) in the ^{13}C NMR spectra. The chemical shift for C3, the new steroidogenic center, is also the same (70.9 ppm) suggesting the same *R*-configuration for **6a** and RM-532-105, as expected.

For compound **6b**, the configuration of the asymmetric C3 carbon was expected to be *S* per the work of Aher et al,[37] but it was addressed and confirmed by NMR analyses. Using the

HMBC experiment, we first identified the methyl, producing a correlation with the carbonyl-17 (18-CH_3), and that producing no correlation (19-CH_3). The 4 correlations observed with 19-CH_3, in combination with ^{13}C NMR data found in literature for 5β-steroids,[38] allowed the identification of the following carbons: 1-CH_2, 10-C, 9-CH and 5-CH. Using the HSQC experiment, the 5-C signal observed at 38.0 ppm was linked to the 5β-H at 1.75 ppm. The 5β-H displays also a NOE correlation with 19-CH_3 (13β-CH_3) but not with the 20-CH_2 located at C3 (Fig. 4A). However, both 4α-H and 4β-H produced NOE correlations with 20-CH_2, which is only possible with the *S* configuration at C3 (3β-OH/3α-CH_2) instead of the *R* configuration (Fig. 4B). The C3-chemical shift (71.5 ppm) for **6b** (3*S* and 5β-H) is also slightly different than the values (both 70.9 ppm) obtained for **6a** and RM-532-105 (both 3*R* and 5α-H).

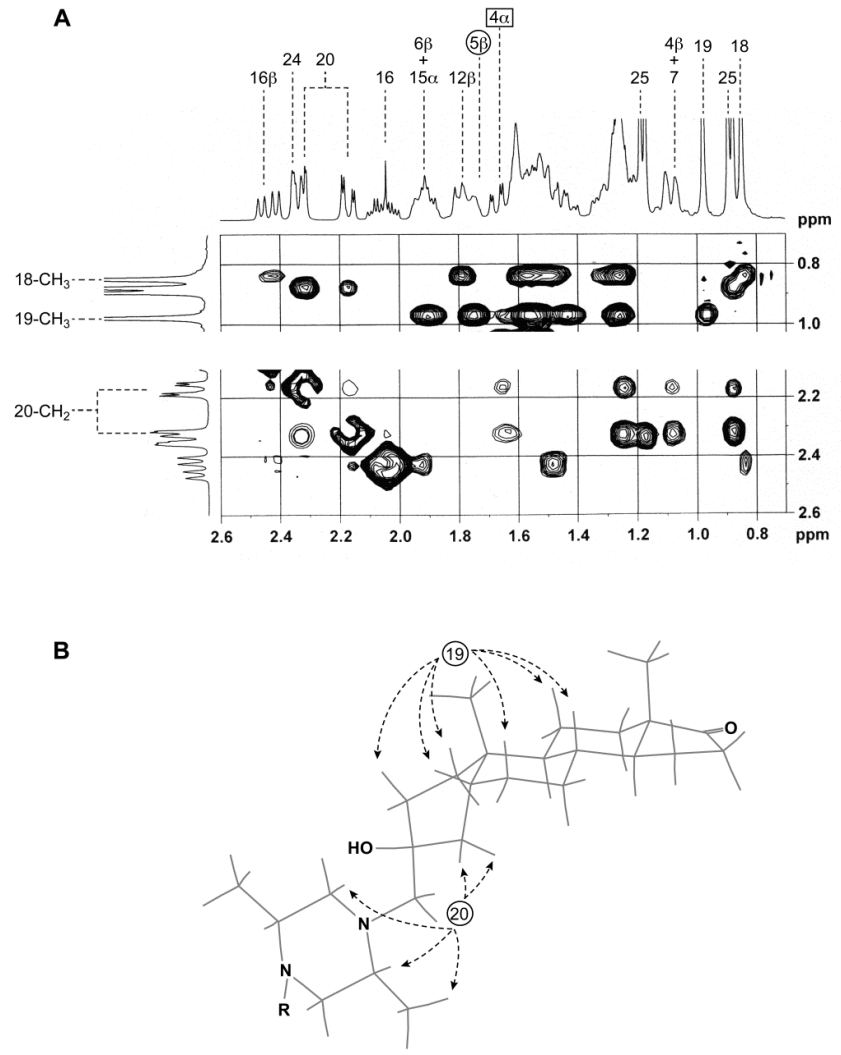
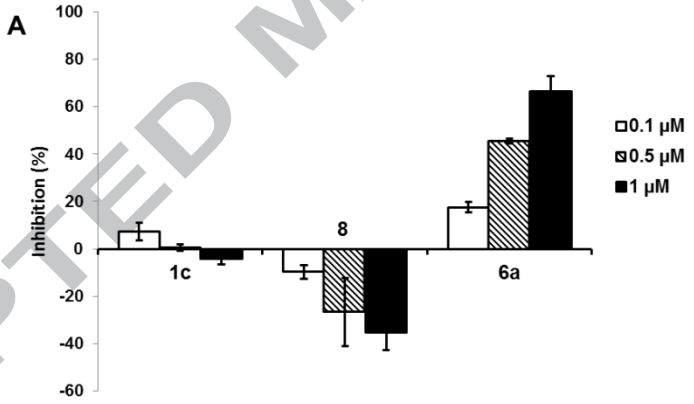


Figure 4. Partial NOESY spectra (**A**) and 3D-representation (**B**) of compound **6b**.

3.2. Biological activity

3.2.1. 17β-HSD3 inhibitory activity

The inhibition of 17β-HSD3 activity was determined using intact LNCaP cells overexpressing 17β-HSD3, by measuring the amount of labeled T formed from natural labeled 4-dione, and then calculating the percentage of inhibition (Fig. 5). The first general observation from this screening study is that the steroid backbone alone (compounds **1c** and **1b**), as well as the side chain alone (compound **8**), did not contribute to the inhibitory activity. However, the combination of these 2 structural elements on the same molecule (compounds **6a** and **6b**) significantly reduced the catalytic activity of the enzyme. Both the 13α-CH_3-derivative **6a** (Fig. 5A) and 5β-H-derivative **6b** (Fig. 5B) inhibited the enzyme at the 3 concentrations tested (0.1, 0.5 and 1 µM). Thus, the presence of the side chain attached to the C3 position of the steroid backbone is crucial to the inhibition of 17β-HSD3 activity.



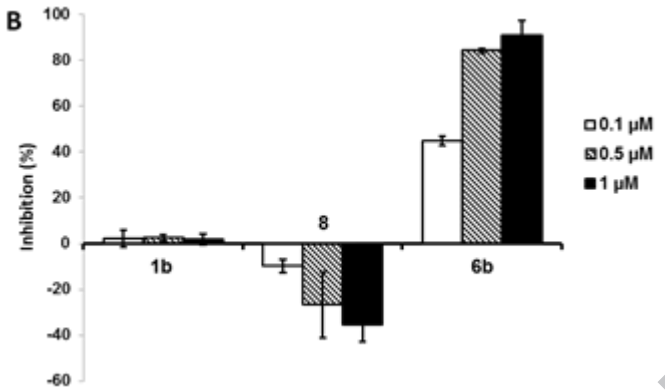


Figure 5. Impact of steroid scaffold (steroids **1c** and **1b**), 3β-side chain (compound **8**) and both structural elements (steroid derivatives **6a** and **6b**) on 17β-HSD3 inhibitory activity (transformation of 4-dione to T in LNCaP cells overexpressing 17β-HSD3). **A**) Modification at the C/D ring junction (13α-CH_3) and **B**) modification at the A/B ring junction (5β-H).

The IC_{50} values were obtained for the newly designed compounds **6a** and **6b,** as well as for the reference RM-532-105 (Fig. 6). All compounds showed a dose-dependent inhibition of 17β-HSD3 activity that afforded IC_{50} values of 1.09, 0.15 and 0.11 µM for **6a**, **6b** and RM-532-105, respectively. Therefore, **6a** (5α/13α-RM-532-105) is 10-fold less potent than the inhibitor used as reference (RM-532-105, with a 5α-H and 13β-CH_3), but interestingly **6b** (5β/13β-RM-532-105) is as almost as potent as the lead inhibitor. Based on these findings, changing the shape of D ring greatly decreases 17β-HSD3 inhibitory activity, whereas the A-ring inversion (*cis-trans* instead *trans-trans* conformation) combined to the inversion of the OH at C3 didn't exhibit a substantial impact on the inhibitory effect. A possible explanation for this might be that the ketone at C17 is a key function interacting with a hydrogen bond donor from an amino acid residue in the binding pocket of 17β-HSD3. Thus, changing the shape of D-ring disrupts this interaction resulting in a lower inhibitory potency. On the other hand, the 2 modifications in A-ring may be tolerated because this change does not affect the interactions performed by the side chain at C3. In fact, this moiety has a high degree of conformational flexibility that can favor interactions in the binding cavity of 17β-HSD3, especially in the hydrophobic pocket, which

seems able to accommodate large substituents and greatly contributed to the inhibitory potency of 3β-substituted-ADT derivatives.[39-41]

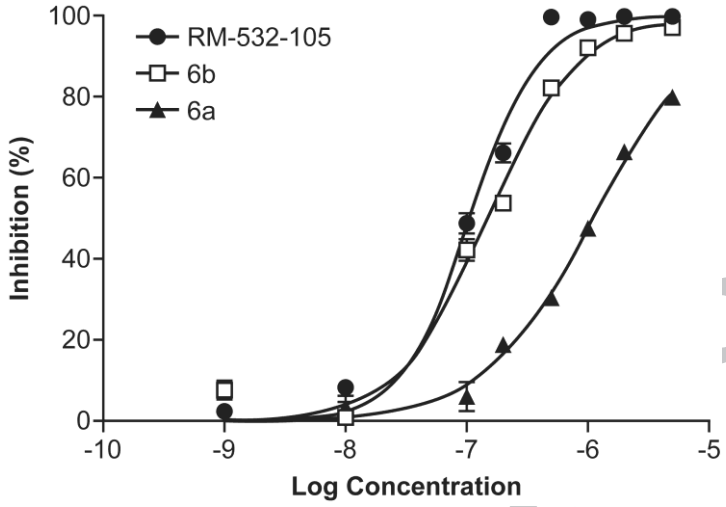


Figure 6. Inhibition of 17β-HSD3 activity by several concentrations of **6a** (5α/13α-RM-532-105, IC_{50} = 1.09 µM), **6b** (5β/13β-RM-532-105, IC_{50} = 0.15 µM) and 5α/13β-RM-532-105 (IC_{50} = 0.11 µM).

3.2.2. Proliferative effect on LAPC-4 cells (AR^+)

An inhibitor of androgen biosynthesis should be devoid of androgenic activity for use in prostate cancer therapy. To investigate whether the synthetized compounds display an androgenic profile, we assessed compounds **6a** and **6b** on the androgen-sensitive prostate cancer LAPC-4 (AR^+) cells and measured the proliferative (androgenic) activity which was expressed as the difference between the cell proliferation (in %) caused by a given compound and the basal cell proliferation fixed at 100% (Fig. 7). The natural androgens DHT and RM-532-105 were also tested as reference compounds. At 10 nM, the potent androgen DHT increased the cell proliferation to 190%, thus clearly demonstrating the androgen-dependence of LAPC-4 cells, which express the wild-type androgen receptor.[42] With percentages of cell growth lower than 100% (96, 48 and 33% at 0.1, 0.5 and 1 µM, respectively), RM-532-105 did not show an androgenic effect. As expected, the isomers of RM-532-105 displayed no androgenic activity with cell growth values of 76.3, 61.6 and 51.0% for **6a** and 87.2%, 72.3% and 28.2 % for **6b** at 0.1, 0.5 and 1 µM, respectively. In fact, these 3 compounds reduced cell proliferation when compared to the control, which suggests a potential cytotoxic activity at least at the 2 higher

concentrations. Compound **6b**, the 5β-isomer of RM-532-105, is however less cytotoxic than the other 2 compounds.

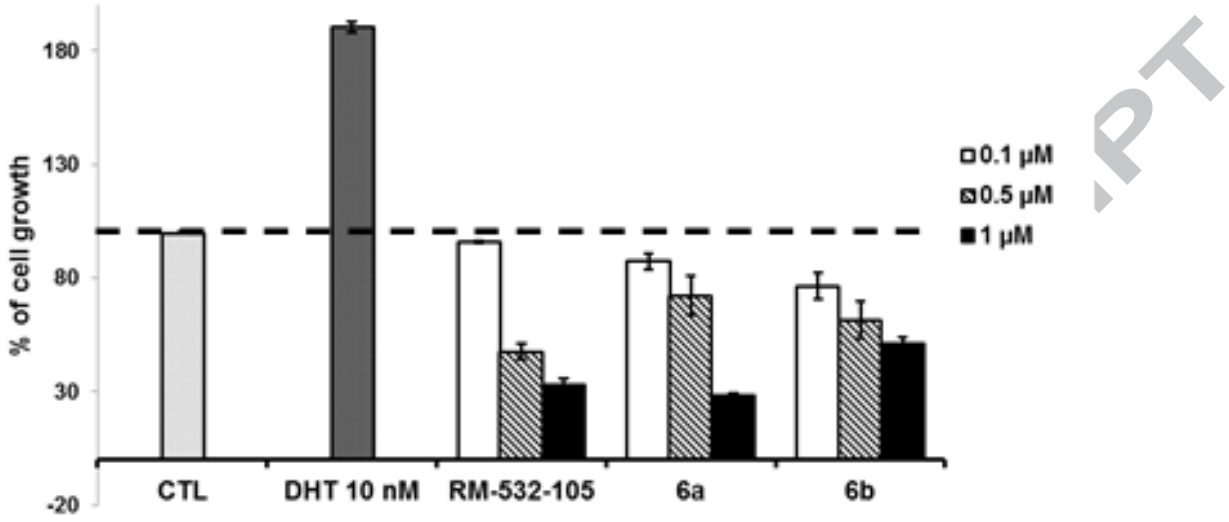


Figure 7. Androgenic profile for **6a** (5α/13α-RM-532-105), **6b** (5β/13β-RM-532-105) and 5α/13β-RM-532-105 on androgen-sensitive LAPC-4 cells.

3.2.3. Metabolic stability

Another criterion for discriminating different potential drug candidates is to evaluate their metabolic stability. Compounds **6a** and **6b** were thus treated for 1 h by a microsomal preparation of human liver in the presence of NADPH as cofactor to measure the impact of the modifications at C5 and C13. In this assay, a compound is transformed by phase-I reactions (oxidation, reduction, hydrolysis) instead of phase-II reactions (glucuronidation, sulfatation, acetylation), and the remaining compound is measured at the end of the incubation time, and expressed in %. As observed in Figure 8, the inversion at C5 (compound **6b**) did not influence metabolic stability. However, with only a tertiary alcohol, the A-ring of RM-532-105, **6a** and **6b** is not favorable to phase-I reactions. Interestingly, compound **6a** is more stable than RM-532-105 and **6b**. This result suggests a less important metabolism on D-ring positions 16 and 17 for **6a** (13α-CH_3) than for **6b** and RM-532-105 (both 13β-CH_3). In fact, the reduction of the C17-carbonyl into alcohol and the hydroxylation at position 16 are 2 reactions involved in the formation of known metabolites of steroidal ketones.[43] Thus, the production of D-ring metabolites by the

enzymes of the cytochrome P450 family found in the liver is probably reduced by the steric hindrance involved in the inversion of 18-CH_3.

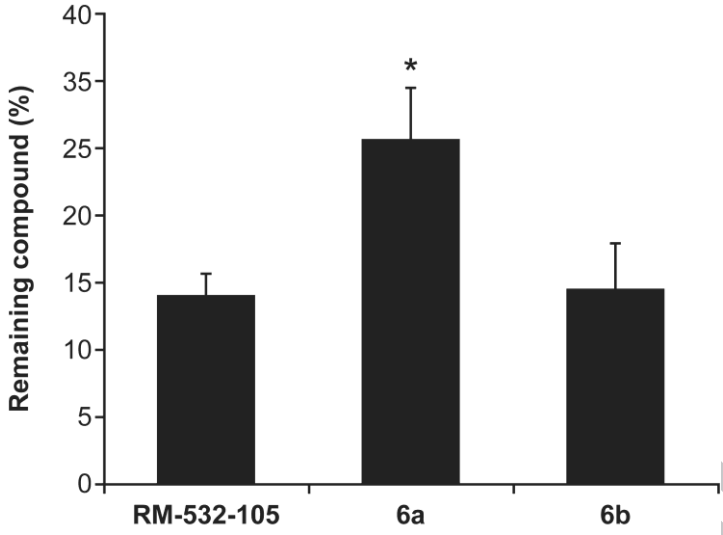


Figure 8. Metabolic stability of **6a** (5α/13α-RM-532-105), **6b** (5β/13β-RM-532-105) and 5α/13β-RM-532-105. The results are expressed as the % of the remaining quantity of the tested compound treated with a microsomal preparation of human liver. The data represent the average of 4 experiments ± SD. * $p<0.05$.

4. Conclusion

Two isomers of 17β-HSD3 inhibitor RM-532-105 (compounds **6a** and **6b**) were prepared and characterized to assess the impact of two structural modifications of the steroid scaffold on biological activities. The biological assessments on LNCaP cells overexpressing 17β-HSD3 and androgen-sensitive prostate cancer LAPC-4 cells allowed the identification of **6b** (5β-H; *cis-trans* A/B fused rings) as a 17β-HSD3 inhibitor without androgenic activity and a weak cytotoxic activity. The 17β-HSD3 inhibitory activity of compound **6a** (13α-CH_3; *trans-cis* C/D fused rings) was however reduced, but its androgenic profile was not affected. Compared to RM-532-105 (5α-H/13β-CH_3), the liver microsomal stability of **6b** (5β-H) was not affected, whereas that of **6a** (13α-CH_3) was improved. This finding has important implications for developing new generations of more potent 17β-HSD3 inhibitors. Moreover, these results justify the exploration

of other structural modifications on the A-ring, since this change seems to be well-tolerated by the 17β-HSD3 enzyme.

Acknowledgments

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Supplementary data

HPLC chromatogram, HRMS data, IR spectra, ^{1}H NMR, ^{13}C (APT), HSQC, HMBC, COSY and NOESY spectra for compounds **6a**, **6b** and **8**.

Supplementary data associated with this article can be found, in the online version, at …….

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