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Synthesis of new derivatives of 21-imidazolyl-16-dehydropregnenolone as inhibitors of 5α-reductase 2 and with cytotoxic activity in cancer cells

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Abstract

The aim of this study was to synthesize several 16-dehydropregnenolone derivatives containing an imidazole ring at C-21 and a different ester moiety at C-3 as inhibitors of 5α-reductase 1 and 2 isoenzymes. Their binding capacity to the androgen receptor (AR) was also studied.

Additionally, we evaluated their pharmacological effect in a castrated hamster model and their cytotoxic activity on a panel of cancer cells (PC-3, MCF7, SK-LU-1).

The results showed that only the derivatives with an alicyclic ester at C-3 showed 5α-R2 enzyme inhibition activity, the most potent of them being 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclohexanecarboxylate with an IC_{50} of 29 nM. This is important because prostatic benign hyperplasia is directly associated with the presence of 5α-R2. However, all the derivatives failed to inhibit 5α-R1 or bind to the AR. These alicyclic ester derivatives decreased the weight and size of androgen-dependent glands in the hamster, indicating they are very active *in vivo* and are not toxic. In addition, the 21-(1H-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-acetate derivative showed the highest cytotoxic activity on the three cancer cell lines studied.

It is therefore important in the synthesis of steroidal compounds to consider the size of the ester moiety at C-3 of the steroid skeleton, which is key in obtaining biological activity, as observed in this experiment.

Key words

16-dehydropregnenolone acetate derivatives, alicyclic ester moiety at C-3 of the steroidal skeleton, 5-α-reductase, cytotoxic activity, cell lines: PC-3, MCF7, SK-LU-1.

1. Introduction

Prostate cancer (PC) and benign prostatic hyperplasia (BPH) are two of the major conditions that currently affect the male population over 45 years old. PC is the second leading cause of cancer death, and BPH is the second cause of surgery in some countries; for example the United States [1]. Both diseases are directly related to an excess of androgens in the body.

The main androgens produced by the body are testosterone (T) and dihydrotestosterone (DHT). Testosterone, the best known androgen, is converted to DHT, a more potent androgen, by the action of 5α-reductase isoenzymes. The activity of these isoenzymes in androgen-dependent tissues has long been known; DHT is responsible for sending a signal to induce prostate growth, which in pathological conditions produces the development of both diseases [2]. The three 5α-reductase isoenzymes are expressed in different human tissues [3]. Type 1 isoenzyme (5α-R1) is expressed in prostate epithelial cells and is directly related to prostate cancer. Type 2 (5α-R2) is associated with development of benign prostatic hyperplasia [4] and is found mostly in the prostate, seminal vesicles and genital skin. Type 3 (5α-R3) is related to hormone-refractory prostate cancer (HRPC) and is found in the brain and pancreas [5-6]. Unfortunately, these three isoenzymes have not been purified and crystallized, due to their unstable nature, and as a result, the 5α-R inhibitors have been designed based on structure-activity studies. Two of these compounds are finasteride and dutasteride. Both are 4-azasteroids currently used in CP and BPH with undesirable secondary antiandrogen effects such as impotence, muscle growth impairment, and gynecomastia [7]. Also, their use for long periods can have a potential hepatoxic effect [8]. Many different compounds are currently being studied with

the aim of decreasing these unwanted effects and enhancing the therapeutic effect for a successful prostate cancer treatment.

Nowadays a variety of many drugs used in the treatment of different steroid-dependent and independent diseases, have a heterocyclic group in their structure, for example the aminoglutethimide and its derivatives; esters of 4-pyridineacetic acid; bis-chlorophenyl-pyrimidine analogues; as well as imidazole and triazole derivatives, such as, liarozole, fadrozole, CGS 18320 B, vorozole and CGS 20267 [9-10]. In particular, various biological studies have demonstrated that some pregnane 21 and 16-derivatives have interesting activity, such as an antiproliferative effect, on different cancer cell lines and 5α-reductase inhibition. [9-21].

On this basis, we synthesized and determined the biological activity of a novel series of 21-(1*H*-imidazole)-5-16-pregnadiene-20-one-2-yl derivatives (**8a−j**; see below) as inhibitors of type 1 and 2 5α-reductase isoenzymes. We determined their androgen receptor (AR) binding capacity and their antiproliferative effect on the following cancer cell lines: PC-3 (prostate), MCF7 (breast) and SK-LU-1 (lung). The pharmacological effect was evaluated in a hamster model.

These experiments were designed to complement a previously reported study of a molecule with a triazole group at C-21 on a pregnane skeleton [12, 15] and to compare the importance of the azole group on the biological activity.

2. Material and methods

2.1. Reagents

Reagents and solvents were purchased from commercial sources and were used without further purification. Melting points (uncorrected) were determined on a Fisher Johns melting point apparatus. Infrared spectra (IR) were recorded on a Perkin-Elmer 200 spectrometer (Perkin-Elmer Life and Analytical Science, Shelton CT, USA). ^{1}H and ^{13}C NMR were taken on a Varian VRX-400 spectrometer (MR resources NC, USA) operating at 400 (^{1}H) and 100 (^{13}C) MHz with tetramethylsilane (TMS) as internal standard (δ=0) in $CDCl_3$ (the abbreviations of signal patterns are as follows: s, singlet; d, doublet, t, triplet; m, multiplet). High resolution mass spectra (HRMS) were obtained with a Thermo DFS spectrometer by direct infusion and using the FAB^+ ionization mode (Thermo Fisher Scientific, San Jose, CA USA).

(1,2,6,7-^{3}H) Testosterone [^{3}H] T specific activity: 95 Ci/mmol and Mibolerone (17a-methyl-^{3}H) [^{3}H] MIB 10 specific activity 70–87 Ci/mmol were provided by Perkin Elmer Life and Analytical Sciences (Boston, MA).

Radioinert T, 5α-DHT, and MIB were supplied by Steraloids (Wilton, NH, USA). Sigma Chemical Co. (St. Louis, MO) provided NADPH. Finasteride was obtained by extraction from Proscar® (Merck, Sharp & Dohme). The tablets were crushed, extracted with chloroform and the solvent was eliminated in vacuum; the crude product was purified by silica gel 60 (63–200 µm) column chromatography (Sigma–Aldrich) with ethyl acetate as eluent. The melting point of the isolated finasteride (252–254 °C) was identical to that reported in the literature.

16-deydropregnenolone acetate and ketoconazole used as a reference compound were purchased from Sigma Life Sciences.

2.2. Synthesis of 16-dehydropregnenolone derivatives (8a−j)

Steroids **8a**−**j** (Fig. 1) were prepared from the commercially available 16-dehydropregnenolone acetate (1) with the synthesis of their intermediate compounds being previously reported by our group. [15] The synthetic pathways for the preparation of **8a–j** are briefly outlined in Fig. 1

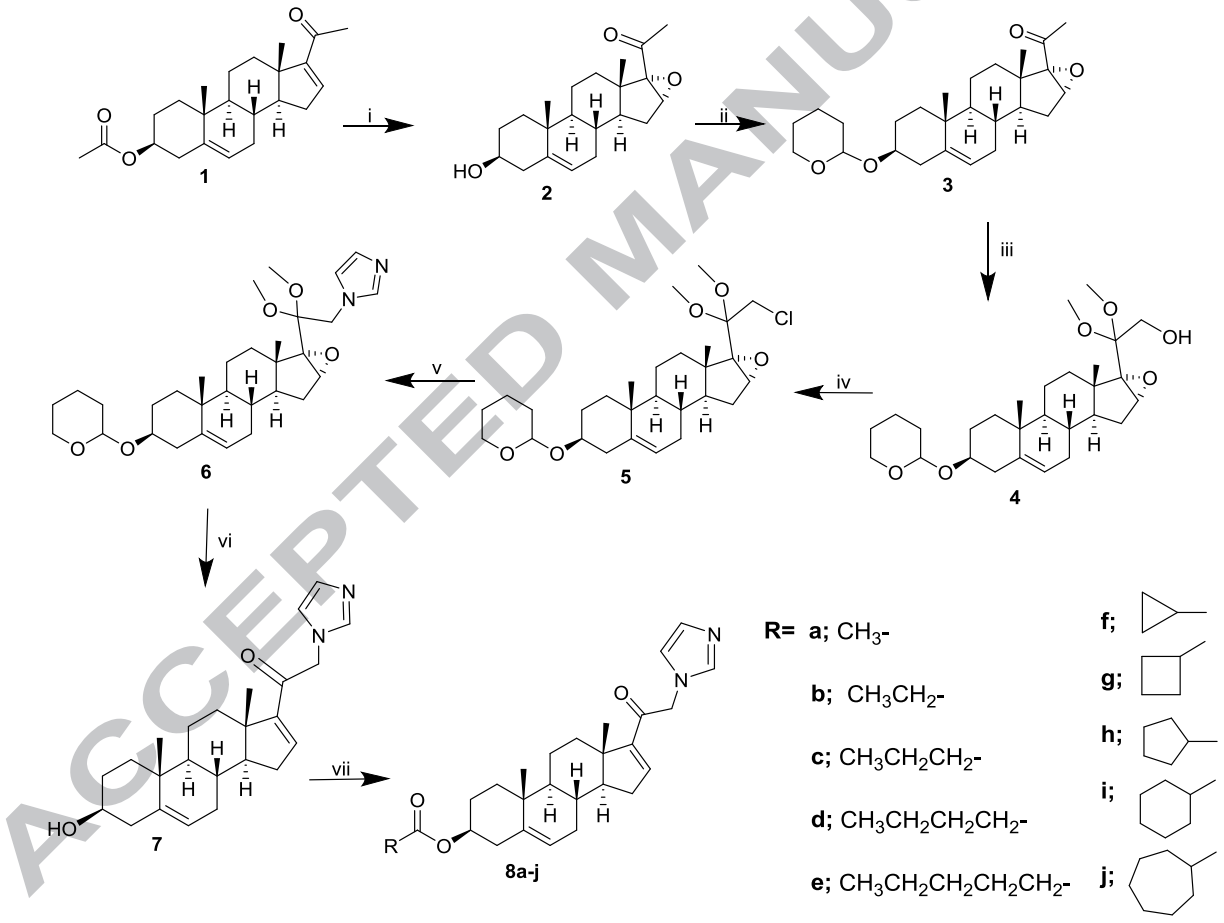


Figure. 1. Figure. 1. Reagents and conditions: (***i***) H_2O_2, NaOH 4N; (***ii***) DHP, p-toluensulphonic acid; CH_2Cl_2(***iii***) NaOH, $C_6H_5I(OAc)_2$, MeOH; (***iv***) $SOCl_2$, Py, CH_2Cl_2; (***v***) 1*H*-imidazole, Cs_2CO_3, 80 °C, 5 h; (***vi***) HCl, $CrCl_2$, CH_3COOH, 20 min.; (***vii)*** *a*) acetic anhi**y**dride, Py; *b-j*) R-COOH, DCC, DMAP, $CHCl_3$.

Synthesis of 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl derivatives (8a−j in Fig. 1)

2.2.1.1. Preparation of 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-acetate (8a in Fig. 1).

The synthesis of this compound was carried out starting with a solution of steroid **7** [15] (0.1 g, 2.62 mmol) in pyridine (0.4 mL) and acetic anhydride (0.4 mL). This mixture was stirred at room temperature for 1 h. After, a solution of water and salt was added and the precipitate formed was filtered. The compound was washed with water 3 times.

Yield 0.10 g (90%), m.p. 105–108 °C, IR (KBr) cm^{-1}: 2937, 1729, 1236 and 1031. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.10 (s, H-18, 3H), 1.30 (s, H-19, 3H), 2.03 (t, ester-H-2′, 3H), 3.90 (m, *J* = 3.2 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.9 Hz, H-6, 1H), 6.73 (s, *J* = 6.7, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.40 (C-18), 20.30 (C-19), 21.0 (C-2′ of ester), 65.60 (C-21), 74.07 (C-3), 121.96 (C-6), 140.03 (C-Het), 140.10 (C-5), 144.21 (C-Het), 147.20 (C-16), 152.04 (C-Het), 156.00 (C-17), 170.67 (ester carbonyl), 199.27 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 422.2569 $C_{26}H_{34}N_2O_3$, found 422.2563.

2.2.1.2. Preparation of 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-propionate (8b) to 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cycloheptanecarboxylate (8j). To a solution of steroid **7** (0. 1 g, 0.262 mmol), DCC (0.110 g, 0.524 mmol) and DMAP (0.064 g, 0.524 mmol) in chloroform (3 mL), the corresponding acid (0.6 mmol) was added. The resulting solution was stirred at room temperature for 2 h. Ethyl acetate (8 mL) was added and the precipitated dicyclohexyl urea formed was filtered. The organic phase was washed three times with 10% aqueous hydrochloric acid, 5%

aqueous sodium bicarbonate, and then water. This organic phase was then dried over anhydrous sodium sulfate. The crude ester was recrystallized from a mixture of chloroform and methanol.

Seven esters were obtained from this reaction: (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-propionate (**8b)**; (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-butyrate (8**c)**; (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-pentanoate (**8d),** (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-hexanoate (**8e**), (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclopropanecarboxylate (**8f**), (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclobutanecarboxylate (**8g**), (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclopentanecarboxylate (**8h**), (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclohexanecarboxylate (**8i**) and (3S)-21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cycloheptanecarboxylate (**8j**).

2.2.1.2.1. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-propionate (8b in Fig. 1).

Yield 0.078 g (68%), m.p. 180–182 °C, IR (KBr) cm^{-1}: 2927, 1731, 1568, 1270 and 1087. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (s, H-18, 3H), 1.20 (s, H-19, 3H), 1.10 (t, H-3′ of ester, 3H), 3.90 (m, *J* = 3.6, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.9 Hz, H-6, 1H), 6.73 (s, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.30 (C-3′ of ester), 15.30 (C-18), 21.30 (C-19), 65.35 (C-21), 71.54 (C-3), 121.01 (C-6), 141.21 (C-5), 144.21 (H-Het), 147.80 (C-16), 151.55 (C-Het), 157.09 (C-17), 194.64 (ester carbonyl), 206.09 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 436.2726 $C_{27}H_{36}N_2O_3$, found 436.2732.

2.2.1.2.2. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-butyrate (8c in Fig. 1).

Yield 0.071 g (60%), m.p. 184–187 °C, IR (KBr) cm^{-1}: 2929, 1729, 1574, 1270 and 1088. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.00 (t, ester-H-4′, 3H), 1.10 (s, H-18, 3H), 1.30 (s, H-19, 3H), 3.90 (m, *J* = 3.9 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.9 Hz, H-6, 1H), 6.73 (s, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.30 (C-3′ of ester), 15.01 (C-18), 23.40 (C-19), 65.30 (C-21), 71.54 (C-3), 121.01 (C-6), 141.21 (C-5), 141.26 (C-Het), 144.89 (C-Het), 148.44 (C-16), 151.55 (C-Het), 157.12 (C-17), 196.64 (ester carbonyl), 206.09 (C-20). HRMS: *m/z* $[M+H]^+$ calculated 450.2882 $C_{28}H_{38}N_2O_3$, found 450.2878.

2.2.1.2.3. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-pentanoate (8d in Fig. 1).

Yield 0.067 g (55%), m.p. 172–175 °C, IR (KBr) cm^{-1}: 2929, 1729, 1574, 1270 and 1087. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (t, ester-H-5′, 3H), 1.01 (s, H-18, 3H), 1.29 (s, H-19, 3H), 3.90 (m, *J* = 3.2 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.9 Hz, H-6, 1H), 6.73 (s, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.01 (C-5′ of ester), 15.30 (C-18), 24.50 (C-19), 63.40 (C-21), 71.54 (C-3), 121.01 (C-6), 141.22 (C-Het), 144.89 (C-Het), 148.45 (C-16), 151.55 (C-Het), 155.71 (C-17), 196.64 (ester carbonyl), 206.09 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 464.3039 $C_{29}H_{40}N_2O_3$, found 464.3033.

2.2.1.2.4. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-hexanoate (9e in Fig. 1).

Yield 0.067 g, (53%), m.p. 169–171 °C, IR (KBr) cm^{-1}: 2931, 1730, 1575, 1270 and 1087. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.67 (t, ester-H-6′, 3H), 0.90 (s, H-18, 3H), 1.02 (s, H-19, 3H), 3.90 (m, *J* = 3.9 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.36 (d, *J* = 5.8 Hz, H-6, 1H), 6.73 (s, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.36 (C-6′), 15.30 (C-18), 22.13 (C-19), 62.31 (C-21), 71.54 (C-3), 121.01 (C-6), 141.21 (C-5), 141.22 (C-Het), 144.89 (C-Het), 148.19 (C-16), 151.55 (C-Het), 155.71 (C-17), 196.64 (ester carbonyl), 206.08 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 478.3195 for $C_{30}H_{42}N_2O_3$, found 478.3189.

2.2.1.2.5. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclopropanecarboxylate (8f in Fig. 1).

Yield 0.065 g, (55%), m.p. 187–189 °C, IR (KBr) cm^{-1}: 2926, 1726, 1624, 1570, 1270 and 1086. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25 (s, H-18, 3H), 1.30 (s, H-19, 3H), 1.65 (m, ester-H-1′, 1H), 3.47 (m, *J* = 3.5 Hz, H-3, 1H), 4.22−4.59 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.4 Hz, H-6, 1H), 6.72 (s, *J* = 6.7 Hz, C-16, 1H), 7.11 (s, H-Het, 1H), 7.35 (s, H-Het, 1H), 7.51 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 10.10 (C-1′ of ester), 13.253 (C-18), 15.00 (C-19), 62.45 (C-21), 73.82 (C-3), 119.23 (C-6), 139.99 (C-Het), 147.21 (C-16), 147.22 (C-Het), 157.00 (C-Het), 157.03 (C-17), 174.44 (ester carbonyl), 204.08 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 448.2726 for $C_{28}H_{36}N_2O_3$, found 448.2730.

2.2.1.2.6. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclobutanecarboxylate (8g in Fig. 1).

Yield 0.069 g, (57%), m.p. 101–103 °C, IR (KBr) cm^{-1}: 2928, 1723, 1624, 1572, 1270 and 1087. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.80 (s, H-18, 3H), 1.10 (s, H-19, 3H), 2.29 (m, ester-H-1′, 1H), 3.90 (m, *J* = 3.7 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.4 Hz, H-6, 1H), 6.73 (m, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.34 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.40 (C-18), 19.01 (C-19), 30.21 (C1′ of ester), 62.32 (C-21), 73.54 (C-3), 121.91 (C-6), 124.60 (C-Het), 128.83 (C-Het), 140.23 (C-5), 144.00 (C-16), 156.99 (C-Het), 157.00 (C-Het), 175.09 (ester carbonyl), 190.36 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 462.2882 for $C_{29}H_{38}N_2O_3$, found 462.2886.

2.2.1.2.7. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclopentanecarboxylate (8h in Fig. 1).

Yield 0.056 g, (45%), m.p. 91–93 °C, IR (KBr) cm^{-1}: 2928, 1724, 1625, 1574, 1270 and 1087. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.10 (s, H-18, 3H), 1.30 (s, H-19, 3H), 2.31 (m, ester-H-1′, 1H), 3.47 (m, *J* = 3.4 Hz, H-3, 1H), 4.65−4.87 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.33 (d, *J* = 5.4 Hz, H-6, 1H), 6.73 (m, *J* = 6.7 Hz, C-16, 1H), 7.04 (s, H-Het, 1H), 7.32 (s, H-Het, 1H), 7.69 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.01 (C-18), 19.00 (C-19), 30.10 (C1′ of ester), 62.30 (C-21), 73.54 (C-3), 121.91 (C-6), 124. 60 (C-Het), 128.83 (C-Het), 140.15 (C-5), 140.15 (C-5), 144.00 (C-16), 156.99 (C-Het), 157.09 (C-aromatic), 175.09 (ester carbonyl), 199.36 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 476.3039 for $C_{40}H_{40}N_2O_3$, found 476.3035.

2.2.1.2.8. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cyclohexanecarboxylate (8i in Fig. 1).

Yield 0.071 g, (55%), m.p. 193–195 °C, IR (KBr) cm^{-1}: 2929, 1726, 1624, 1591, 1270 and 1080. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.24 (s, H-18, 3H), 1.40 (s, H-19, 3H), 2.30 (m, ester-H-1′, 1H), 3.45 (m, *J* = 3.7 Hz, H-3, 1H), 4.62−4.82 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.4 Hz, H-6, 1H), 6.73 (m, *J* = 6.7 Hz, C-16, 1H), 7.10 (s, H-Het, 1H), 7.35 (s, H-Het, 1H), 7.51 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 20.01 (C-18), 21.00 (C-19), 30.10 (C1′ of ester), 62.21 (C-21), 73.91 (C-3), 121.02 (C-6), 141.21 (C-Het), 141.27 (C-5), 141.45 (C-16), 144.89 (C-Het), 154.41 (C-Het), 157.09 (C-17), 170.66 (ester carbonyl), 200.87 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 490.3195 for $C_{31}H_{42}N_2O_3$, found 490.3199.

2.2.1.2.9. 21-(1*H*-imidazol-1-yl)-20-oxopregna-5,16-dien-3β-yl-cycloheptanecarboxylate (8j in Fig. 1).

Yield 0.071 g, (53%), m.p. 180–182 °C, IR (KBr) cm^{-1}: 2917, 1725, 1626, 1564, 1260 and 1115. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.01 (s, H-18, 3H), 1.20 (s, H-19, 3H), 2.30 (m, ester-H-1′, 1H), 3.49 (m, *J* = 3.4 Hz, H-3, 1H), 4.62−4.82 (ABq, *J*= 16.4 Hz, H-21, 2H), 5.35 (d, *J* = 5.4 Hz, H-6, 1H), 6.75 (m, *J* = 6.7 Hz, C-16, 1H), 7.10 (s, H-Het, 1H), 7.35 (s, H-Het, 1H), 7.51 (s, H-Het, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.40 (C-18), 20.00 (C-19), 30.11 (C1′ of ester), 62.10 (C-21), 73.91 (C-3), 121.02 (C-6), 121.12 (C-Het), 141, 27 (C-5), 144.50 (C-16), 144.52 (C-Het), 154.51 (C-17), 157.09 (C-Het), 170.66 (ester carbonyl), 200.87 (C-20). HRMS: *m/z* $[M+H]^+$ calculated for 504.3352 for $C_{32}H_{44}N_2O_3$, found 504.3358.

2.2. Animal and human tissues

2.2.1. Type 1 5α-R isoenzyme isolated from rat liver

All procedures involving animals were approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM, Xochimilco).

Two adult (8-month old) rats were obtained by the Metropolitan University in Xochimilco from Animal Care Facility, Mexico.

The adult rats weighing 500 g had been fasted overnight to decrease glycogen levels before their livers were extirpated for use as a source of 5α-R1 [22]. To prepare microsomes, the livers (30 g) were minced in one volume of buffer A (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose, 0.1 mM DTT; Sigma-Aldrich, Mexico City, Mexico) with an IKA_A11 basic tissue mill (IKA Laboratory Equipment, Mexico City, Mexico). Unless otherwise specified, the following procedures were carried out at 4 °C.

The tissue was homogenized and the suspension was centrifuged (13,200 rpm; 20 min; 0 °C) (Beckman L70 K ultracentrifuge), and the pellet was discarded. The supernatant was filtered through a nylon mesh filter (pore size 11 lm, distributed by OEM-Membrane Solution, Dallas TX) and centrifuged again (10,000 rpm; 60 min, 4 °C); the microsomal pellet was suspended in five volumes of buffer A with a homogenizer. The protein concentration was measured by the Bradford method [23].

The suspension was re-centrifuged (13,200 rpm; 20 min, 4 °C) and the pellet was suspended in buffer A to give a final concentration of 20 mg protein/mL. The microsomal suspension was stored at −70°C prior to the preparation of the sample steroid.

2.2.2. Type 2 5α-R isoenzyme isolated from human prostate

Four hours after a 53-year-old patient had died from myocardial infarction, his normal prostate was extirpated in the Pathology Department of the Mexico City General Hospital. The Ethical Committee of the General Hospital in Mexico City approved this protocol.

Human prostate was used in this experiment because this tissue is an abundant source of 5α-R2, but not of 5α-R1; the AR study in this model is very complex.

The tissue was rinsed and immediately chilled in ice-cold 150 mM NaCl, and stored at −20 °C. The frozen human prostate was thawed on ice, rinsed, and minced in buffer A (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose, 0.1 mM DTT; Sigma-Aldrich, Mexico City, Mexico) with an IKA® A11 basic tissue mill (IKA Laboratory Equipment, Mexico City, Mexico). Unless otherwise specified, the following procedures were carried out at 4 °C. The human prostate tissue was homogenized in two volumes of buffer A with a tissue homogenizer Ultra-Turrax IKA, T18 basic (Wilmington, NC). The homogenates were centrifuged (1500g; 60 min) [24] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA). The pellets were suspended in buffer A, and stored at −70°C. This suspension had a final concentration of 5 mg protein/mL, as determined by the Bradford method [23] and was used as source of 5α-R2 isozyme.

2.2.3. Rat prostate cytosol as source of AR

In this study we used rats because the prostate gland is bigger than that of the hamster and there is no difference in the binding activity of the AR between rat and hamster cytosol [25].

In order to evaluate the binding of steroids **8a−j** to the androgen receptor, the prostates of 50 adult rats (8 months old; 500 g) were removed, blotted, weighed and soaked

in cold TEMD (40 mM tris–HCl, 3 mM EDTA and 20 mM sodium molybdate, dithiothreitol 0.5 mM, 10% glycerol at pH 8) before use. All procedures were carried out in an ice bath at 0 °C. The tissues were homogenized with a tissue homogenizer (Teckmar, Cincinnati, OH) in one volume of buffer TEMD plus protease inhibitors (2 mM PMSF, 10 lg/mL antipain, 5 mM leupeptin) [26] in an ice bath with a tissue homogenizer; subsequently the homogenates were centrifuged (14,000 rpm, 60 min) [24] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA). The cytosolic fraction obtained from the supernatant liquid of the rat prostate homogenate described above was stored at −70 °C. The protein concentration (6 mg of protein in 200 lL) was determined by the Bradford method.

2.2.4. *In vivo* experiments

For the experiments *in vivo*, adult male golden hamsters (2.5 months old; 150–200 g) were used. After gonadectomies had been performed on the hamsters under isoflurane anesthesia, the castrated hamsters were allowed to recover for 30 days prior to experimentation [25]. The castrated hamsters and a group of eight intact hamsters were housed in a room with controlled temperature (22 °C) and light-dark periods of 12 h; the hamsters were fed with food and water *ad libitum*. Thirty days post gonadectomy, the hamsters were separated into groups consisting of four animals per group. The hamsters were treated for six days, and thereafter sacrificed with CO_2 [24]. This experiment was carried out twice under the same conditions.

2.2.5. Cell line characteristics

The panel of cells used in this study was: PC-3 (human prostate cancer cell line), MCF7 (human breast cancer cell line), and SK-LU-1 (human lung cancer cell lines), supplied by the National Cancer Institute (USA).

2.3. Biological activity of 8a−j compounds

2.3.1. *In vitro* experiments

The effect of steroids **8a−j** (Fig. 1) on the activity of 5α-R1 and 5α-R2 was evaluated by following the conversion of T to DHT, under the same conditions that we described previously [27].

The 50% inhibitory concentration (IC_{50}) of **8a−j** was determined using a range of concentrations (1×10^{-10} to 1×10^{-4} M) of each derivative. The transformation of T to DHT in the presence of **8a−j** was calculated from radioactive compounds present in each sample, observed in the lanes of the chromatographic plate. The data were plotted using SigmaPlot software 12 inhibition curves [24].

2.3.2. *In vivo* experiments

2.3.3.1. Weight of the prostate, seminal vesicles and diameter of the pigmented spot

For six consecutive days, each of the steroid derivatives **8a−j** (2 mg/kg body weight (BW)) dissolved in 200 µL of sesame oil, together with 1 mg/kg (BW) of testosterone, was administered by subcutaneous (SC) injection to a group of gonadectomized hamsters (four animals per derivative). Three groups of gonadectomized animals were kept as control; the first group was injected SC with 200 µL sesame oil, the second group with 1 mg/kg (BW) of testosterone, and the third group with 1 mg T plus 1 mg/kg (BW) of finasteride also

prepared in sesame oil. Additionally, one group of four intact hamsters was used as the intact control. On the seventh day, the animals were sacrificed with CO_2 and the diameters of the flank organs were measured using a Vernier Caliper; the prostate and seminal vesicles of each hamster were dissected and weighed.

Two separate experiments were performed for each group of steroid treated hamsters. The results were analyzed using one-way analysis of variance and Dunnett's method to compare means, using JMP IN 5.1 software (JMP, Statistical Discovery, and Cary, NC, USA).

2.3.3. Competitive studies on AR

The competitive studies of steroids **8a−j** on AR were carried out using the same procedure described by our group previously [28]. The IC_{50} of each compound was calculated from plots of concentration versus percentage of binding using SigmaPlot 12. Mibolerone (MIB) was used as a positive control.

2.3.4. Cytotoxicity assay

Cytotoxic activity of the steroid derivatives (**8a−j**) on three cancer cell lines (PC-3, MCF7, and SK-LU-1) was evaluated. The procedure has been previously reported [29].

Cell growth inhibition was calculated according to the following expression:

$$\text{cell growth inhibition } (\%) = \frac{100 - (\text{sample absorbtion})}{\text{vehicle absorbtion}} \times 100$$

3. Results

3.1. Chemistry

The yield for the formation of **8a** from the reaction involving acetic anhydride and pyridine was 90%, while compounds **8b–j** were obtained in moderate yields of 45–68% using the Steglish esterification method [30].

8b–j were isolated and characterized by ^{1}H NMR, ^{13}C NMR and mass spectrometry. Also, results obtained from the melting point determination of each compound showed a maximum two degree difference, indicating that these steroids were pure.

3.2. Effect of derivatives as inhibitors of the activity of 5α-R isoenzymes

Table 1 shows the IC_{50} values for derivatives **8a−j** required to inhibit 50% of the activity of 5α-reductase isoenzymes.

These results showed that the chain derivative with the smallest ester at C-3 and alicyclic derivatives also present at C-3 of the steroidal skeleton showed greater selectivity as 5α-R2 inhibitors than the other derivatives studied. However their potency to inhibit this enzyme was lower than that of finasteride (IC_{50} = 8.5 nM) in all cases. None of the prepared compounds was able to inhibit the activity of the 5α-R1 enzyme.

3.3. Weight of the prostate, seminal vesicles and diameter of the pigmented spot

After castration, the weight of the hamster prostate and seminal vesicles decreased ($p < 0.05$) compared to the normal weight of the respective glands, as did the diameter of the pigmented spot. Treatment with vehicle did not change these effects. However, treatment with 1 mg/kg of T for 6 days significantly increased ($p < 0.05$) the weight of prostate, seminal vesicles and diameter of the pigmented spot as well as the weight of the prostate and seminal vesicles (Table 2). When T (1 mg/kg) and finasteride were injected

together, the weight of the prostate, seminal vesicles and diameter of the flank organs decreased significantly ($p < 0.05$) (Table 2). This reduction in the weight of these glands and diameter of the pigmented spot was comparable to that produced by finasteride (Table 2).

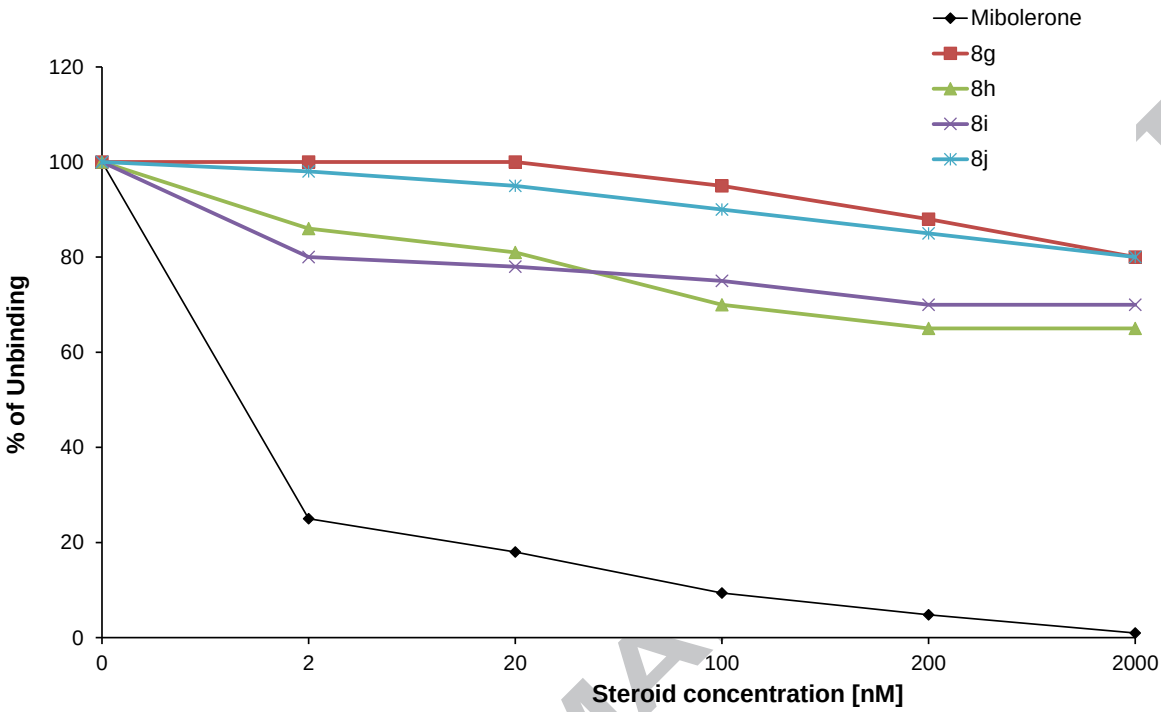
Table 2 shows that when T was administered together with finasteride or steroids **8g–j**, the weight of the prostate and seminal vesicles decreased significantly ($p < 0.05$). In addition, **8g–j** showed a significant ($p < 0.05$) reduction in diameter of the pigmented spot. The remainder of the compounds studied did not display any comparable effect (Table 2).

3.4. Competitive studies on AR

This study showed that compounds **8a−j** did not displace the tritium-labeled MIB from the AR. MIB is a very potent AR agonist and exhibited an IC_{50} value of 1 nM. Figure 2 below shows only the graphs of AR unbinding for compounds **8g–j** to illustrate the behavior of steroids that did not bind to this receptor.

These results show that the steroidal derivatives are not androgenic types and for this reason cannot bind on the active site of the AR.

Figure 2

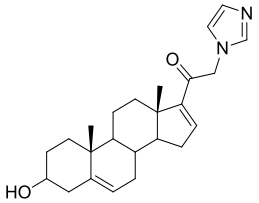
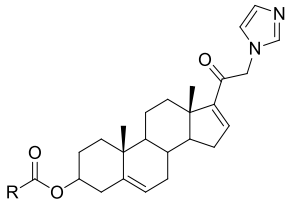


3.5. Cytotoxic activity on cancer cell lines (PC-3, MCF7 and SK-LU-1)

The effect of the synthesized compounds (**8a–j**) on the growth of three cancer cell lines; PC-3 (prostate), MCF7 (breast) and SK-LU-1 (lung), was determined using the SBR assay method. The values of the antiproliferative effect are shown in Table 1. Ketoconazole and 16-dehydropregenolone acetate were used as controls in this study.

Ketoconazole was used as a positive control, because its interesting cytotoxic activity *in vitro* studies has been previously reported [16; 31-34]. Compounds **7** [12,15] and **8a** showed higher cytotoxic activity than 16-dehydropregenolone acetate in the three cancer cell lines (Table 1). Furthermore, **8a** showed higher toxicity than **8b–j** in these three cancer cell lines. This may be because these steroids do not dissolve well in the culture medium containing a dimethyl sulfoxide (DMSO) concentration of 0.05%.This idea is supported by the calculated log P value for each one of these derivatives. (Table 1)

Table 1. Biological activity of novel derivate compounds on growth inhibition in human cancer cell lines at 50 μM, IC_{50} value on 5α-R isoenzymes and AR binding assay.

Structure	Compound	R	Log*P* Calc[a]	PC-3	MCF-7	SKLU-1	5α-R1 IC_{50} nM	5α-R2 IC_{50} μM	RBA AR %
Ketoconazole	–	–	–	88.48±3.9	95.16±4.8	73.39±1.6	–		
Finasteride	–	–	4.00	NA	NA	NA	630	0.0085	NA
16-DHP	**1**	–	4.51	21.4±11.5	25.5±1.2	12.8±3.5	NA	NA	NA
	**7**	–	3.11	96.2±1.3	89.4±2.3	74.8±1.5	NA	118.8	NA
	8a	$-CH_3$	3.82	79.6 ± 8.7	77.9 ± 0.8	96.7 ± 1.3	NA	122	NA
	8b	$-C_2H_5$	4.18	22.6 ± 5.2	0	16.8 ± 1.0	NA	NA	NA
	8c	$-C_3H_7$	4.74	0	0	25.2 ± 0.1	NA	NA	NA
	8d	$-C_4H_9$	5.24	23.7 ± 2.9	51.3 ± 8.3	39.2 ± 8.2	NA	NA	NA
	8e	$-C_5H_{11}$	5.75	17.2 ± 3.6	28.1 ± 4.9	28.5 ± 7.4	NA	NA	NA
	**8f**		4.48	NA	NA	NA	NA	0.711	NA
	8g		4.65	NA	NA	NA	NA	0.0764	NA
	8h		5.39	NA	NA	NA	NA	0.035	NA
	8i		5.90	NA	NA	NA	NA	0.0293	NA
	8j		6.40	NA	NA	NA	NA	NA	NA

Results are expressed as a percentage of inhibition ±standard error. NA: This compound was not active.

[a] The Log*P* property was calculated by Molinspiration (http://www.molinspiration.com/).

Table 2. Weight of prostate, seminal vesicle glands, and diameter of the pigmented spot of the flank organs ± standard deviation from castrated hamsters receiving different treatments for 6 days.

Treatment	Weight of Prostate (mm)±standard error	Weight of Seminal Vesicles (mm)±standard error	Diameter of the Pigmented Spot (mm)±standard error
Control	**50.4 ± 9.9**	**96.11 ± 12.5**	**2.03 ± 0.63**
T	**98 ± 12.6**	**200.6 ± 55**	**5.65 ± 1.12**
T+F	**69.6 ± 21.2**	**161.4 ± 44.5**	**3.57 ± 0.89**
T+8a	**85.80 ± 19.41**	**200.50 ± 32.79**	**3.06 ± 0.08**
T+8b	**88.40±16.10**	**231.70±25.08**	**1.80±0.38**
T+8c	**83.43±8.46**	**217.45±59.28**	**1.98±0.8**
T+8d	**80.93±6.06**	**198.33±22.20**	**2.94±0.99**
T+8e	**71.85±16.06**	**198.33±22.20**	**3.36±0.40**
T+8f	**77.1±17**	**160.9±15.8**	**3.4±0.47**
T+8g	**71.3±9***	**145.2±32.1***	**3.0±0.45***
T+8h	**65.1±15.8***	**143.7±50.66***	**2.0±0.4***
T+8i	**54.3±4.9***	**143.68±50.7***	**2.4±0.3***
T+8j	**70.8±5.3**	**143.6±29.89***	**2.8±0.5***

The control animals were treated with vehicle only.

* Statistically significant values.

4. Discussion

Our research group previously synthesized several steroidal derivatives with an ester moiety at C-3 and several azole derivatives. These steroids demonstrated high antiproliferative activity on different cancer cell lines [28-29] and an inhibitory effect on 5α-reductase type 1 and 2 activity [35].

Recently, we also demonstrated that the presence of a triazole group at C-21 with a linear ester moiety at C-3 on the steroidal skeleton enhanced 5α-R1 inhibitory activity. In addition, compound 20-oxo-21-(1*H*-1,2,4-triazole-1-yl)pregna-5,16-dien-3β-yl-acetate presented a high cytotoxic effect on the SK-LU-1 cancer cell line. Banday et al. similarly identified a series of novel D-ring substituted 1,2,3-triazolyl 20-keto pregnane derivatives as antiproliferative agents on DU-145 and PC-3 cancer cell lines [19].

This report serves as an extension of our earlier study where it was demonstrated that two pregnane derivatives with a triazole or imidazole moiety at C-21, inhibited the three studied cancer cell lines proliferation; [15] particularly the 3β-hydroxy-21- (1H-imidazol-1-yl) pregna-5,16-dien-20one; I-OH (**7**) showed a good PC3 cells antiproliferative effect. However docking studies indicated this steroid is unable to binding to the pocket of the AR, so that this activity cannot explained by this way. In order to understand the action of imidazole derivatives in prostate tissue, here we report the *in vivo* and *in vitro* activity of ten different novel 16-dehydropregnenolone derivatives with an imidazole group at C-21 and a linear or alicyclic ester moiety at C-3.

Although all these novel steroidal derivatives (**8a–j**) have an ester moiety at C-3, they are noteworthy because they show a very different biological activity, allowing other

possible therapeutic applications. Interestingly, only **8a** shows higher activity on PC-3, which is satisfactory as the intent was to obtain specific compounds for each type of cancer.

Furthermore, only derivatives with an alicyclic ester at C-3 demonstrated 5α-R2 inhibitory activity, with compounds **8h** and **8i** showing the highest inhibitory activity on this isoenzyme. The pharmacological activity of the compounds was also demonstrated by a decrease in weight and size of the androgen-dependent glands studied. However, these derivatives with an aliphatic group at C-3 were unable to bind to the androgen receptor as docking studies had predicted, [15] hence the pharmacological effect observed was due to their activity as inhibitors of 5α-R2 activity. This is important because prostatic benign hyperplasia is directly associated with the presence of 5α-R2. [4, 7] In addition, the fact that these compounds were not able to bind the androgen receptor increases their therapeutic value by generating the possibility of only causing minor side effects. The overall data suggests that the size of the ester moiety could be related to the cytotoxic effect of these compounds.

The antiproliferative activity of these compounds can also be attributed to the nature of the azole group [36-37, 10]. This group has a sextet of μ-electrons, giving the azole a higher nucleophilic character [38] that enables formation of a strong hydrogen bond between the steroidal compound and active polar groups in the cells. Because of this, a stronger cytotoxic effect is produced [39].

In conclusion, the results presented here indicate that 16-dehydropregnenolone derivatives were successfully synthesized, and presented an inhibitory effect on 5α-R2, as well as an important pharmacological effect that was non-cytotoxic in the treatment-time. Furthermore, the compounds showed interesting cytotoxic activities on different cancer cell

lines, especially on PC-3, an androgen-independent prostate cancer cell line [40-41], showing therapeutic potential for cancer treatment.

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Conflict of Interest

The authors declare that that are no real or perceived conflicts of interest arising from intellectual, personal, or financial circumstances of the research. Additionally, all authors are aware of, and approve the contents and order of authorship of the manuscript.

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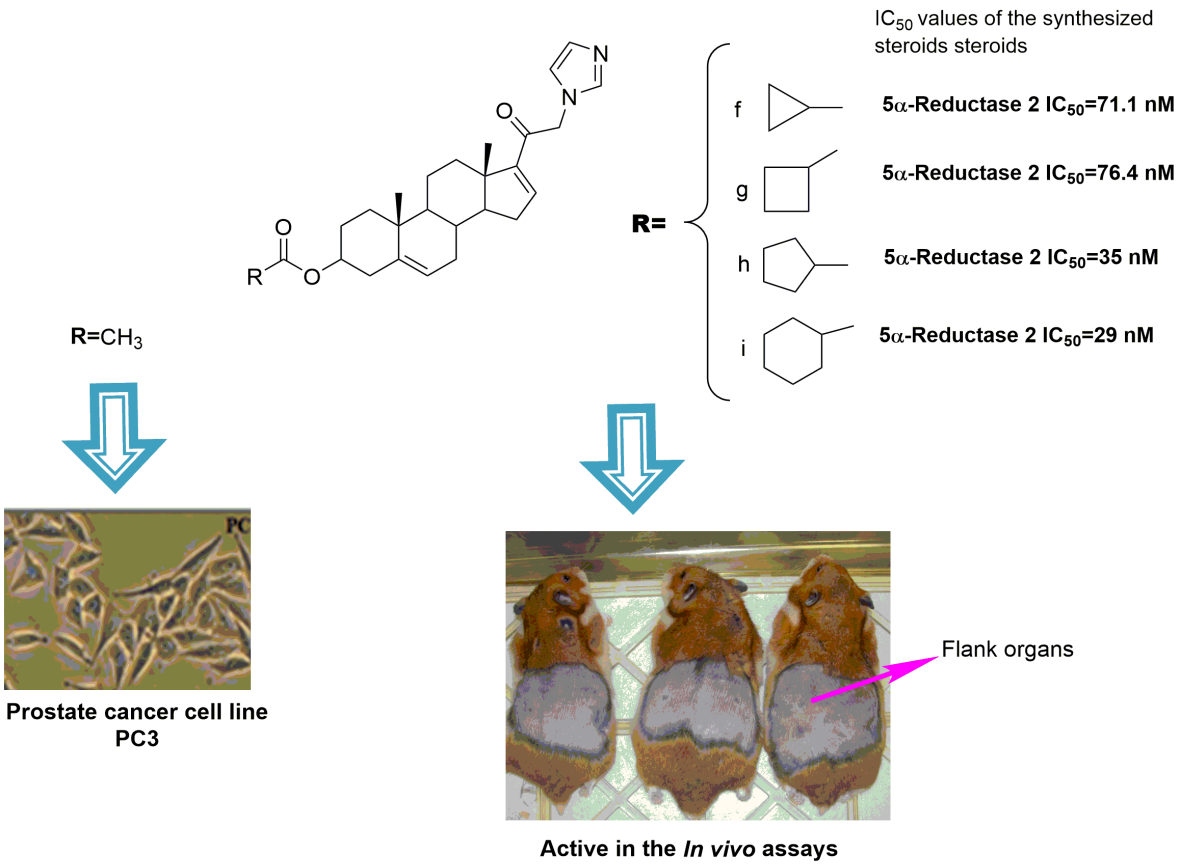
Graphical abstract



GRAPHYCAL ABSTRACT

Highlights

1. Ten novel 21-imidazolyl-16-dehydropregnenolone derivatives were synthesized.
2. These steroids have different ester moiety at C-3 to compare their activity.
3. Steroids with alicyclic ester at C-3 showed 5α-R2 inhibition and *in vivo* activity.
4. Only derivatives with an alicyclic ester at C-3 exhibited anticancer activity.
5. The ester moiety's size matters for enzyme selectivity and anticancer activity.