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Synthesis and biological activity of two pregnane derivatives with a triazole or imidazole ring at C-21

Aylin Viviana Silva-Ortiz[a], Eugene Bratoeff[a,1], María Teresa Ramírez-Apan[c], Rocío García-Becerra[b], David Ordaz-Rosado[b], Nancy Noyola-Martínez[b], Rafael Castillo-Bocanegra[a], David Barrera[b,*]

[a] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Mexico, D.F. 04510, Mexico
[b] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez, Sección XVI, México, D.F. 14080, Mexico
[c] Instituto de Química, Universidad Nacional Autónoma de México, México, D.F. 04510, Mexico

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ABSTRACT

Pregnane derivatives are studied as agents for the treatment of different hormone-dependent diseases. The biological importance of these steroids is based on their potential use against cancer. In this study, we report the synthesis, characterization and biological activity of two pregnane derivatives with a triazole (3β-hydroxy-21-(1*H*-1,2,4-triazol-1-yl)pregna-5,16-dien-20-one; **T–OH**) or imidazole (3β-hydroxy-21-(1*H*-imidazol-1-yl)pregna-5,16-dien-20-one; **I–OH**) moieties at C-21. These derivatives were synthesized from 16-dehydropregnenolone acetate. The activity on cell proliferation of the compounds was measured on three human cancer cells lines: prostate cancer (PC-3), breast cancer (MCF7) and lung cancer (SK-LU-1). The cytotoxic and antiproliferative effects of **T–OH** and **I–OH** were assessed by using SBR and XTT methods, respectively. The gene expressions were evaluated by real time PCR. In addition, results were complemented by docking studies and transactivation assays using an expression vector to progesterone and androgen receptor.

Results show that the two compounds inhibited the three cell lines proliferation in a dose-dependent manner. Compound **I–OH** downregulated the gene expression of the cyclins D1 and E1 in PC-3 and MFC7 cells; however, effect upon Ki-67, EAG1, BIM or survivin genes was not observed. Docking studies show poor interaction with the steroid receptors. Nevertheless, the transactivation assays show a weak antagonist effect of **I–OH** on progesterone receptor but not androgenic or antiandrogenic actions.

In conclusion, the synthesized compounds inhibited cell proliferation as well as genes key to cell cycle of PC-3 and MCF7 cell lines. Therefore, these compounds could be considered a good starting point for the development of novel therapeutic alternatives to treat cancer.

1. Introduction

It is well known that the steroids play a pivotal role in several processes, such as the differentiation, development, growth, as well as physiological and reproductive functions in the human body. Steroid hormones are molecules that share as structural feature a tetracyclic nucleus formed by three ciclohexanes and one cyclopentane. They differ by the oxidation state, the chains and functional groups attached to this four-ring structure which change their chemical functions and biologic specificity [1].

Generally, many of the cellular effects of steroids are mediated via the classical genomic mechanism, where the steroids are taken from circulation to cells and bind to specific receptors, two hormone-receptor complexes dimerize and are translocated from cytoplasm to nucleus to recognize the hormone-response elements and induce changes in the transcriptional expression of target genes [2]. In addition, several non-genomic actions have been described. In this respect, ion channels, membrane-associated steroid receptors, enzyme-linked receptors and cytoplasmic proteins activate intracellular signaling cascades that in turn activate second messengers [3]. In fact, the steroid effects may

Abbreviations: AR, androgen receptor; PR, progesterone receptor; VDR, vitamin D receptor; P_4, progesterone.

* Corresponding author at: Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez, Sección XVI, Tlalpan, 14080 México, D. F., México.
E-mail addresses: barrera1912@gmail.com, vidadav@hotmail.com (D. Barrera).

[1] Deceased.

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occur using one or both mechanisms in a tissue-dependent manner.

Indeed, the literature describes numerous examples of the natural and synthetic compounds used for the treatment of both sex hormone-dependent and independent cancer tumors [4–9]. However, the resistance to therapies impedes the successful treatment. Consequently, the design of compounds with a specific activity for each molecular target should be performed.

In this sense, the replacement of one or more carbon atoms in a steroid molecule by nitrogen atoms affects the chemical properties of the steroid and changes its biological activity. Since 1980′s, azasteroids had received much attention for the development of new compounds that are being used for the treatment of hormone-dependent diseases such as adenocarcinomas, offering an important strategy for tumor control [10]. Actually, many drugs used in the treatment of a number of androgen-independent, androgen-, estrogen-, and other steroid-dependent diseases, have in its structure a heterocyclic group. Some interesting compounds in the pharmaceutical market are for example aminoglutethimide and its derivatives; esters of 4-pyridineacetic acid; bis-chlorophenyl-pyrimidine analogues; as well as imidazole and triazole derivatives, such as ketoconazole, liarozole, fadrozole, CGS 18320 B, vorozole and CGS 20267 [11].

In particular, pharmacological studies have displayed that some pregnane derivatives have a variety of bioactivities, such as cytotoxicity and immunoregulation among others, whereas some synthetic steroids have been previously described as regulators of apoptosis or implicated in cellular proliferation [12–17]. In this study, we synthesized two steroidal derivatives from 16-dehydropregnenolone acetate with triazole (3β-hydroxy-21-(1*H*-1,2,4-triazol-1-yl) pregna-5,16-dien-20-one; **T–OH**) or imidazole (3β-hydroxy-21-(1*H*-imidazol-1-yl) pregna-5,16-dien-20-one; **I–OH**) moieties at C-21 (Fig. 1). The compounds were tested *in vitro* to determine the effect on cell growth, and upon gene expression of proliferative and apoptotic proteins using a panel of human cancer cell lines: PC-3 (prostate), MCF7 (breast) and SK-LU-1 (lung).

In order to rationalize and visualize at the molecular level the important features of these pregnane derivatives and their implication in ligand-protein interaction we used docking tools to calculate the thermochemical parameters between **T–OH** and **I–OH** *versus* progesterone, androgen and vitamin D receptors (PR, AR and VDR, respectively). Additionally, transactivation assays were performed with **I–OH** compound to determine its agonistic or antagonistic actions.

2. Materials and methods

2.1. Reagents

Reagents and solvents were purchased from commercial sources. Melting points (uncorrected) were determined on a Fisher Johns melting point apparatus (Fisher Johns, Mexico City, Mexico). 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 ($\delta = 0$) 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 FAB^+ ionization mode (Thermo Fisher Scientific, San Jose, CA USA).

16-dehydropregnenolone acetate, progesterone (P_4), RU486, 5α-dihydrotestosterone (5α-DHT), flutamide (FLUT) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Ketoconazol was purchased from Sigma Life Science (St. Lois, MO, USA). Cell culture medium (DMEM and RPMI) was obtained from Gibco-Invitrogen. TRIzol and oligonucleotides for real time polymerase chain reaction (qPCR) were from Invitrogen (CA, USA). The probes, capillaries, the TaqMan Master reagents, the Transcriptor First Strand cDNA synthesis kit, the cell proliferation assay (XTT) were purchased from Roche (Roche Applied Science, IN, USA). The ^{3}[H]chloramphenicol (specific activity 38.9Ci/mmol) was purchased from DuPont NEN Research products (Perkin-Elmer, Boston, MA) and radioactivity was determined in a Beckman LS6500 scintillation system (Beckman Instruments, CA) using Biodegradable Counting Scintillant (Amersham, CA) as counting solution.

2.2. Synthesis of 16-Dehydropregnenolone derivates (*T–OH* and *I–OH*)

The synthetic pathways for the preparation of **T–OH** and **I–OH** are outlined in Fig. 2. These compounds were prepared from the commercially available 16-dehydropregnenolone acetate (**1**) as follows:

2.2.1. 16α,17α-Epoxy-3β-hydroxypregn-5-en-20-one (*2*)

A solution of steroid **1** (1 g, 2.82 mmol), sodium hydroxide 4 N (2 mL, 8 mmol) and H_2O_2 30% (4 mL, 135 mmol) in hot methanol (66 mL) was stirred at room temperature for 4 h. After this time the methanol was evaporated and the product was washed with water and air dried to give a white solid.

Yield 0.665 g (72%), m.p. 197–199 °C, IR (KBr) cm^{-1}: 3457, 1692, 1642 and 1042. ^{1}H RMN (400 MHz, $CDCl_3$) δ: 1.00 (s, H-18, 3H), 1.03 (s, H-19, 3H), 2.01 (s, H-21, 3H), 3.47 (m, $J = 3.4$ Hz, H-3, 1H), 3.50 (s, OH, 1H), 3.66 (s, H-16, 1H), 5.32 (t, $J = 5.3$ Hz, H-6, 1H). 13C RMN (100 MHz, $CDCl_3$) δ: 15.38 (C-18), 19.30 (C-19), 27.51 (C-21), 60.55 (C-16), 71.16 (C-17), 71.75 (C-3), 121.13 (C-6), 141.27 (C-5), 205.07 (C-20). HRMS cal. for $C_{21}H_{30}O_3$ 330.2195, found 330.2187.

2.2.2. 3β-(Tetrahydro-2H-pyran-2-yloxy)-16α,17α-epoxypregn-5-en-20-one (*3*)

A solution of steroid **2** (1 g, 2.42 mmol), 3,4-dihydro-2*H*-pyran (30 mL) and *p*-toluensulfonic acid monohydrate (0.100 mg, 0.058 mmol) in dichloromethane (40 mL), was stirred at room

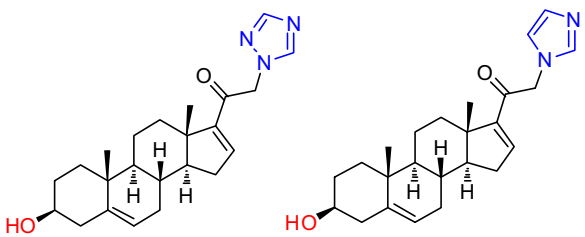

Fig. 1. Molecular structures of **T–OH** and **I–OH**.

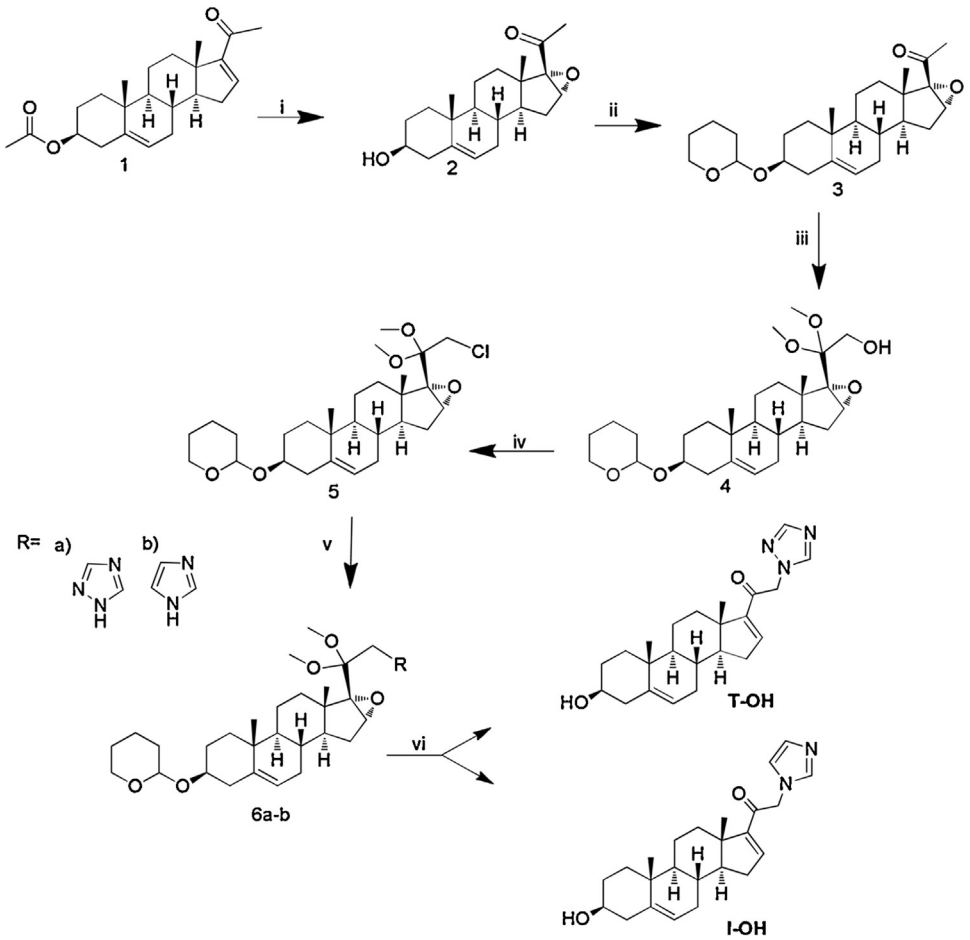

Fig. 2. Synthesis of two 16-dehydropregnenolone acetate derivates.

temperature for 1 h. Then, the solvent was removed with the rotaevaporator and the product was recrystallized from methanol.

Yield 0.88 g (71%), m.p. 114–116 °C, IR (KBr) cm^{-1}: 2935, 1703, 1438 and 1024. ^1H RMN (400 MHz, $CDCl_3$) δ: 1.00 (s, H-19, 3H), 1.02 (s, H-19, 3H), 2.01 (s, H-21, 3H), 3.47 (m, *J* = 3.4 Hz, H-3, 1H), 3.66 (s, H-16, 1H), 3.89 (d, H-5′ of protector group, 2H), 4.69 (t, H-1′ of protector group, 1H), 5.31 (1H, d, *J* = 5.3 Hz, H-6). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.37 (C-18), 19.28 (C-19), 27.65 (C-21), 60.63 (C-5 of protector group), 62,96 (C-16), 75,99 (C-17), 76.06 (C-3), 120.92 (C-6), 141.38 (C-5), 205.06 (C-20). HRMS cal. for $C_{26}H_{38}O_4$ 414.2770, found 414.2774.

*2.2.3. 20,20-Dimethoxy-3β-(tetrahydro-2H-pyran-2-yloxy)-16α,17α-epoxypregn-5-en-21-ol (**4**)*

A solution of steroid **3** (1 g, 2.11 mmol), (diacetoxyiodo) benzene (1.2 g, 3.72 mmol) and sodium hydroxide (1 g, 25 mmol) in methanol (21 mL) and dichloromethane (10 mL) was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure, and the residue was purified by a column chromatography packed with basic aluminum oxide eluted with a mixture of hexane and acetone (95:5).

Yield 0.751 g (65%), m.p. 139–141 °C, IR (KBr) cm^{-1}: 3504, 2937, 1083 and 1030. ^1H RMN (400 MHz, $CDCl_3$) δ: 1.00 (s, H-18, 3H), 1.24 (s, H-19, 3H), 3.25 (s, OCH_3, 6H), 3.43 (m, *J* = 3.4, H-3, 1H), 3.44 (s, OH, 1H), 3.48 (s, H-21, 2H), 3.67 (s, H-16, 1H), 3.89 (d, H-5′ of protector group, 2H), 4.69 (t, H-1′ of protector group, 1H), 5.32 (d, *J* = 5.3 Hz, H-6, 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.27 (C-18), 19.41 (C-19), (CH_3 of protector group), 50.49 (OCH_3), 60.63 (C-5′ of protector group), 62.69 (C-16), 63.02 (C-21), 76.08 (C-3), 76.02 (C-17), 97.13 (C-20), 101.95 (C-1′ of protector group), 121.22 (C-6), 141.29 (C-5). HRMS cal. for $C_{28}H_{44}O_6$ 476.3138, found 476.3134.

*2.2.4. 21-Choloro-20,20-dimethoxy-3β-(tetrahydro-2H-pyran-2-yloxy)-16α,17α-epoxypregn-5-ene (**5**)*

To a cold solution of steroid **4** (0.5 g, 1.01 mmol) in a mixture of dry dichloromethane (4 mL) and pyridine (0.5 mL) was added dropwise thionyl chloride (0.15 mL) under a nitrogen atmosphere, and the mixture was stirred at room temperature for 20 min. Chloroform (50 mL) was added to stop the reaction and then it was evaporated in vacuum. The crude product was recrystallized from methanol.

Yield 0.418 g (80%), m.p. 101–103 °C, IR (KBr) cm^{-1}: 2932, 1670, 1065 and 1039. ^1H RMN (400 MHz, $CDCl_3$) δ: 1.02 (s, H-18, 3H), 1.23 (s, H-19, 3H), 3.25 (s, OCH_3, 6H), 3.43 (m, *J* = 3.4, H-3, 1H), 3.48 (s, H-21, 2H), 3.67 (s, H-16, 1H), 3.89 (d, H-5′ of protector group, 2H), 4.69 (t, H-1′ of protector group, 1H), 5.32 (d, *J* = 5.3 Hz, H-6, 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.26 (C-18), 19.50 (C-19), 50.45 (OCH_3), 59.51 (C-21), 68.30 (C-16), 76.08 (C-3), 60.63 (C-5′ of protector group), 97.13 (C-20), 101.95 (C-1′ of protector group), 121.22 (C-6), 141.29 (C-5). HRMS cal. for $C_{28}H_{43}ClO_5$ 494.2799, found 494.2793.

*2.2.5. 20,20-Dimethoxy-16α,17α-epoxy-3β-(tetrahydro-2H-pyran-2-yloxy)-21-(1H-1,2,4-triazol-1-yl) pregn-5-ene (**6a**)*

A mixture of 1,2,4-triazole (0.031 g, 0.443 mmol), K_2CO_3 (0.123 g, 0.89 mmol) and compound **5** (0.1 g, 0.202 mmol) was dissolved in dry DMF (5 mL) and the solution was heated at 90 °C under nitrogen for 3 h. Cold water was added and the resulting

solid was filtered. The compound was purified by a column chromatography with florisil.

Yield 0.078 g (73%), m.p. 124–125 °C, IR (KBr) cm^{-1}: 2934, 1379, 1275 and 1026. ^1H RMN (400 MHz, $CDCl_3$) δ: 0.87 (s, H-18, 3H), 0.98 (s, H-19, 3H), 3.28 (s, OCH_3, 6H), 3.46 (m, *J* = 3.4, H-3, 1H), 3.48 (s, H-21, 2H), 3.68 (s, H-16, 1H), 3.90 (d, H-5′ of protector group, 2H), 4.70 (t, H-1′ of protector group, 1H), 5.32 (d, *J* = 5.3 Hz, H-6, 1H), 7.98 (s, H-Het., 1H), 8.27 (s, H-Het., 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.20 (C-18), 19.43 (C-19), 49.66 (C-16), 50.47 (OCH_3), 58.52 (C-21), 62.68 (C-5′ of protector group), 76.04 (C-3), 97.13 (C-20), 101.93 (C-1′ of protector group), 121.16 (C-6), 121.23 (C-Het.), 141.31 (C-5), 141.48 (C-Het.). HRMS cal. for $C_{30}H_{45}N_3O_5$ 527.3359, found 527.3355.

*2.2.6. 3β-Hydroxy-21-(1H-1,2,4-triazol-1-yl) pregna-5,16-dien-20-one (**T–OH**)*

Compound **6a** (0.1 g, 0.19 mmol) was hydrolyzed and the epoxide group at C-16 was eliminated at the same time with a reaction carried out in acetic acid (4 mL), chromous chloride (0.08 g, 0.65 mmol) and two drops of 36% hydrochloric acid at room temperature for 20 min the mixture was diluted with cold water (150 mL) and the precipitate was filtered and dried. The product was purified by silica gel column chromatography using a mixture of hexane and acetone (9:1).

Yield 0.370 g (77%), m.p. 120–122 °C, IR (KBr) cm^{-1}: 3355, 2927, 1709, 1670, 1276 and 1051. ^1H RMN ($CDCl_3$) δ: 0.90 (s, H-18, 3H), 1.20 (s, H-19, 3H), 3.25 (m, *J* = 3.5 Hz, H-3, 1H), 3.52 (s, OH, 1H), 4.18 (s, 2H, H-21), 5.34 (d, *J* = 5.3 Hz, H-6, 1H), 6.74 (s, H-16, 1H), 7.50 (s, H-Het., 1H), 7.70 (s, H-Het., 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.32 (C-18), 20.01 (C-19), 62.15 (C-21), 71.78 (C-3), 121.04 (C-6), 141.23 (C-5), 141.38 (C-16), 141.47 (C-Het.), 144.83 (C-Het.), 196.66 (C-20). HRMS cal. for $C_{23}H_{31}N_3O_2$ 381.2416, found 381.2409.

*2.2.7. 21-(1H-Imidazol-1-yl)-20,20-dimethoxy-16α,17α-epoxy-3β-(tetrahydro-2H-pyran-2-yl)-pregn-5-ene (**6b**)*

This compound was obtained by the procedure described in the section 2.2.5 using 1*H*-imidazole (0.04 g, 0.61 mmol) and Cs_2CO_3 (0.40 g, 1.21 mmol).

Yield 0.083 g (78%), m.p. 90–92 °C, IR (KBr) cm^{-1}: 2935, 1377, 1258 and 1028. ^1H RMN (400 MHz, $CDCl_3$) δ: 1.00 (s, H-18, 3H), 1.28 (s, H-19, 3H), 3.28 (s, OCH_3, 6H), 3.44 (m, *J* = 3.4, H-3, 1H), 3.67 (s, H-16, 1H), 3.90 (d, H-5′ of protector group, 2H), 4.04 (s, H-21, 2H), 4.71 (t, H-1′ of protector group, 1H), 5.35 (d, *J* = 5.3 Hz, H-6, 1H), 7.12 (d, H-Het., 1H), 7.71 (d, H-Het., 1H), 8.27 (s, H-Het., 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.30 (C-18), 19.90 (C-19), 49.52 (C-16), 50.00 (OCH_3), 55.5 (C-21), 63.02 (C-5′ of protector group), 76.10 (C-3), 97.09 (C-17), 101.97 (C-1′ of protector group), 115.01 (C-20), 121.23 (C-6), 141.29 (C-Het.), 141.31 (C-5), 141.89 (C-Het.), 151.55 (C-Het.). HRMS cal. for $C_{31}H_{46}N_2O_5$ 526.3407, found 526.3411.

*2.2.8. 3β-Hydroxy-21-(1H-imidazol-1-yl) pregna-5,16-dien-20-one (**I–OH**)*

The procedure to prepare this compound is the same as that previously described in section 2.2.6.

Yield 0.053 g (73%), m.p. 179–181 °C, IR (KBr) cm^{-1}: 3334, 2930, 1707, 1668, 1277 and 1051. ^1H RMN ($CDCl_3$) δ: 0.95(s, H-18, 3H), 1.30 (s, H-19, 3H), 3.52 (m, *J* = 3.5 Hz, H-3, 1H), 3.66 (s, OH, 1H), 4.25 (s, 2H, H-21), 5.32 (d, *J* = 5.3 Hz, H-6, 1H), 6.74 (s, H-16, 1H), 7.04 (d, H-Het., 1H), 7.35 (d, H-Het., 1H), 7.78 (s, H-Het., 1H). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 15.30 (C-18), 19.9 (C-19), 61.31 (C-21), 71.69 (C-3), 121.01 (C-6), 141.21 (C-5), 144.30 (C-16), 141.24 (C-Het.), 144.89 (C-Het.), 151.55 (C-Het.), 154.40 (C-17), 206–09 (C-20). HRMS cal. for $C_{24}H_{32}N_2O_2$ 380.2464, found 380.2470.

Reagents and conditions: (***i***) H_2O_2, NaOH 4N, 4 h; (***ii***) DHP, *p*-toluensulfonic acid, 2 h; (***iii***) NaOH, $C_6H_5I(OAc)_2$, MeOH, 3 h; (***iv***) $SOCl_2$, Py, CH_2Cl_2, 20 min; (*v*) *a*) 1,2,4-triazole, K_2CO_3, 80 °C, 5 h; *b*) 1*H*-imidazole, K_2CO_3, 80 °C, 5 h; (***vi***) HCl, $CrCl_2$, CH_3COOH, 20 min.

2.3. Cell culture

PC-3, MCF7, SK-LU-1 and HeLa human cell lines were supplied by National Cancer Institute (NCI), USA. These lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2-95% air. Experimental procedures were performed with different supplemented medium as described in each section.

2.4. Cell cytotoxic assay with SRB

The cytotoxicity of 16-dehydropregnenolone acetate and **I–OH** was determined using the protein-binding dye SRB in microculture assay at a single concentration for the studied compounds and compared with Ketoconazole and **T–OH** previously reported [16].

The compounds were screened *in vitro* on PC-3, MCF7 and SK-LU-1, these cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B (Gibco®), and 1% of nonessential amino acids (Gibco®). The viability of these cells exceeds 95% as determined with trypan blue.

Cells were removed from the tissue culture flask and diluted with fresh media. From this cell suspension, 100 μL containing 5000 cells per well, were seeded in 96-wells tissue culture plates (Costar) and the material was incubated at 37 °C for 24 h. Furthermore, another plate was prepared with culture medium only and it was incubated for 1 h. Next, 100 μL of a solution of the compounds obtained by diluting the stocks were added to each well.

Cells were exposed for 48 h to the compounds at concentration of 50 μM. Then, the cells were fixed by the addition of cold 50% aqueous trichloroacetic acid and the plates were incubated at 4 °C for 1 h, washed with tap H_2O and air-dried. The trichloroacetic-acid-fixed cells were stained by the addition of 0.4% SRB as described [15]. Free SRB solution was removed by washing with 1% aqueous acetic acid. The samples were air dried, and the bound dye was solubilized with 100 μL (10 mM) of unbuffered Tris base. The plates were placed on a vortex for 5 min, and the absorption was determined at 515 nm using an ELISA plates reader (Bio-Tek, Winooski, VT, USA). Cell growth inhibition was calculated according to the following expression:

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

Ketoconazole was used as control for this assay. This compound is an antimycotic drug that has previously been described as an agent that inhibits cytochrome P450 enzymes and has shown cytotoxic activity *in vitro* studies [18–20].

2.5. Cell proliferation assay with XTT method

The PC-3 cells were incubated in RPMI 1640, MCF7 in DMEM-HG and the SK-LU-1 in DMEM-F12 medium, all supplemented and with phenol red. The proliferation was determined using the colorimetric XTT assay Kit (Roche®). The **T–OH** and **I–OH** proliferative effects were evaluated at different concentrations and its inhibitory concentrations 20% and 50% (IC_{20} and IC_{50}) were determined.

For this assay, 1000 cells were seeded in 96-well tissue culture plates by sextuplicate. After 24 h, culture medium was removed and cells were incubated with **T–OH** or **I–OH** at different concentrations (1×10^{-10}–1×10^{-4} M). Ethanol (0.1% v/v) was used as vehicle (V). The cells were incubated at 37 °C for 4 h (control plate) or 4 days (evaluation plates) using the colorimetric XTT assay according to manufacturer's instructions. The absorbance at 492 nm was determined in a microplate reader (BioTek, Winooski,

VT, USA). The IC_{20} and IC_{50} values were calculated by means of a scientific graphing software (Origin 5, OriginLab Corporation, Northampton MA) using a non-linear regression analysis with sigmoidal fitting based in a dose-response curve as described [21].

2.6. Gene expression analysis by qPCR

The cancer cells were incubated in the presence of different **I–OH** concentrations (1×10^{-9}–1×10^{-4} M) or its vehicle.

Afterwards, medium was aspired and total RNA was extracted using Trizol reagent and 1 μg was reverse transcribed using the transcriptor RT system (Roche, Germany).

The qPCR was carried out using the LightCycler Taqman Master System and the LightCycler 2.0 Instrument from Roche (Roche Diagnostics, Mannheim, Germany). The following protocol was used: Taq DNA polymerase activation and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles, each cycle consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C.

The genes studied related with cell proliferation were cyclin D1 (CCND1), cyclin E1 (CCNE1), Ki-67 and the potassium channel Ether-à-go-go (EAG1), while for apoptosis were BIM and survivin. Additionally, in order to establish a possible binding of the compounds to the VDR, the expression of CYP24A1 gene was evaluated with different concentrations of **I–OH** (1×10^{-9}–1×10^{-6} M), in absence and presence of calcitriol (1×10^{-8} M). Expression levels were calculated after normalization to the housekeeping gene β-actin (internal control). Probes and primers were designed with the Universal Probe Library Assay Design Center from Roche, and they are reported in Table 1.

Data obtained from qPCR were analyzed using the statistical software Sigma Plot 12.0. The representative differences for dose-response were determined by One-Way ANOVA followed by appropriate post-hoc test (Holm-Sidak method for pair-wise comparisons). Data were expressed as the mean ± standard deviation (S.D.) and the differences were considered statistically significant at $P \leq 0.01$.

2.7. Plasmid and transfection in HeLa cell line

HeLa cells were plated the day before transfections, at 6 well plates in DMEM-HG medium without red phenol, supplemented with 5% stripped FBS, 100 U/mL penicillin and 100 μg/mL streptomycin [22]. Transfections were performed in triplicate using PolyFect™ (QIAGEN Inc., Valencia, CA) following the protocol provided by the manufacturer. The cells were co-transfected with 1.0 μg of the expression vector pLEN-hPRB or pSVhAR.BHEXE containing the coding sequence of the PR-B and AR, respectively; and 0.5 μg of reporter plasmid PRE-E1b-CAT which contains an oligonucleotide with a progesterone/androgen response element upstream of the adenovirus E1bTATA box fused to the chloramphenicol acetyltransferase (CAT) gene [23]. Twenty-four hours later, the cells were treated with different concentrations of **I–OH** alone, or in combination with P_4 and 5α-DHT, or the antagonists RU486 and FLUT. The plates were incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Ethanol was used as vehicle. The CAT activity was determined using 5 μg of protein, 10 μg of butyryl coenzyme-A (Sigma), 2×10^5 cpm of xylene-extracted [^{3}H] chloramphenicol in 0.25 M Tris–HCl, pH 8.0. The reporter plasmid and expression vectors used to transfect HeLa cells were kindly provided by Dr A. J. Cooney (Baylor College of Medicine).

2.8. Docking studies

To characterize the molecular interactions between the receptors and the synthetized compounds we performed a docking study. Considering the structural origin of these compounds, the progesterone receptor (PR: NR3C3) and androgen receptor (AR: NR3C4) were used as templates for the docking analysis. Additionally, vitamin D receptor (VDR: NR1I1) was evaluated.

The X-ray crystal structure of PR, AR and VDR of Petit-Topin, He and Moras complexes were used, these were obtained from Protein Data Bank (PDB) codes: 3D90, 1XQ3 and 1S19 respectively. PR (A/B chains) was co-crystalized with 13-β-ethyl-17-alpha-ethynyl-17β-hydroxygon-4-en-3-one [24], AR was co-crystalized with (17β)-17-hydroxy-17-methylestra-4,9,11-trien-3-one [25], and the VDR was co-crystalized with calcipotriol at the active site [26].

The geometry optimizations of these compounds were submitted by Molecular Mechanics using the MMFF94x force-field as implemented in the Spartan 10 program (Wave-function Inc. Irvine, CA), this optimized the structure conformers and the minimum energy of each compound were filtered. The torsional root and branches of the ligands were chosen utilizing MGLTools 1.5.4. Docking calculations were performed with AutoDock 4.2 [27] and Glide 5.7 softwares [28]. To perform Glide docking the ligands and the receptors were prepared with Maestro v9.8. [29].

A three-dimensional grid box of size 70Å × 70Å × 70 Å, with 0.375 Å spacing and centered at the amino acid GLN725 in PR, GLN711 in the AR and SER278 in VDR was performed to establish the possible interaction of the compounds with each receptor.

The parameters used in the docking study were: number of GA runs (25), the population (150), the energy evaluations (2,500,000), and the maximum number of top individuals that automatically survive (27,000). The best binding mode of each molecule was selected based on the lowest free binding energy. All molecular graphics figures were prepared with PyMOL [30].

3. Results

3.1. Chemistry

The synthetic pathways for the preparation of **T–OH** and **I–OH** are outline in Fig. 2. Commercially available 16-dehydropregnenolone acetate (**1**) was treated with hydrogen peroxide and sodium hydroxide 4 N to form the 16β,17β-epoxy compound **2** with a 71% yield. The hydroxyl group at C-3 was protected with 3,4-dihydro-

Table 1
Probes and primers used to the qPCR.

Gen/accession number	L-primer	R-primer	Probe number[a]
CCND1/NM_053056.2	gaagatcgtcgccacctg	gacctcctcctcgcscttct	67
CCNE1/NM_001238.1	ggccaaaatcgacaggac	gggtctgcacagactgcat	36
Ki-67/X65550.1	ggtgtgcagaaaatccaaaga	actgtccctatgacttctggttg	73
EAG1/AF078741.1	cctggaggtgatccaagatg	ccaaacacgtctccttttcc	49
BIM/AY305716.1	gctgtggaggctgaatcc	tcggctgcttggtaattattc	63
Survivin/AB154416.1	gcccagtgtttcttctgctt	aaccggacgaatgcttttta	11
CYP24A1/NM_000782.3	catcatggccatcaaaacaa	gcagctcgactggagtgac	88
β-actin/NM_001101.3	ccaaccgcgagaagatga	ccagaggcgtacagggatag	64

[a] From the universal probe library (Roche).

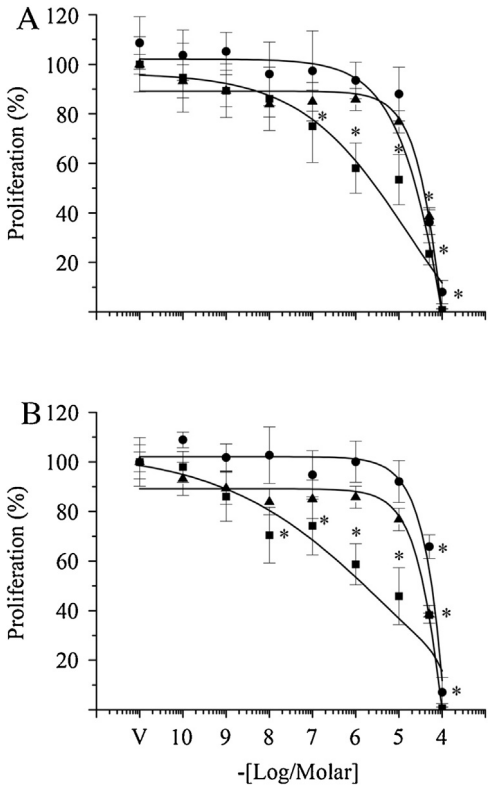

Fig. 3. Effects of **T–OH** and **I–OH** on proliferation of three different cancer cells lines. PC-3 (■), MCF7 (●) and SK-LU-1 (▲) were incubated with different concentrations of **T–OH** (A) and **I–OH** (B) during 4 days. Cell growth assays by the XTT colorimetric method were performed. Results are the mean ± S.D. of sextuplicate and represented at least three different experiments. The vehicle (V) data were normalized to 100% for each experiment. $^{*}P \leq 0.01$ *vs.* V.

2*H*-pyran and *p*-toluenesulfonic acid monohydrate in dichloromethane to provide compound **3** with a 65% yield.

The oxidation of **3** with (diacetoxyiodo) benzene and sodium hydroxide in methanol generated the alcohol in C-21 thus affording compound **4** with a regular yield of 65%.

Treatment of **4** with thionyl chloride gave chlorinated compound **5** (80%)**.** Similarly, it has been reported others methods to perform this same reaction [16,31].

Compound **6a** with a triazole ring at C-21, and **6b**, with an imidazole ring at C-21, were prepared in 73% and 78% respectively from **5** potassium carbonate or cesium carbonate and 1,2,4-trizole or imidazole, respectively in DMF 80 °C for 5 h. The 16β,17β-epoxy elimination and C-3 hydrolysis were carried out with chromium chloride (II), hydrochloric acid and acetic acid affording **T-OH** and **I-OH** with 77% and 73% yield, respectively.

All synthesized compounds were isolated and the melting points showed a maximum of two degree difference. The desired compounds and intermediates were characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry.

3.2. Cytotoxic and metabolic effect

In this study, using SRB method was determined the cytotoxic effect of 16-dehypregnenolone acetate and **I–OH** at 50 μM concentration and compared with Ketoconazole and **T–OH** previously reported [16]. Similarly to Ketoconazole, **I–OH** showed a high cytotoxic activity in the three cancer cells lines: PC-3, MCF7 and SK-LU-1 compared with the precursor that had a weak effect. Likewise, **I–OH** was more effective than **T–OH** in PC-3 and MCF7 cells (data not shown).

Interestingly, using XTT method the compounds inhibited in a concentration-dependent manner the cell cancer growth (Fig. 3). As can be seen in Table 2, the IC_{20} and IC_{50} were calculated and the order to inhibition by **T–OH** and **I–OH** was: PC-3 > MCF7 > SK-LU-1. Nevertheless, PC-3 cells were more sensitive to **I–OH** antiproliferative effect compared with **T–OH**, whereas in MCF7 and SKLU-1 cell lines, an equipotential effect was observed.

3.3. Effects of *I–OH* on different genes involved in proliferation or apoptosis

Since **I–OH** had better antiproliferative effect than **T–OH** gene expression studies were performed with **I–OH** for CCND1, CCNE1, Ki-67, EAG1, BIM, and survivin on three cell lines.

As depicted in Fig. 4, the gene expression of CCND1 and CCNE1 decreased in a concentration-dependent manner both in PC-3 and MCF7 line cells whereas Ki-67, EAG1, BIM and survivin gene expression was not affected significantly by **I–OH** (data not shown). In SK-LU-1 cell line a significant response on these genes was not observed (data not shown).

3.4. Molecular docking

The biological importance of pregnane derivatives lies on their structural similarities with P_4, and, at the same time, with some androgen derivatives, giving them progestogenic and androgenic

Table 2
Inhibitory concentrations (IC_{20} and IC_{50}) values of **T–OH** and **I–OH** on a panel cancer cell lines studied.

Compound	IC	PC-3 (mol/L)	MCF7 (mol/L)	SK-LU-1 (mol/L)
T–OH	20	3.1×10^{-7}	4.1×10^{-5}	1.1×10^{-4}
	50	1.7×10^{-5}	3.6×10^{-4}	2.3×10^{-4}
I–OH	20	4.3×10^{-9}	4.1×10^{-5}	1.3×10^{-4}
	50	2.0×10^{-6}	1.9×10^{-5}	1.8×10^{-4}

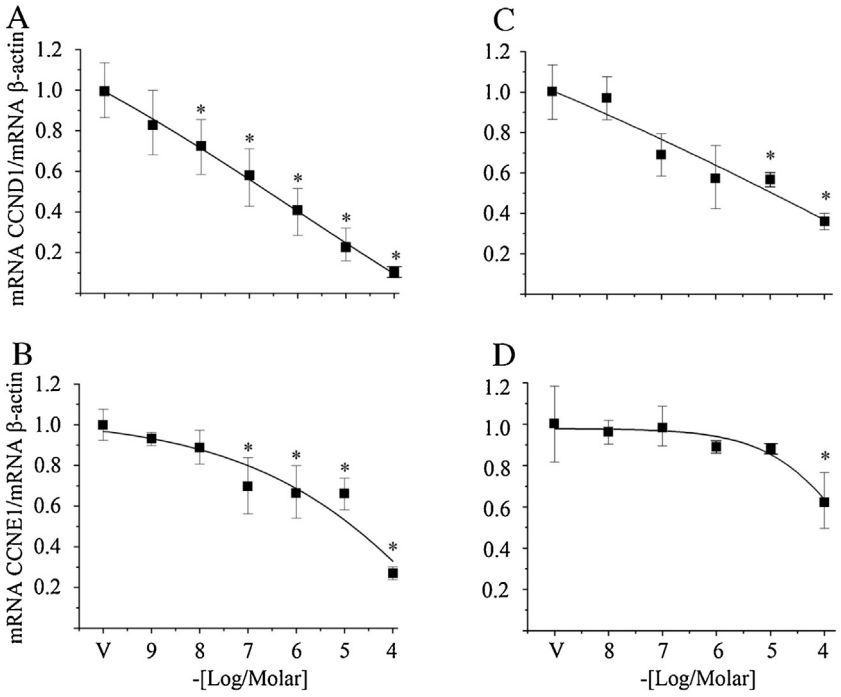

Fig. 4. Regulation of CCND1 and CCNE1 gene expression by **I–OH**. The PC-3 (A, B) and MCF7 (C, D) cells were incubated with different concentrations of **I–OH**. The mRNA was extracted and the qPCR was performed. Data are expressed as mean ± S.D. of three independent experiments per triplicate. Values were normalized by β-actin mRNA used as housekeeping gene. Data were normalized to 1 for vehicle-treated cells (V). $^{*}P \leq 0.01$ *vs.* V.

properties. In order to rationalize and visualize at molecular level the importance of the structural features of **T–OH** and **I–OH** and their implication in ligand-protein interaction, both compounds were docked into PR, AR and VDR model. The VDR was included because the synthesized compounds in this work also share a geometry, hydrogen bonding, binding free energy and structural chemical similitudes with the calcitriol (1,25-dihydroxyvitamin D_3), the hormonally active form of vitamin D [32].

In Table 3 is presented the lowest binding energy and cluster size conformation using AutoDock and Glide Score of the compounds and the natural ligands of each receptor.

As expected, the binding energies for P_4, 5α-DHT and calcitriol showed highly affinity for their specific receptors as previously was reported [33,34] but not for the others compounds. Likewise, the Glide was a more effective tool to visualize and compare the binding energies between the natural ligands with its receptor.

Generally, **T–OH** and **I–OH** into PR, AR and VDR had similar energy but minor clusters size compared with P_4, 5α-DHT and calcitriol, respectively. The Fig. 5 shows the proposed binding models for these binding energies. The results suggested that the compounds induce conformational changes on PR and these modifications possibly block P_4 interactions with the GLN725 amino acid in the B chain [25]. On the other hand, none compound binds suitably in the binding pocket of the AR.

Interestingly, the analyses using VDR demonstrated that both compounds were docked into the VDR ligand binding domain, with similar interactions that the calcitriol. The interactions observed are based in hydrogen bonds with TYR 143, HIS 305 and HIS 397 residues. Strong interactions are considered those with less than 4 angström (Å) between atoms, suggesting an interaction with the VDR in live systems.

3.5. Transfection studies on HeLa cell line

To address the question as to whether **I–OH** elicits agonistic or antagonistic actions comparable to those previously observed for P_4 and 5α-DHT or RU486 and FLUT respectively, we used *in vitro* reporter gene assays. In general, results showed that **I–OH** did not stimulate reporter gene transcription, which discarded the progestagenic (Fig. 6A) and androgenic activity by **I–OH** (data not shown). However, **I–OH** partially inhibited the P_4 effect on the transcriptional activity indicated an antiprogestagenic effect (Fig. 6B) whereas no effect of **I–OH** was observed upon reporter gene activation induced by 5α-DHT (data not shown). These results indicated that **I–OH** did not act as antiandrogenic compound.

Unfortunately, we do not have VDR plasmid, therefore gene CYP24A1 expression was evaluated which is a gene highly induced

Table 3
Binding energies and cluster size of **T–OH** and **I–OH** in the PR, AR and VDR model.

Compound	PR			AR			VDR		
	Glide score ΔG_{bind} (Kcal/mol)	AutoDock ΔG_{bind} (Kcal/mol)	Cluster size	Glide score ΔG_{bind} (Kcal/mol)	AutoDock ΔG_{bind} (Kcal/mol)	Cluster size	Glide score ΔG_{bind} (Kcal/mol)	AutoDock ΔG_{bind} (Kcal/mol)	Cluster size
P_4	−12.2	−9.42[a]	17	−5.8	−6.94[a]	5	−8.1	−9.28[a]	11
5α-DHT	−5.8	−6.86[a]	4	−10.8	−8.99[a]	21	−7.5	−8.68[a]	11
Calcitriol	−5.2	−6.25[a]	3	−5.1	−6.21[a]	3	−13.3	−9.91[a]	17
T–OH	−7.3	−8.45	7	−5.4	−6.37[a]	9	−9.8	−10.63[a]	8
I–OH	−7.4	−7.47	6	−7.4	−8.23[a]	8	−8.5	−9.61[a]	7

[a] Lowest energy conformation for each compound.

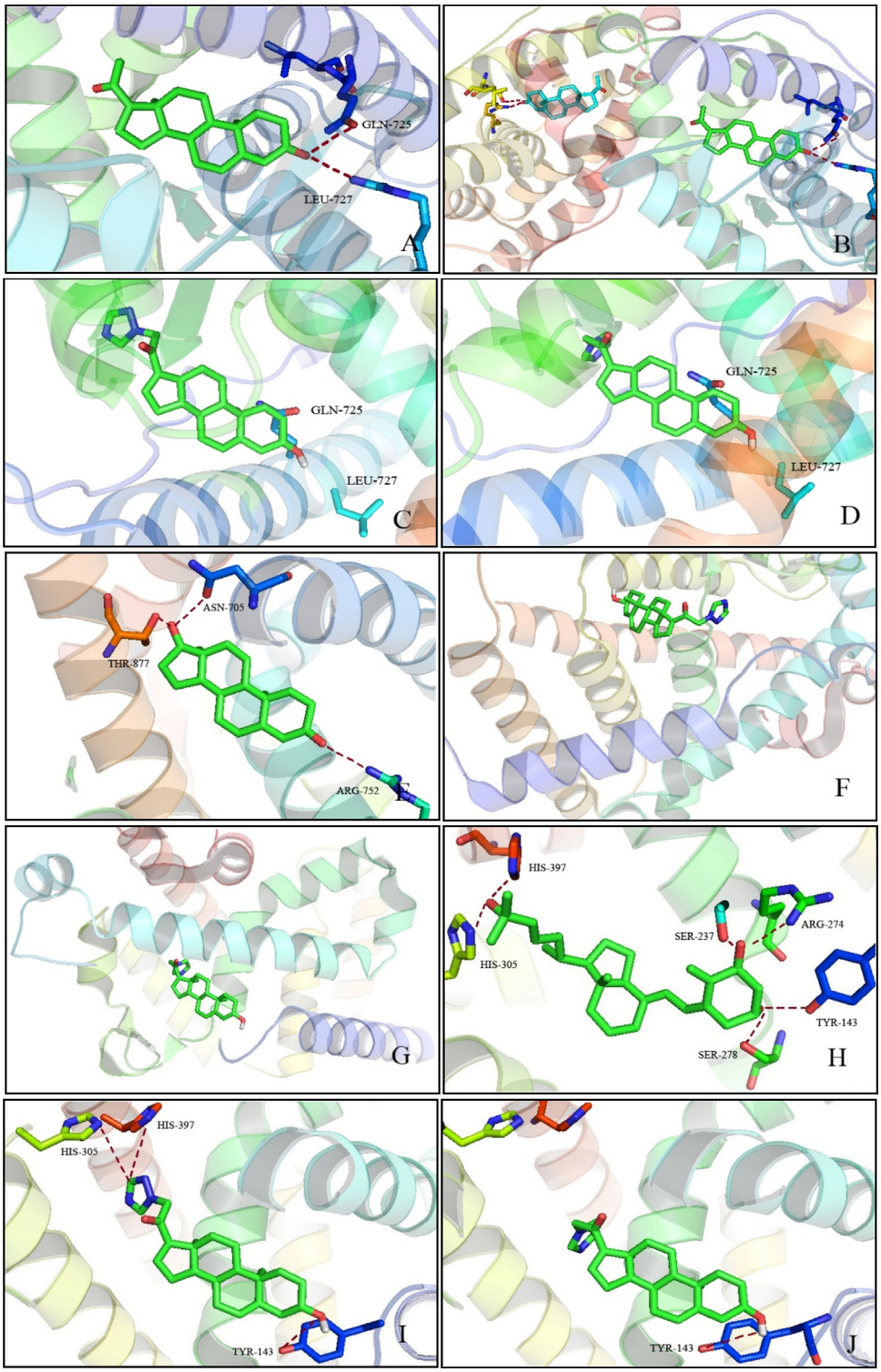

Fig. 5. Molecular docking studies that show binding interactions of **T–OH** and **I–OH** with hormonal receptors. PR: A) P_4, B) P_4 on chain A and chain B of PR, C) **T–OH** and D) **I–OH** on PR in B chain; AR: E) 5α-DHT, F) **T–OH** and G) **I–OH**; VDR: H) Calcitriol, I) **T–OH**, J) **I–OH**. A, D and G were the natural ligands used as controls to compare the energies and the spatial distribution.

by calcitriol. However, **I–OH** was incapable to induce the mRNA of this enzyme (data not shown).

4. Discussion

Over the last years, a large number of molecules have been modified and synthetized as potential inhibitors of proliferative process to be used as anticancer treatment, and the steroids are not the exception [10–17,35–37]. Particularly, Bratoeff E, et al., showed that a synthetic compound from dehydroepiandrosterone with an ester moiety at C-3 and an imidazole group at C-17 (16-formyl-17-(1*H*-imidazol-1-yl) androsta-5,16-diene-3β-yl) inhibited both the biological activity of 5α-reductase type 1 as well as cell proliferation of the human prostate cancer cell line; PC-3 [15].

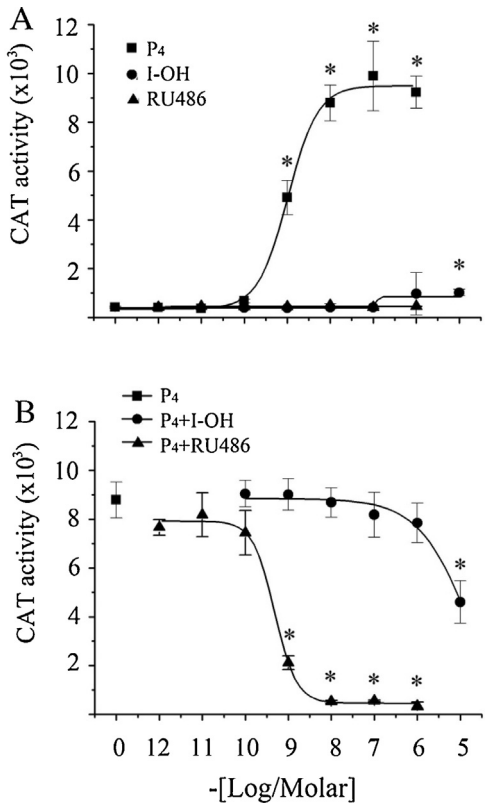

Fig. 6. Antiprogestagenic activity of **I–OH**. HeLa cells transfected were incubated in absence or presence of different concentrations of **I–OH**, P_4 and RU486 (A). Cells were treated with P_4 (1×10^{-8} M) alone or plus increased concentration of **I–OH** or RU486 (B). The results are the mean ± S.D. of the transactivation of PR-B of three independent experiments per triplicate. $^*P \leq 0.01$ *vs.* 0.

Similarly, Banday A. et al., reported a series of novel D-ring substituted 1,2,3-triazolyl 20-keto pregnenane derivatives. Among these compounds 21-{4-[(4-acetylphenoxy) methyl]-1*H*-1,2,3-triazol-1-yl}-3-hydroxypregn-5-en-20-one was the most active, especially against proliferation of DU-145 and PC-3 cell lines [17]. Recently, was reported the synthesis and biological activity of new 16-dehydropregnenolone derivatives with a triazole moiety at C-21 [16]. Herein, we compared two compounds that contain each one a triazole and imidazole moiety at C-21, respectively. As expected, these compounds showed antiproliferative effects on PC-3, MCF-7 and SK-LU-1 cancer cell lines. Interestingly, **I–OH** compound had higher antiproliferative activity than **T–OH,** showing a preferential inhibition upon PC-3 cells. These results show that the imidazole moiety into the molecular structure of the steroid improved its biological activity.

In addition, we demonstrated that CCND1 and CCNE1 gene were downregulated by **I–OH** treatment in PC-3 and MCF7 cells. These cyclins are key components of the core cell cycle machinery; CCND1 is a key positive regulator of the G1-S phase transition and its overexpression is implicated in genesis and progression of some neoplasms [38]. Likewise, it has been reported that upregulation CCND1 might be related to the evolution of androgen-independent disease in prostate cancer [39,40]. In the same way, the CCNE1 protein is tightly related as an endpoint of several regulatory pathways that are critical for growth control and frequently are altered in cancer cells. CCNE1 in conjunction with its kinase subunit Cdk2 regulates essential processes at the G1–S-phase boundary of the cell cycle. CCNE1 gene is undetectable in G0 and G1 phases of the cell cycle, but it rises sharply during of time that precedes each entry into S phase [41,42].

On the basis of these findings, we suggest that **I–OH** avoids the accumulation and activity of cyclin dependent kinase 4 (Cdk4)/CCND1 complex, and hyperphosphorylation of the retinoblastoma protein as well as affects the regulation of the progression through the restriction point R at the end of the G1-phase to allow cells to enter S-phase [43,44]. On the other hand, Ki-67, EAG1, BIM and survivin genes were not significantly altered, which indicates that these proteins are not involved in the antiproliferative effect by **I–OH**. Collectively, this study proposes that **I–OH** affects directly the cell cycle progression of both MCF7 and PC-3 cells. In the case of SK-LU-1, none gene studied was significantly modified, thus others molecular targets deserve further investigation to this cell type.

Lastly, to elucidate the potential interaction between **T–OH** and **I–OH** with hormonal receptors we use molecular docking simulations. Results suggest that these synthetic compounds bind to the hormone binding pocket into PR and induce conformational changes to interfere with natural agonist activity by competitive binding and thus have antagonist action [45]. Indeed, the experimental results from transfection assays with the PR in HeLa cells showed weak antiprogestagenic properties by **I–OH**. The functional significance of this is unknown but has been showed that P_4 and some synthetic derivatives had a proliferative action in some cancers and inducing the gene CCND1 expression. In fact, in the progression from normal to malignant breast cells, the expression of the two PR isoforms (A and B) is altered, and more aggressive breast cancers are associated with a predominance of either one of isoforms [46]. Contrary to P_4, some synthetic pregnane derivates abolished the hyper-proliferative process and downregulated the cyclins expressions. It is possible that **T–OH** and **I–OH** act as antagonist of PR blocking cyclins activation during cancer, which deserves further study.

Conversely, the transfection assays with the AR, **I–OH** did not have androgenic or antiandrogenic effect compared with 5α-DHT or FLUT respectively. These results demonstrated that effect of **I–**

OH is AR-independent pathway and this corresponds with the effect observed in the PC-3 cells that is lacking of AR and was the most sensitive cell line for this treatment.

Considering, that the synthesized compounds also share structural and chemical similitudes with the calcitriol, a hormone with antiproliferative effects, that use VDR signaling to affect target genes in multiple cancer cells. Additionally, we complete docking study using the VDR. Although the cluster size is lower, these values indicated a possible interaction with the pocket domain into VDR. Nonetheless, the experimental result showed that **I–OH** was not able to induce the mRNA expression of CYP24A1, a gene highly inducible by calcitriol via VDR, which discards totally, that the antiproliferative effect uses the VDR pathway. Taken together, these data are consistent with previous computer-based quantitative structure-activity relationship studies of ligand-receptor interactions [33,34].

It is worth to mention that antiproliferative activity of these compounds can also be attributed to the nature of the azole groups [10,12,47]. These groups are highly soluble in water and are classified as aromatic compounds because have a sextet of π-electrons, these characteristics given to the azol group a higher nucleophilic character [48]. Therefore, these groups allowed strong hydrogen bonds formation of the steroidal compound with polar active groups in the cells and as a result of this [49], a stronger cytotoxic effect.

On the other hand, it has been reported that a number substituted imidazoles act as selective inhibitors of nitric oxide synthase and Heme oxygenases, crucial enzymes that are involved in the proliferative process [50,51]. Alternatively, the biological activities of the imidazole pharmacophore are related to the downregulation of intracellular Ca^{++} and K^{+} fluxes that as consequence interference with translation initiation [50,52]. This last part showed some interesting possibilities that deserve more research to understand its mechanism biological.

In summary, results presented herein show that the 16-dehydropregnenolone derivatives were successfully synthesized and had antiproliferative activity upon three cancer cell lines. Interestingly, the PC-3 cells were more sensitive to **I–OH** antiproliferative effect. In addition, we also demonstrated that **I–OH** downregulated the mRNA expression of two cyclins key to cell cycle machinery both in PC-3 and MCF7, suggesting that antiproliferative effect of **T–OH** and **I–OH** involved the inhibition of CCND1 and CCNE1 in these cells. On the other hand, the experimental results demonstrated that **I–OH** had an antagonist effect upon PR and discard completely the AR or VDR pathways as possible action mechanism upon antiproliferative process. Nevertheless, the modification of structure steroidal by imidazole and triazole moieties suggests that these compounds use alternative routes to inhibit this process which deserve further investigation. As a concluding remark, these steroidal molecules, could offer a therapeutic alternative for patients with cancer.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.02.013.

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