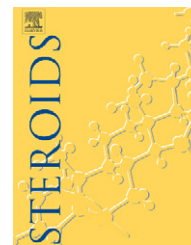


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Synthesis and evaluation of novel 17-indazole androstene derivatives designed as CYP17 inhibitors

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ABSTRACT

A series of novel 1H- and 2H-indazole derivatives of the commercially available dehydroepiandrosterone acetate have been synthesized and tested for inhibition of human cytochrome 17 α -hydroxylase-C_{17,20}-lyase (CYP17), androgen receptor (AR) binding affinity, and cytotoxic potential against three prostate cancer (PC) cell lines.

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1. Introduction

CYP17 is an endoplasmic reticulum membrane-bound multifunctional enzyme that exhibits 17 α -hydroxylase and C_{17,20}-lyase activities on a single active site, both of which are crucial for human physiology [1–5]. The hydroxylase activity is involved in the conversion of pregnenolone to 17 α -hydroxypregnenolone and progesterone to 17 α -hydroxyprogesterone whereas the lyase activity is responsible for the side-chain cleavage of these hydroxy derivatives to afford dehydroepiandrosterone (DHEA) and androstenedione (AD), respectively. DHEA and AD are androgen precursors and

can be further metabolized in steroidogenic tissues to more potent androgens such as testosterone and dihydrotestosterone (DHT).

The testis and the adrenal cortex are the two sites thought to produce most of the androgenic steroids in humans. The testis are responsible for about 90–95% of circulating androgens whereas the adrenals account for the remaining 5–10% [6].

In the adult prostate, androgens act directly on epithelial cells to maintain structural and functional viability. The secretory epithelial cells express the AR and require chronic androgenic stimulation for survival and functional integrity.

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Glandular involution occurs as a result of cell apoptosis when androgen levels drop below a threshold (as is the case in medical or surgical castration) [7].

Androgen deprivation as therapy for advanced PC was introduced by Huggins et al. in 1941 [8,9] and ever since it has been the mainstay for advanced PC treatment. At least 80% of the human prostate cancers show a favourable response to androgen deprivation as evidenced by the disappearance of symptoms or a decline of prostate specific antigen (PSA) levels [10,11]. However, relapses are seen invariably when tumors emerge as androgen-independent and apoptosis-resistant [7]. Mechanisms that may mediate this adaptation include AR amplification, AR mutation, alterations in the balance between transcriptional coactivators and corepressors, and activation of signal transduction pathways that by-pass the AR [12,13]. Gene amplification and amino acid substitutions in the AR are detected at a high frequency in recurrent tumors. These changes confer growth advantage to the tumor cells due to either hypersensitivity of AR to low, castrate-level androgens or a realignment of the receptor conformation, leading to altered ligand specificity that enables antiandrogens, adrenal androgens and non-androgen steroids to act agonistically to increase AR activity [7].

Enhanced intracellular conversion of adrenal androgens to testosterone and DHT has also been reported as an important mechanism for disease progression [14]. It may explain why the available AR antagonists do not have substantial activity against the androgen-independent tumor cells that emerge subsequent to androgen deprivation therapy, seeing that the AR antagonists will have a much lower affinity for the AR than the natural substrates. Inhibition of CYP17 is therefore a valuable approach for the treatment of androgen-dependent diseases such as PC as a means of inhibiting androgen biosynthesis both in the testis and adrenals. It should be noted that PC is a leading cause of mortality being the second most common cause of cancer-related death in both the USA and Australia (behind lung cancer), and the third most common cause of cancer-related death in the European Union (behind lung cancer and colorectal cancer) [15–17].

Several steroidal and non-steroidal compounds have been synthesized and evaluated as CYP17 inhibitors [18–23]. Out of these compounds, ketoconazole 1 (Fig. 1), an imidazole fungicide that has inhibitory activity towards CYP17 [24,25], has been used clinically in high dose (400 mg, every 8 h) for the treatment of advanced PC [26–28]. However, the fact that it concomitantly inhibits other steroidal P450 enzymes causing significant side effects [29] has limited its use. A recent investigation of the efficacy of low dose ketoconazole (200 mg, three times daily) found clinical benefit equal to high dose treatment, with a reduction in side effects [30]. Ketoconazole is still currently used alone or in combination with glucocorticoids as secondary hormonal therapy for hormone-refractory PC (HRPC) [31].

A common approach to the synthesis of potent steroidal inhibitors of CYP17 has been the design of substrate-like molecules bearing a heterocycle at the C17 position with privileged heteroatoms (N, S, O) which can interact as the sixth ligand with the heme iron of the enzyme. One of such compounds, abiraterone acetate 2 (Fig. 1), reported to be a very potent inhibitor of the enzyme [32], has successfully under-

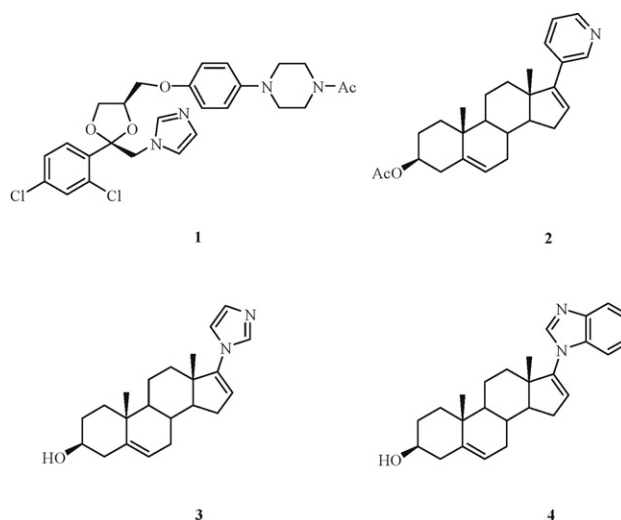


Fig. 1 – CYP17 inhibitors.

gone Phase I clinical trials for PC treatment [33,34] and the first set of results of an open-label Phase II clinical trial have just been reported. Thus, 11 out of 18 patients have had PSA declines $\geq 50\%$ at 3 months with 5 patients having a PSA decline $\geq 90\%$ when 1000 mg of the drug were administered orally once daily to chemotherapy-naive castration-resistant PC (CRPC) patients, resistant to luteinizing hormone-releasing hormone (LHRH) analogues, antiandrogens, and frequently diethylstilbestrol (DES) and steroids [35].

Another class of interesting steroidal inhibitors has been reported in which theazole group is attached to the C17 of the steroid nucleus through a nitrogen atom [36–38]. Both compound 3 (code named VN/85-1) (Fig. 1) and compound 4 (VN/124-1) potentially inhibit CYP17. VN/124-1 has also been shown to have antiandrogenic properties against the androgen-dependent LAPC4 human prostate tumor xenograft, being actually more effective than castration in suppressing its growth [38].

Herein we report the synthesis and biological evaluation of novel 17-indazole androstene derivatives designed as CYP17 inhibitors. Other than their CYP17 inhibitory potential, their ability to bind to the AR, and cytotoxicity against three PC cell lines has also been evaluated.

2. Experimental

2.1. Chemistry

2.1.1. General

Dehydroepiandrosterone acetate, indazole, bis(triphenylphosphine)rhodium(I)carbonyl chloride, 1,3-bis(diphenylphosphino)propane, aluminum isopropoxide, and *N*-methylpiperidone were obtained from Sigma-Aldrich Co. All solvents used were previously dried and purified according to standard procedures. For TLC analysis, Kieselgel 60HF₂₅₄/Kieselgel 60G was used. Melting points were determined using a BUCHI Melting Point B-540 apparatus and are uncorrected. IR spectra were obtained using a JASCO

FT/IR-420 spectrophotometer. NMR spectra were obtained using a Bruker Digital NMR-Avance 300 apparatus or a Varian Inova 500 apparatus, in CDCl₃ with MeSi₄ as the internal standard. Mass spectra were recorded on a Finnigan Polaris Q GC/MS Benchtop Ion Trap mass spectrometer. HRMS were determined on a Bruker 12T APEX-Qe FTICR-MS with an Apollo II ion source and an Advion Triversa Nanomate system. Elemental analysis was carried out on a Fisons Instruments EA 1108 CHNS-O elemental analyzer.

2.1.2. Synthesis of 17-(indazole) androstene derivatives

2.1.2.1. 17-Chloroandrosta-5,16-dien-3 β -yl acetate (**6**) and 17-chloro-16-formylandrosta-5,16-dien-3 β -yl acetate (**7**). Details of the synthesis of these compounds were reported previously [37,39].

2.1.2.2. 16-Formyl-17-(1H-indazol-1-yl)androsta-5,16-dien-3 β -yl acetate (**8**) and 16-formyl-17-(2H-indazol-2-yl)androsta-5,16-dien-3 β -yl acetate (**9**). A mixture of **7** (2 g, 5.32 mmol), indazole (943 mg, 7.98 mmol), and K₂CO₃ (2.2 g, 15.94 mmol) in dry DMF (40 ml) was heated at 80 °C under N₂ atmosphere for 6 h. The mixture was then concentrated under reduced pressure. Water (60 ml) and dichloromethane (200 ml) were added and the mixture was left under magnetic stirring for a couple of hours. The aqueous phase was extracted another two times with dichloromethane (2 × 50 ml). The organic phase was then washed with water (60 ml), brine (60 ml), dried with anhydrous MgSO₄, filtered, and concentrated under reduced pressure to give a yellowish oil. This oil was subjected to flash chromatography with chloroform/petroleum ether 40–60 °C (7:3) and afforded compound **8** (1.27 g; 52%): m.p. (acetone/*n*-hexane) 185–189 °C; IR 1248, 163, 1661, 1721 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.08 (s, 3H, 18-H₃), 1.27 (s, 3H, 19-H₃), 2.04 (s, 3H, 3 β -OAc), 4.61 (m, 1H, 3 α -H), 5.43 (m, 1H, 6-H), 7.25 (m, 1H, aromatic-H), 7.45 (m, 2H, aromatic-H), 7.78 (m, 1H, aromatic-H), 8.24 (m, 1H, aromatic-H), 9.72 (s, 1H, CHO); EI-MS *m/z* (%): 458 (12) M⁺, 398 (26), 225 (25), 185 (39), 157 (100), 143 (16), 91 (22), 77 (14); Anal. calcd. for C₂₉H₃₄N₂O₃: C 75.95, H 7.47, N 6.11, found: C 75.89, H 7.29, N 5.90. A second fraction afforded compound **9** (539 mg; 22%): m.p. (acetone/*n*-hexane) 190–192 °C; IR 1245, 1629, 1661, 1731 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.09 (s, 3H, 18-H₃), 1.28 (s, 3H, 19-H₃), 2.04 (s, 3H, 3 β -OAc), 4.62 (m, 1H, 3 α -H), 5.43 (m, 1H, 6-H), 7.13 (m, 1H, aromatic-H), 7.34 (m, 1H, aromatic-H), 7.68 (m, 1H, aromatic-H), 7.72 (m, 1H, aromatic-H), 8.19 (m, 1H, aromatic-H), 10.15 (s, 1H, CHO); EI-MS *m/z* (%): 458 (32) M⁺, 398 (74), 383 (48), 293 (24), 225 (56), 185 (54), 157 (100), 130 (20); Anal. calcd. for C₂₉H₃₄N₂O₃: C 75.95, H 7.47, N 6.11, found: C 76.02, H 7.53, N 5.88.

2.1.2.3. 17-(1H-Indazol-1-yl)androsta-5,16-dien-3 β -yl acetate (**10**). A mixture of bis(triphenylphosphine)rhodium(I) carbonyl chloride (325.4 mg, 0.47 mmol) and 1,3-bis(diphenylphosphino)propane (420.7 mg, 1.02 mmol) in dry xylenes (95 ml) was stirred at 80 °C under N₂ for 15 min when a fine yellow precipitate formed. Compound **8** (1.2 g, 2.62 mmol) was added, and the mixture was refluxed under N₂ for 10 days, then cooled, and concentrated under reduced pressure. The resulting oil was dissolved in dichloromethane (600 ml) and treated with charcoal. The organic phase was

washed with water (60 ml), dried with anhydrous MgSO₄, filtered, and concentrated under reduced pressure to give a yellowish oil. This oil was subjected to flash chromatography with chloroform/petroleum ether 40–60 °C (7:3) to give compound **10** (700 mg; 62%): m.p. (acetonitrile/THF) 154–156 °C; IR 1241, 1620, 1728 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.09 (s, 3H, 18-H₃), 1.18 (s, 3H, 19-H₃), 2.04 (s, 3H, 3 β -OAc), 4.61 (m, 1H, 3 α -H), 5.43 (m, 1H, 6-H), 5.86 (m, 1H, 16-H), 7.18 (m, 1H, aromatic-H), 7.39 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.74 (m, 1H; aromatic-H), 8.07 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 124.3 (C3a), 139.9 (C7a), 140.1 (C5), 150.6 (C17), 170.5 (CH₃CO); EI-MS *m/z* (%): 430 (20) M⁺, 370 (100), 355 (40), 195 (26), 157 (85), 144 (32), 119 (28), 91 (38); Anal. calcd. for C₂₈H₃₄N₂O₂: C 78.1, H 7.96, N 6.51, found: C 78.40, H 7.60, N 6.36.

2.1.2.4. 17-(1H-Indazol-1-yl)androsta-5,16-dien-3 β -ol (**11**). Compound **10** (400 mg; 0.93 mmol) was dissolved in methanol (7.5 ml) at room temperature under Ar. KOH (10%) in methanol (2.5 ml) was added and the mixture was left under magnetic stirring for 1.5 h. After this time the mixture was concentrated under reduced pressure. Dichloromethane (150 ml) and water (20 ml) were added, and left to agitate for a couple more hours. The aqueous phase was extracted with dichloromethane (2 × 100 ml). The organic phase was then washed with water (20 ml), dried with anhydrous MgSO₄, filtered, and evaporated to dryness to give compound **11** (297.1 mg; 82%): m.p. (acetone) 189–191 °C; IR 1625, 3257 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.08 (s, 3H, 18-H₃), 1.18 (s, 3H, 19-H₃), 3.55 (m, 1H, 3 α -H), 5.41 (m, 1H, 6-H), 5.86 (m, 1H, 16-H), 7.18 (m, 1H, aromatic-H), 7.39 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.74 (m, 1H; aromatic-H), 8.07 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 124.3 (C3a), 139.9 (C7a), 141.0 (C5), 150.6 (C17); EI-MS *m/z* (%): 388 (100) M⁺, 373 (35), 237 (29), 195 (24), 181 (14), 157 (26), 119 (22), 91 (17); Anal. calcd. for C₂₆H₃₂N₂O: C 80.37, H 8.3, N 7.21, found: C 80.46, H 8.07, N 7.0.

2.1.2.5. 17-(1H-Indazol-1-yl)androsta-4,16-dien-3-one (**12**). A mixture of **11** (470 mg; 1.21 mmol), *N*-methylpiperidone (1.7 ml), and toluene (28 ml) was distilled off ca. 10 ml. Aluminum isopropoxide (453 mg; 2.22 mmol) was then added, and the mixture was refluxed under N₂ for 5 h. The mixture was then concentrated under reduced pressure. Ethyl acetate (20 ml) and water (50 ml) were added and the mixture was left under magnetic stirring for a couple of hours. The aqueous phase was further extracted with ethyl acetate (2 × 100 ml). The organic phase was washed with 5% HCl (50 ml), 10% NaHCO₃ (50 ml), and water (50 ml), dried with anhydrous MgSO₄, filtered, and evaporated to dryness to afford compound **12** (390 mg; 83%): m.p. (acetone/*n*-hexane) 196–197 °C; IR 1621, 1672 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.20 (s, 3H, 18-H₃), 1.24 (s, 3H, 19-H₃), 5.76 (brs, 1H, 4-H), 5.85 (m, 1H, 16-H), 7.17 (m, 1H, aromatic-H), 7.40 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.74 (m, 1H, aromatic-H), 8.07 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 124.4 (C3a), 139.9 (C7a), 150.4 (C17), 171.0 (C5), 199.5 (C3); EI-MS *m/z* (%): 386 (100) M⁺, 371 (75), 253 (22), 211 (16), 183 (21), 157 (30), 119 (18), 81 (14); HRMS calcd 387.2431 (C₂₆H₃₀N₂OH⁺), found 387.2435.

2.1.2.6. 17-(2H-Indazol-2-yl)androsta-5,16-dien-3 β -yl acetate (13). The method followed that described for compound 10 but using compound 9 (700 mg; 1.53 mmol). The resulting oil was subjected to flash chromatography with chloroform/petroleum ether 40–60 °C (7:3) which afforded compound 13 (308 mg; 47%): m.p. (acetone) 144–146 °C; IR 1243, 1620, 1728 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.09 (s, 3H, 18-H₃), 1.19 (s, 3H, 19-H₃), 2.04 (s, 3H, 3 β -OAc), 4.62 (m, 1H, 3 α -H), 5.43 (m, 1H, 6-H), 6.14 (m, 1H, 16-H), 7.06 (m, 1H, aromatic-H), 7.27 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.71 (m, 1H; aromatic-H), 8.11 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 121.7 (C3a), 140.0 (C5), 148.9 (C7a), 151.7 (C17), 170.5 (CH₃CO); EI-MS *m/z* (%): 430 (14) M⁺, 370 (100), 355 (56), 209 (18), 195 (24), 157 (57), 105 (16), 91 (13); Anal. calcd. for C₂₈H₃₄N₂O₂: C 78.1, H 7.96, N 6.51, found: C 78.5, H 7.6, N 6.2.

2.1.2.7. 17-(2H-Indazol-2-yl)androsta-5,16-dien-3 β -ol (14). The method followed that described for compound 11 but using compound 13 (255.8 mg; 0.59 mmol) to afford compound 14 (216.8 mg; 94%): m.p. (acetonitrile/THF) 224–226 °C; IR 1629, 3352 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.08 (s, 3H, 18-H₃), 1.19 (s, 3H, 19-H₃), 3.55 (m, 1H, 3 α -H), 5.40 (m, 1H, 6-H), 6.17 (m, 1H, 16-H), 7.07 (m, 1H, aromatic-H), 7.27 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.71 (m, 1H; aromatic-H), 8.11 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 121.7 (C3a), 141.2 (C5), 148.9 (C7a), 151.70 (C17); EI-MS *m/z* (%): 388 (100) M⁺, 373 (68), 369 (23), 223 (22), 209 (26), 195 (32), 181 (18), 157 (86); Anal. calcd. for C₂₆H₃₂N₂O: C 80.37, H 8.3, N 7.21, found: C 80.2, H 8.16, N 7.15.

2.1.2.8. 17-(2H-Indazol-2-yl)androsta-4,16-dien-3-one (15). The method followed that described for compound 12 but using compound 14 (450 mg; 1.16 mmol) to afford compound 15 (370 mg; 83%): m.p. (acetone) 169–172 °C; IR 1629, 1674 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz): δ 1.21 (s, 3H, 18-H₃), 1.25 (s, 3H, 19-H₃), 5.77 (brs, 1H, 4-H), 6.14 (m, 1H, 16-H), 7.07 (m, 1H, aromatic-H), 7.27 (m, 1H, aromatic-H), 7.64 (m, 1H, aromatic-H), 7.72 (m, 1H; aromatic-H), 8.11 (m, 1H, aromatic-H); ¹³C NMR (CDCl₃, 75 MHz): δ 121.7 (C3a), 148.9 (C7a), 151.6 (C17), 170.6 (C5), 199.4 (C3); EI-MS *m/z* (%): 386 (72) M⁺, 371 (62), 223 (39), 195 (36), 157 (100), 145 (25), 119 (35), 91 (42); HRMS calcd 387.2431 (C₂₆H₃₀N₂OH⁺), found 387.2431.

2.2. Biology

2.2.1. General

The human PC cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (Rockville, MD). 293T cells were the gift of Dr. Yun Qiu (UMB, Maryland), and LAPC4 cells were provided by Dr. Charles L. Sawyers (UCLA School of Medicine).

RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), trypsin/EDTA (0.25%/0.02%), and penicillin/streptomycin (P/S) were obtained from Gibco-BRL. Fetal Bovine Serum (FBS), charcoal-stripped serum (CSS), and trypsin/versene were obtained from Biofluids Inc. Poly-L-lysine, triamcinolone acetate, ketoconazole, DHT, and MTT powder were obtained

from Sigma-Aldrich Co. Casodex was kindly provided by Astra-Zeneca Inc. Scintiverse BD Cocktail (Scintanalyzed) fluid was obtained from Fisher Scientific. VN/85-1 was prepared as previously reported [37].

The synthetic androgen methyltrienolone [³H]R1881, with a specific activity of 72 Ci/mmol, was purchased from Perkin-Elmer. [21-³H₃]-17 α -hydroxypregnenolone, with a specific activity of 13.61 μ Ci/ μ mol, was prepared as described by Akhtar et al. [40].

The calcium phosphate transfection kit was purchased from Promega (Promega Profection Mammalian Transfection System). Tox-2 kit (XTT based) was purchased from Sigma-Aldrich Co.

293T and 293T-CYP17 cells were routinely maintained in DMEM supplemented with 10% FBS and 1% P/S solution. LNCaP and PC-3 cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% P/S solution. LAPC4 cells were grown in RPMI supplemented with 15% FBS, 1% P/S solution, and 10 nM DHT.

The pCDNA3Hmod17(His)₄ construct was designed as previously reported [41].

Radioactivity measurements were performed in a Tri-carb 2100 TR liquid scintillation analyzer. Absorbance and luminescence measurements were made using a Victor 1420 Multilabel counter.

2.2.2. In vitro CYP17 assay (C_{17,20}-lyase activity)

The *in vitro* C_{17,20}-lyase inhibitory activities of the compounds were evaluated using the acetic acid releasing assay (AARA) [37,38,41–49] with 293T cells that were transfected with the pCDNA3Hmod17(His)₄ construct using the calcium phosphate method (Promega Profection Mammalian Transfection System), to express the human enzyme.

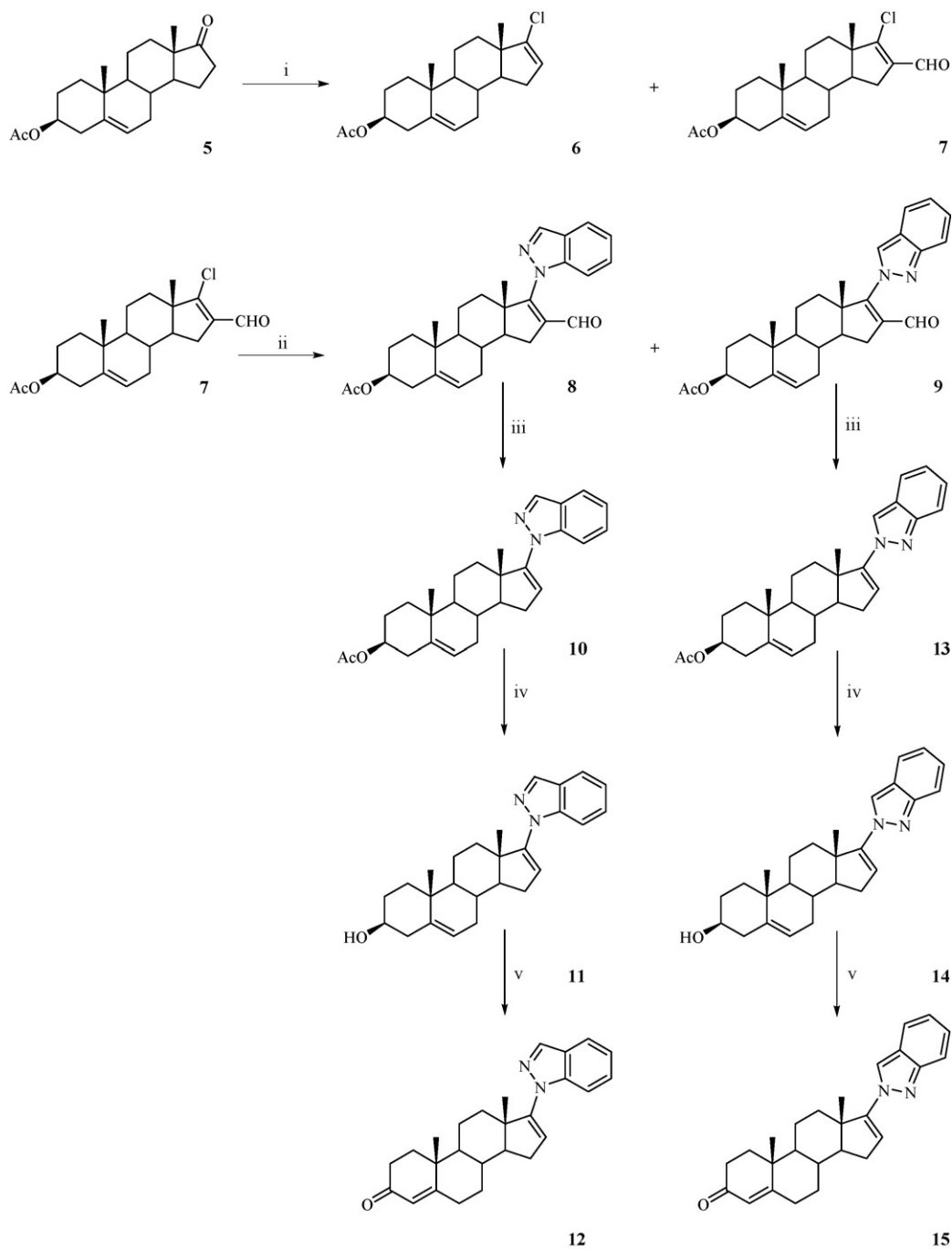
Briefly, 100 mm plates were coated with poly-L-lysine (0.05 mg/ml) for 30 min, rinsed twice with sterilized distilled water, and allowed to dry for 2 h. 293T cells were then plated in DMEM at a density sufficient for achieving approximately 60% confluency on the following day, for transfection. Three hours prior to transfection the DMEM was renewed on the plate. On a small eppendorf flask, 10 μ g of pCDNA3Hmod17(His)₄ were added to sterile, deionized water and vortexed briefly. A 2 M CaCl₂ solution (62 μ l) was added to bring the final volume up to 500 μ l. This mixture was added drop-wise with light vortex to 500 μ l of HEPES solution and incubated at room temperature for 30 min. The solution was vortexed again and dripped into the 100 mm plate. The medium was changed 18 h later and enzyme activity was assayed as described below 48 h after transfection.

293T-CYP17 cells were grown to 80% confluency and divided evenly in 6-well plates. On the following day, cells were washed with DPBS and incubated with clear DMEM (with 5% CSS and 1% P/S) containing a saturating concentration of [21-³H₃]-17 α -hydroxypregnenolone. The test compounds were then added in the desired concentrations and the plates were left to incubate for 18 h at 37 °C. The steroids were extracted with 2 ml of chloroform at 4 °C. After 2 h, the aqueous phase was collected and charcoal suspension was added to a 2.5% final concentration. Following a 30-min incubation at 4 °C, an aliquot of the aqueous supernatant was removed and radioactivity measured by liquid scintillation counting.

2.2.3. Competitive AR binding assay

Competitive binding assays with the synthetic androgen methyltrienolone [^3H]R1881 were performed essentially as previously described [50,51]. LNCaP or LAPC4 cells were transferred to clear RPMI medium (with 5% CSS and 1% P/S) 3 days before the start of the experiment. 24-Well plates were coated with poly-L-lysine (0.05 mg/ml) for 30 min, rinsed with sterilized distilled water, and dried for 2 h. The cells were then plated ($2-3 \times 10^5$ cells/well) and allowed to attach. The following day the medium was replaced by clear RPMI (with

1% P/S added) containing a saturation concentration (5 nM) of [^3H]R1881, triamcinolone acetonide (1 μM), and the desired concentrations of the test compounds. Following a 2 h incubation period at 37 $^\circ\text{C}$, cells were washed twice with ice-cold DPBS, and solubilized in DPBS containing 0.5% SDS and 20% glycerol. Extracts were removed and cell associated radioactivity counted in a scintillation counter. All results represent an average of a minimum of three wells. To determine the EC_{50} values of the test compounds, a minimum of eight concentrations of each test compound was used. EC_{50} values were



Scheme 1 – Synthesis of 17-indazole compounds. (i) POCl_3 -DMF, CH_3Cl , Ar, reflux; (ii) indazole, K_2CO_3 , DMF, N_2 , 80 $^\circ\text{C}$; (iii) $(\text{PPh}_3)_2\text{RhCOCl-Ph}_2\text{P}(\text{CH}_2)_3\text{PPh}_2$, xylenes, N_2 , reflux; (iv) KOH (10%) in methanol, N_2 , rt; (v) $\text{Al}(\text{i-PrO})_3$, *N*-methylpiperidone, toluene, reflux.

determined by non-linear regression with Graphpad Prism software.

2.2.4. Cell culture and viability assay

To determine the effect of steroids and novel compounds on cell proliferation on LNCaP and LAPC4 cells, each cell type was transferred into clear RPMI medium (with 5% CSS and 1% P/S) 3 days prior to the start of the experiments. The cells were then plated on previously coated 96-well plates (2.5×10^3 cell/well) and after a 24 h attachment period, the medium was aspirated and replaced by new medium with the novel compounds (0.1–20 μM). The medium was changed every 3 days and the number of viable cells was compared by XTT assay on the 7th day. Briefly, a 20% solution of XTT in clear medium was added to each of the wells in the plates and after a 4 h incubation period, the plates were read at 450 nm on a Victor 1420 Multi-label counter. All results represent the average of a minimum of three wells.

For PC-3 cells, 1.5×10^4 cells/well were plated in 24-well plates in RPMI medium (with 10% FBS and 1% P/S). After a 24 h attachment period, the cells were treated with the novel compounds (0.1–20 μM). The medium was changed every 3 days and the number of viable cells was compared by MTT assay on the 7th day. Briefly, 0.5 mg/ml MTT in clear RPMI medium (with 5% CSS and 1% P/S) was added to each well and incubated at 37 °C for 4 h. Following incubation, the medium was aspirated completely with care taken not to disturb the formazan crystals. DMSO (400 μl) was used to solubilize these crystals. After slight shaking, the plates were immediately read at 540 nm on a Victor 1420 Multilabel counter. All results represent an average of a minimum of three wells. To determine the EC_{50} values of the test compounds, PC-3 cells were incubated with a minimum of eight concentrations of each test compound. EC_{50} values were determined by non-linear regression with Graphpad Prism software.

3. Results and discussion

3.1. Chemistry

3.1.1. Synthesis of 17-indazole androstene derivatives

The synthesis of the new 17-indazole androstene derivatives is outlined in Scheme 1. It started with the Vilsmeier-Haack reaction of the commercially available dehydroepiandrosterone acetate **5** with phosphorous oxychloride (POCl_3) and dimethylformamide (DMF), as reported previously [37,39]. The major reaction product 17-chloro-16-formylandrosta-5,16-diene-3 β -yl acetate **7** was then treated with indazole in the presence of K_2CO_3 and DMF at 80 °C, under N_2 to afford a mixture of the 1H-**8** and 2H-indazole **9** substituted compounds which were separated by flash chromatography on silica gel. The 1H-indazole derivative **8** was found to be the major reaction product being isolated in 52% yield (compound **9** was isolated in 22% yield).

Each of the obtained 17-indazole compounds **8** and **9** was separately decarbonylated to afford compounds **10** and **13** in 62 and 47% yield, respectively. This decarbonylation was performed with *in situ* generated $\text{Rh}(1,3\text{-bis}(\text{diphenylphosphino})\text{propane})_2^+\text{Cl}^-$ catalyst $[\text{Rh}(\text{dppp})_2]^+$

Cl^-] in refluxing xylenes [37,52], after the use of both the Wilkinson catalyst $[\text{RhCl}(\text{PPh}_3)_3]$ and 10% palladium on activated charcoal, other known methods of decarbonylation, failed. Hydrolysis of the 3 β -acetoxy group to obtain the corresponding 3 β -hydroxy derivatives **11** and **14** was achieved with KOH (10%) in methanol, in 82 and 94% yield. Modified Oppenauer oxidation of substrates **11** and **14** using aluminum isopropoxide and *N*-methylpiperidone afforded the final 3-keto derivatives **12** and **15**, again in good yields (83% in both cases).

The peak pattern of the carbons seen on the ^{13}C NMR spectra of compounds **13**–**15** (2H-series) is consistent with data reported in the literature for 2H-substituted compounds whereas for compounds **10**–**12** (1H-series) the spectra correlated well with that of 1H-substituted derivatives [53]. In the 2H-series, the two quaternary carbons of the indazole ring C3a and C7a are seen at ppm values of 121–123 ppm and 148–153 ppm, respectively. For our compounds C3a was identified at 121 ppm and C7a at 148 ppm. The 1H-series typically has C3a at higher ppm values (123–126 ppm) whereas C7a is seen at lower ppm values of 137–148 ppm. Thus, in our 1H-series we identified C3a at 124 ppm and C7a at 139 ppm.

3.2. Biology

3.2.1. CYP17 inhibition ($\text{C}_{17,20}$ -lyase)

The AARA uses $[21\text{-}^3\text{H}_3]17\alpha\text{-hydroxypregnenolone}$ as substrate and $\text{C}_{17,20}$ -lyase activity is measured by the amount of $[^3\text{H}]$ acetic acid released during the side-chain cleavage of the substrate to DHEA. This assay proved comparable in terms of accuracy and reliability to the previous HPLC procedures [41,42]. Moreover, kinetic analysis of CYP17 in human testicular microsomes showed that the $\text{C}_{17,20}$ -lyase activity is half that of the 17 α -hydroxylase activity thus implying that inhibition of the $\text{C}_{17,20}$ -lyase activity will actually characterize the inhibition of the entire enzyme [41]. Human kidney 293T cells were chosen for the assay because they showed rapid growth

Table 1 – $\text{C}_{17,20}$ -lyase inhibition and AR binding for 17-indazole compounds

Compound	$\text{C}_{17,20}$ -lyase $\text{IC}_{50}^{\text{a}}$ 293T	LNCaP $\text{EC}_{50}^{\text{b}}$ (nM)	LAPC4 $\text{EC}_{50}^{\text{b}}$ (nM)
10	c	d	d
11	c	d	d
12	c	d	5000
13	c	d	d
14	c	d	d
15	c	d	5000
Ketoconazole	49 nM	NT	NT
VN/85-1	1.29 nM	NT	NT
Casodex	NT ^e	971	4500

^a IC_{50} is the concentration of inhibitor required to inhibit the enzyme activity by 50%.

^b EC_{50} is the concentration of compound needed for a 50% displacement of $[^3\text{H}]$ R1881 from the AR.

^c Less than 30% effective at 10 μM .

^d Less than 30% binding at 5 μM .

^e NT = Not tested.

in culture medium and high transfection efficiency for the human CYP17 plasmid.

The synthesized compounds were found not to inhibit C_{17,20}-lyase significantly when compared to both ketoconazole and VN/85-1. Thus, all tested compounds (10-15) were less than 30% effective at 10 μM whereas IC₅₀ values of 49 and 1.29 nM were determined for ketoconazole and VN/85-1, respectively, under the same assay conditions (Table 1). Reasons that may account for this lack of inhibitory activity are

Table 2 – Effect of the 2H-indazole series on PC-3 cell proliferation

Compound	PC-3 EC ₅₀ (μM)
13	5.4
14	8.3
15	1.9

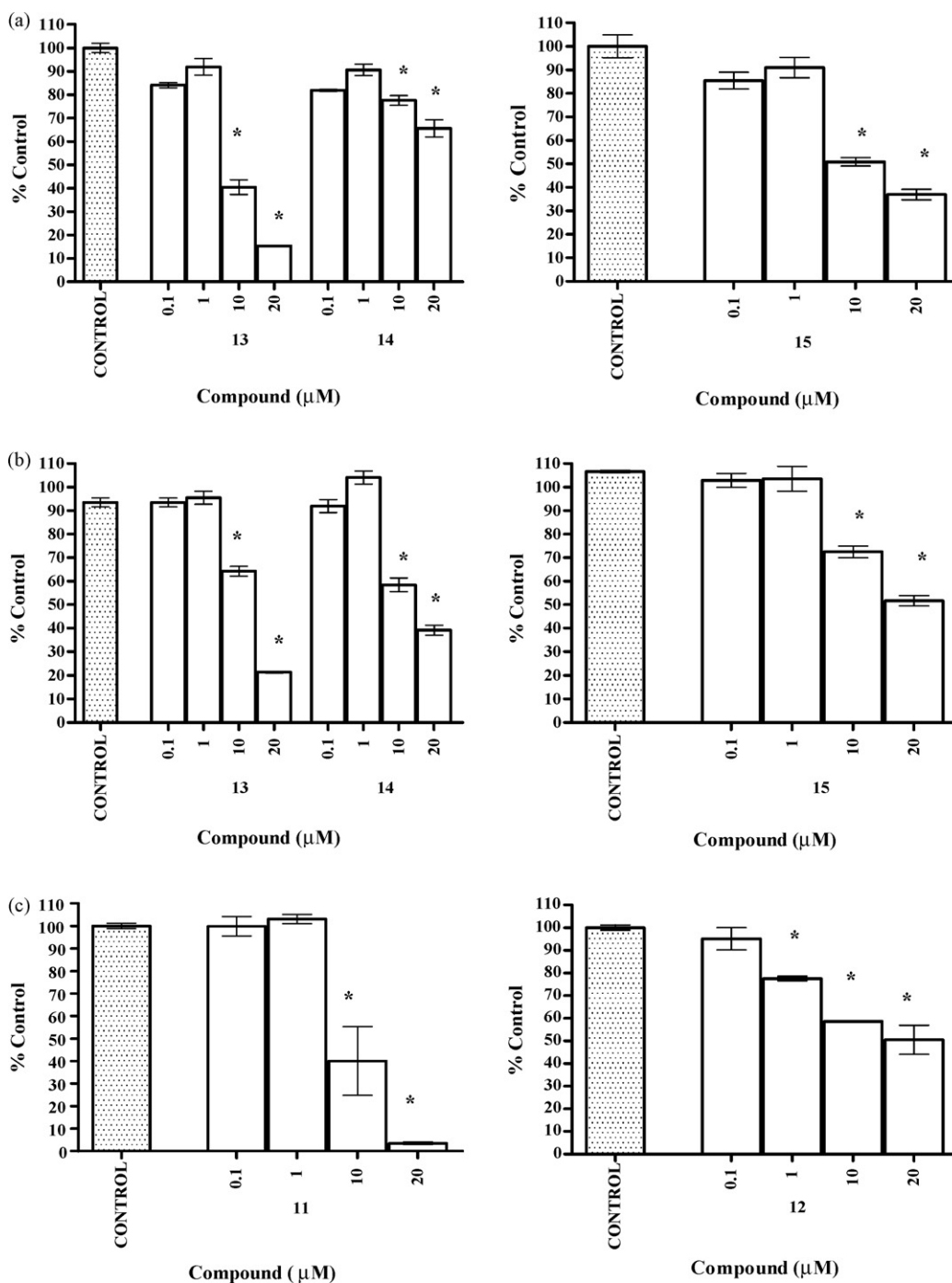


Fig. 2 – Effect of selected compounds on (a) LNCaP, (b) LAPC4, and (c) PC-3 cell proliferation. The percentage (compared to control) of growth inhibition after 7 days of treatment was determined with the XTT (LNCaP and LAPC4) or MTT (PC-3) assay using 0.1–20 μM of compound, as described in Section 2. One-way ANOVA with a Dunnet Post Test was used to analyze the data: *p < 0.01.

the positioning of the N atoms in the heterocycle ring that may not permit good interaction with the enzyme's active site or even the bulkiness of the indazole ring.

3.2.2. AR binding

Following the observation that several compounds designed as CYP17 inhibitors have been shown to bind to the AR and interfere with its function [37,38,45,54], we decided to determine if the synthesized indazole androstene derivatives could bind to the AR. LAPC4 and LNCaP cells were chosen which express the wild-type (wt-) and mutated receptor, respectively. Competitive binding of the compounds to the AR was evaluated using a saturating concentration of the radiolabeled androgen [³H]R1881 in the presence of different concentrations of test compound.

Compounds 10, 11, 13 and 14 showed less than 30% binding to both types of AR at 5 μM (Table 1). However, compounds 12 and 15, having the Δ⁴-3-ketone system in common, bound to the wt-AR with similar affinity when compared to Casodex, an antiandrogen currently used in PC treatment, showing an EC₅₀ of 5 μM (4.5 μM for Casodex).

3.2.3. PC cell toxicity

The potential of the synthesized compounds to inhibit cell proliferation was studied on LNCaP, LAPC4 and PC-3 cell lines. All compounds were screened at 0.1, 1, 10, and 20 μM and the more active were chosen for EC₅₀ calculation. The results are depicted in Table 2 and Fig. 2.

We identified compounds 13–15 in the 2H-indazole series that significantly inhibited the proliferation of LAPC4 and LNCaP cells mostly at 10 and 20 μM (Fig. 2a and b), and were concomitantly effective against PC-3 cell proliferation. Their EC₅₀ values for PC-3 cell proliferation were 5.4, 8.3, and 1.9 μM, respectively (Table 2). In the 1H-indazole series, compound 10 was only active at 20 μM for all PC cell lines. Compounds 11 and 12 were exclusively cytotoxic towards PC-3 cells, showing 50% inhibition of cell proliferation at 10 and 20 μM, respectively (Fig. 2c).

Thus, seeing that there is not a very high affinity of the compounds towards the AR, they probably act on PC cells through mechanisms other than the ones mediated by the AR such as apoptosis or cell cycle arrest. This is more evident for PC-3 cells that are human PC cells derived from bone metastases and do not express the AR at all.

In summary, the synthesized compounds were found not to inhibit C_{17,20}-lyase activity significantly when compared to both ketoconazole and VN/85-1. They also did not display affinity towards the LNCaP mutated AR at the concentrations tested. Compounds 12 and 15, having the Δ⁴-3-ketone system in common, bound to the wt-AR in the same extension as Casodex. However, only moderate inhibition of LAPC4 cell proliferation was seen with compound 15 at higher concentrations, suggesting that other mechanisms that are non-AR mediated account for this effect. The 2H-indazole series (13–15) was particularly effective against PC-3 cells whereas compounds 11 and 12 of the 1H-indazole series were exclusively toxic towards them. Because PC-3 cells lack the AR, it is likely that mechanisms such as apoptosis or cell cycle arrest account for inhibition of proliferation on this particular cell line.

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