Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 2, April – June 2014, p. 609 - 625

ANTI-ANDROGENIC, ANTI-PROSTATE CANCER ACTIVITIES AND EGFR, VEGFR-2 KINASE INHIBITORS OF SOME STEROIDAL DERIVATIVES

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In the present study, forty one steroid candidates containing a pyrazole ring were screened for their anti-androgenic and anti-prostate cancer activities. Also, in the same time these compounds were screened for their EGFR and VEGFR-2 kinase inhibitor potencies comparable to that of the Delphinidin. Initially, all the candidates were less toxic than the reference drug concerning LD_{50} values. Some of the compounds exhibited better anti-androgenic, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities than the reference drugs Bicalutamide and Delphinidin, respectively. The detailed anti-androgenic, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities and toxicity (LD_{50}) of the synthesized compounds were reported.

(Received January 8, 2014; Accepted April 28, 2014)

Keyword: Synthetic steroids; anti-androgenic, Anti-prostate cancer, EGFR and VEGFR-2 kinase inhibitor activities.

1. Introduction

The steroid candidate, such as cortisone (steroid hormone, 17-hydroxy-11-dehydrocorti-costerone) (Fig. 1), it is one of the main hormones released by the adrenal gland in response to stress and which suppress the antibody-forming lymphocyte cells. Also, it have been used to prolong human organ transplants and also prevent antigens from entering cells and thereby prevent local allergic inflammation reactions [1,2].

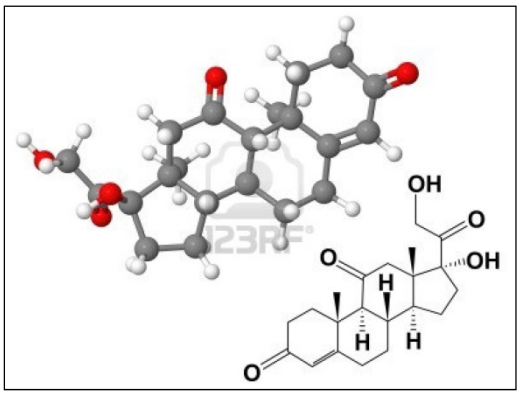

Fig. 1. Chemical structure of Cortisone

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In our previous work, we found that certain of substituted steroidal and terpenoidal derivatives showed anti-androgenic, anabolic, and antioxidant activities [3-5]. Some of new steroidal derivatives fused with heterocyclic moiety have been synthesized and used as 5α-reductase, aromatase inhibitors, anti-inflammatory, anti-alzheimer, anti-arthritic and immunosuppressive [6-12] agents.

Most signal transduction pathways were mediated by protein kinases, which was leads to proliferation of cancer cells as well as angiogenesis and growth of solid tumors such as prostate, colon, breast, and gastric cancers [13]. The VEGF family of receptors consists of three protein tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) and two non-protein kinase co-receptors (neuropilin-1 and neuropilin-2). These components are key intermediates in tumor angiogenesis and in the formation of new blood vessel networks required to supply nutrition and oxygen for tumor growth [14]. Vascular endothelial growth factor receptor-2 (VEGFR-2, KDR) is the main mediator that plays important roles in regulating vascular permeability, migration, endothelial cell proliferation and angiogenesis under physiological conditions mediated by the vascular endothelial growth factor (VEGF) [15]. Although VEGFR-2 has lower affinity for VEGF than VEGFR-1, VEGFR-2 exhibits robust protein tyrosine kinase activity in response to its ligands. VEGFR-2 is expressed at abnormally high levels in a large variety of human solid tumors [14,16]. There is much evidence that direct inhibition of the kinase activity of VEGFR-2 will result in a reduction in angiogenesis and the suppression of this signaling pathway has become an inhibiting method of tumor growth. Therefore, inhibition of VEGFR-2 is an attractive strategy in the treatment of cancers [17]. This research has led to the development of an USAFDA approved anti-VEGF antibody, bevacizumab (Avastin) [18], as well as three small molecule inhibitors of VEGFR-2 kinase, i.e. sorafenib (BAY-43-9006) [19], sunitinib (Su-11248) [20] and pazopanib [21] (Fig. 2). Some other small molecule, such as indolin-2-one, quinolinones, imidazopyridines, benzimidazoles, quinazolines, quinolyl-thienyl chalcones, phthalazines and quinoline amides have been reported as potent inhibitors of VEGFR-2 and angiogenesis [22-24].

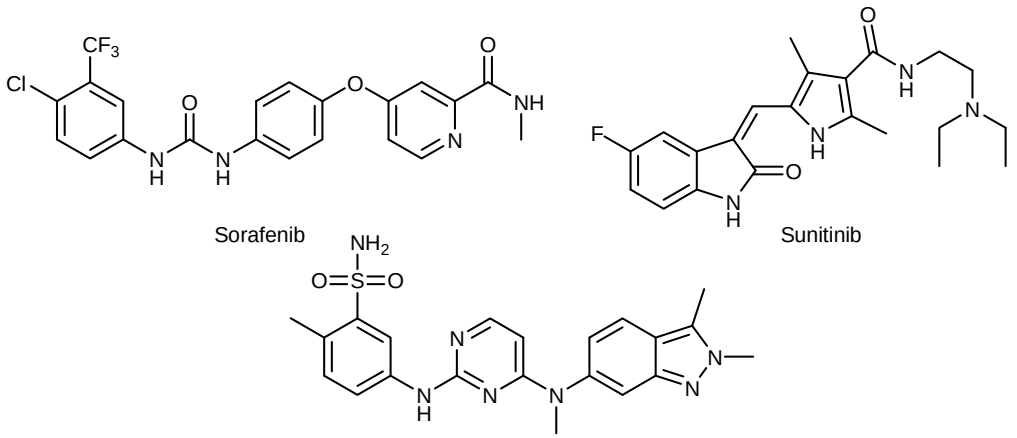


Fig. 2: VEGFR-2 tyrosine kinase inhibitors

2. Experimental

Evaluation of transcriptional activity for human androgen receptor [25]

(a) Establishment of CHO Cells Stably Transfected with Human Androgen Receptor Gene and MMTV-Luciferase Reporter Gene or SV40-Luciferase Gene: Chinese hamster ovary (CHO) cells were maintained in Alpha-modified Eagle's medium supplemented with 10% Fetal Bovine Serum (FBS). The culture medium of neomycin-resistant clone cells was supplemented with 10% dextran-coated charcoal-stripped FBS (DCC-FBS) and 500μg/ml of neomycin. The CHO cells were transfected at 40-70% confluence in 10-cm petri dishes with a total of 20μg DNA (pMAMneoLUC; MMTV-luciferase reporter plasmid and pSG5-hAR; human androgen receptor

expression plasmid, or SV40-LUC; SV40-luciferase reporter plasmid containing neomycin resistant gene) by calcium phosphate mediated transfection. The stable transfected cells were selected in the culture medium supplemented with neomycin. The selected clone was designated as AR/CHO#3 (human AR gene and MMTV-luciferase reporter gene integrated CHO cell) or SV/CHO#10 (SV-40-luciferase reporter gene integrated CHO cell), respectively.

(b) Activities of the Tested Compounds to Inhibit Androgen Receptor Mediated Transcription Induced by DHT (AR Antagonistic Activity): The stable transfected AR/CHO#3 or SV/CHO#10 cells were plated onto 96 well luminoplates (Packard) at a density of $2X10^4$ cells/well, respectively. Four to eight hours later, the medium was changed to the medium containing DMSO, 0.3 nM of DHT, or 0.3 nM of DHT and the tested compound. At the end of incubation, the medium was removed and then cells were lysed with 20µl of lysis buffer [25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-cyclohexanediamine-tetraacetic acid, 10% glycerol and 1% TritonX-100]. Luciferase substrate [20 mM Tris–HCl (pH 7.8), 1.07 mM $(MgCO_3)_4Mg\text{-}(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.47 mM luciferin, 0.53 mM ATP] was added and luciferase activity was measured with a ML3000 luminometer (Dynatech Laboratories). AR antagonistic activities were calculated by formula below;

$$\text{AR antagonistic activity (\%)} = 100(I\text{-}X)/(I\text{-}B)$$

I: (luciferase activity of AR/CHO#3)/(luciferase activity of SV/CHO#10) in the presence of 0.3 nM of DHT

B: (luciferase activity of AR/CHO#3)/(luciferase activity of SV/CHO#10) in the presence of DMSO

X: (luciferase activity of AR/CHO#3)/(luciferase activity of SV/CHO#10) in the presence of 0.3 nM of DHT and the tested compound

The concentration of compounds showing 50% of AR antagonistic activities, IC_{50} values, were obtained by nonlinear analysis using Statistical Analysis System (SAS).

In vivo evaluation of antiandrogenic activities in castrated immature rats [25]

Treated with Androgen Male Wistar rats were obtained from the Animal House Colony, Research Institute of Ophthalmology, Giza, Egypt. Prepubertal male rats aged 3 weeks were castrated by the scrotal route under ether anesthesia. Three days after the castration, testosterone propionate (TP, 0.5 mg/kg, s.c.) was administered once daily for 5days alone or in combination with the tested compound (10-30 mg/kg, p.o.). TP was dissolved in cotton seed oil containing 5% ethanol. The tested compound was suspended with 0.5% methylcellulose. The rats were sacrificed by excessive chloroform anesthesia 6 h after final dosing, and both ventral prostates and seminal vesicles-coagulate glands were removed and weighed. The antiandrogenic activity was expressed as a percentage of inhibition of the TP effect (TP-treated rats were arbitrarily assigned a value of 0% and vehicle-treated rats a value of 100%).

In vitro anti-tumor screening on different prostate cell lines [26, 27]

Compounds were subjected to in vitro disease-oriented primary antitumor screening. Different prostate cell lines of tumor cell lines were utilized. The tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96-well micro-titer plates in 100 mL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the micro-titer plates were incubated at 37^oC, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition. Experimental drugs were solubilized in DMSO at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 mg mL^{-1} gentamicin. Additional four 10-fold or 1/2 log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 mL of these different

drug dilutions were added to the appropriate microtiter wells already containing 100 mL of medium, resulting in the required final drug concentrations. Following drug addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 mL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 mL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 mL of 80% TCA (final concentration, 16% TCA). The parameter used here is GI_{50} which is the log10 concentration at which PG is 50, was calculated for each cell line.

Anti-prostate cancer screening anti-androgenic bioassay in human prostate cancer cells [28]

Human prostate cancer LNCaP and PC-3 cells were maintained in RPMI medium and Dulbecco's minimum essential medium (DMEM), respectively. Both media were supplemented with penicillin (25 units/mL), streptomycin (25 μg/mL), and 10% fetal calf serum. For the androgen receptor transactivation assay, an androgen-dependent reporter gene transcription test was employed as the primary screening for potential antiandrogen identification.

This assay was first performed in LNCaP cells, which express a clinically relevant mutant AR. Once anti-androgenic activity was detected in the LNCaP AR transactivation assay, compounds were re-examined for their potential activity against wild type AR. Wild type AR transactivation assay was performed in PC-3 host cells, which lack an endogenous, functional AR. The method and conditions of cell and gene transfection have been described previously. In brief, cells were plated in 24-well tissue culture dishes for 24 (PC-3 cells) or 48 (LNCaP cells) h prior to transfection. Subsequently, LNCaP cells were transfected with a reporter gene, MMTV-luciferase, which contains MMTV-LTR promoter and androgen receptor binding element, and PRL-SV40, which served as an internal control for transfection efficiency. PC-3 cells were transfected with a wild type AR expression plasmid, pSG5AR, in addition to the above-mentioned MMTV-luciferase reporter gene and PRL-SV40 internal control. SuperFect (Qiagen, Chatsworth, CA) was employed as the transfection reagent following manufacturer's recommendations. At the end of a five-hour transfection, the medium was changed to DMEM or RPMI supplemented with 10% charcoal dextran-stripped, i.e., androgen-depleted, serum. After 24 h, the cells were treated with 1 nM of DHT and/or test compounds at the designated concentration for another 24 h. The cells were harvested for luciferase activity assay using Dual Luciferase Assay System (Promega, Madison, WI). The derived data were expressed as relative luciferase activity normalized to the internal luciferase control. Cells cultured in medium containing DHT (androgen), as a positive control, induced a marked reporter gene expression. Test compounds capable of significantly suppressing this DHT-induced reporter gene expression were identified as potential antiandrogens.

EGFR and VEGFR-2 kinase activity assays by ELISA [29]

The assay was performed in 96-well plates pre-coated with 20 μg mL^{-1} poly (Glu, Tyr)4:1 (Sigma) as a substrate. In each well, 85 μL of an 8 μM ATP solution and 10 μL of the compound were added at varying concentrations. Sorafenib was used as a positive control for VEGFR-2 and EGFR kinase, and 0.1% (v/v) DMSO was the negative control. Experiments at each concentration were performed in triplicate. The reaction was initiated by adding 5 μL of VEGFR-2 or EGFR kinase. After incubation for 1 h at 37 °C, the plate was washed three times with PBS containing 0.1% Tween 20 (T-PBS). Next, 100 μL of anti-phosphotyrosine (PY99; 1 : 500 dilution) antibody was added. After 1 h of incubation at room temperature, the plate was washed three times. Goat anti-mouse IgG horseradish peroxidase (100 μL; 1 : 2000 dilution) diluted in T-PBS containing 5 mg mL^{-1} BSA was added. The plate was reincubated at room temperature for 1 h, and washed as

before. Finally, 100 µL of developing solution (0.03% H_2O_2, 2 mg mL^{-1} o-phenylenediamine in citrate buffer 0.1 M, pH 5.5) was added and incubated at room temperature until color emerged. The reaction was terminated by the addition of 100 µL of 2 M H_2SO_4, and A492 was measured using a multiwell spectrophotometer (VERSAmax™).The inhibition rate (%) was calculated using the equation : Inhibition rate (%) = [1-(A_{492}/ $A_{492Control}$)]X100%

Determination of acute toxicity (LD_{50})

The LD_{50} was determined by using rats. They were injected with different increasing doses of the synthesized compounds. The dose that killed 50% of the animal was calculated according to *Austen* et al. 1961 [30].

3. Results and discussion

Chemistry

In continuation of our previous work, a series of steroidal arylidine and pyazoline candidates **1-11** (Figs. 3 & 4) were synthesized before [31]. Herein, we report the activities of these compounds for evaluation as anti-androgenic, anti-prostate cancer and EGFR, VEGFR-2 kinase inhibitor agents.

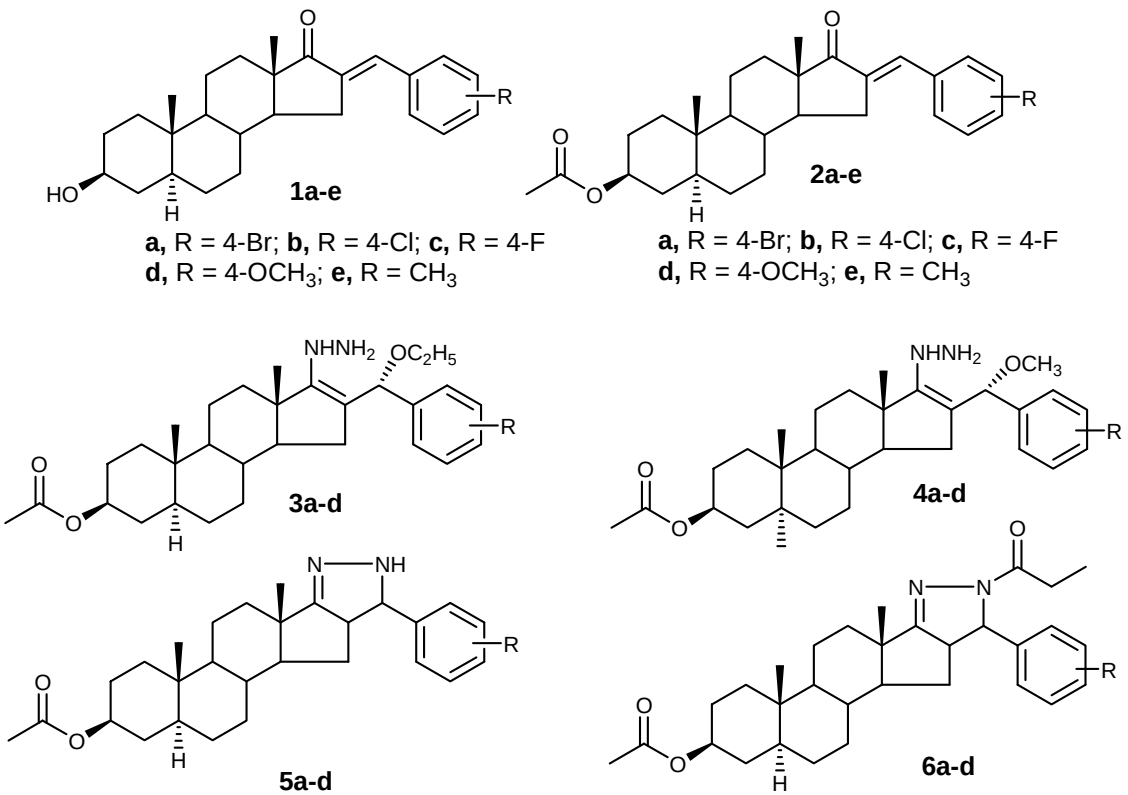
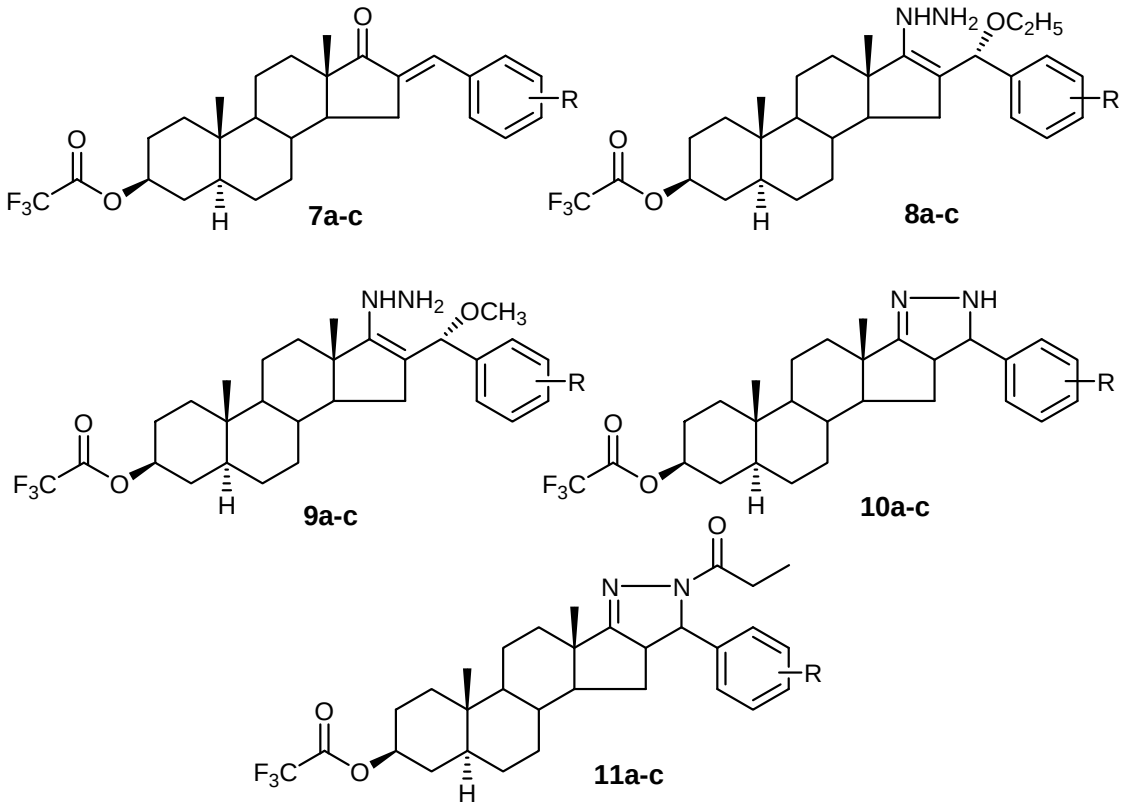


Fig. 3: Chemical structure for compounds 2-6



a, R = 4-Br; **b**, R = 4-F; **c** R = CH_3

Fig. 4: Chemical structure for compounds 7-11

Pharmacological Activities

Anti-androgenic activities

All the synthesized compounds were tested for their transcriptional activity for Human androgen receptor, the authors found that all the compounds except derivatives **(1a-e, 2a-e)** and **(7a-c)** having *in vitro* androgen Receptor (AR) Antagonistic Activities (Table 1). The obtained good data tabulated in Table 1 prompted the author to screen these compounds for their *in vivo* anti-androgenic activities in Castrated Immature Rats, the authors found that all the tested compounds except derivatives **(1a-e** and **2a-e)** and **(7a-c)** having anti-androgenic activities (Table 2).

Table 1: In vitro Androgen Receptor (AR) antagonistic activities of synthesized compounds 1-11

Compound No	IC_{50} (μM)a
Bicalutamide	$0.8900 \pm 2.1 \times 10^{-3}$
1a	Inactive
1b	Inactive
1c	Inactive
1d	Inactive
1e	Inactive
2a	Inactive
2b	Inactive
2c	Inactive
2d	Inactive
2e	Inactive
3a	$0.0098 \pm 4 \times 10^{-7}$

Compound No	IC_{50} (μM)a
3b	$0.0089\pm3x10^{-7}$
3c	$0.0081\pm4x10^{-7}$
3d	$0.0107\pm6x10^{-7}$
4a	$0.0050\pm4x10^{-7}$
4b	$0.0046\pm6x10^{-7}$
4c	$0.0041\pm5x10^{-7}$
4d	$0.0055\pm4x10^{-7}$
5a	$0.0013\pm1x10^{-7}$
5b	$0.0012\pm1.Ex10^{-7}$
5c	$0.0011\pm1.2x10^{-7}$
5d	$0.0015\pm2x10^{-7}$
6a	$0.0026\pm4x10^{-7}$
6b	$0.0023\pm3x10^{-7}$
6c	$0.0021\pm3x10^{-7}$
6d	$0.0028\pm4x10^{-7}$
7a	Inactive
7b	Inactive
7c	Inactive
8a	$0.0130\pm8x10^{-7}$
8b	$0.0118\pm7x10^{-7}$
8c	$0.0143\pm9x10^{-7}$
9a	$0.0067\pm3x10^{-7}$
9b	$0.0061\pm4x10^{-7}$
9c	$0.0073\pm3x10^{-7}$
10a	$0.0018\pm2x10^{-7}$
10b	$0.0016\pm2x10^{-7}$
10c	$0.0019\pm2x10^{-7}$
11a	$0.0034\pm5x10^{-7}$
11b	$0.0031\pm5x10^{-7}$
11c	$0.0038\pm4x10^{-7}$

[a] Compounds were tested for their ability to inhibit AR mediated transcriptional activation using a reporter assay. IC_{50} values data represent mean values for 8 separate experiments. Average and averages, n = 8,

Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

Table 2: In vivo anti-androgen activities of synthesized compounds 1-11

Compound No	% (inhibition)	ED_{50} μM
Bicalutamide	95.00±0.23	1.60±0.001
1a	Inactive	Inactive
1b	Inactive	Inactive
1c	Inactive	Inactive
1d	Inactive	Inactive
1e	Inactive	Inactive
2a	Inactive	Inactive

Compound No	% (inhibition)	ED_{50} µM
2b	Inactive	Inactive
2c	Inactive	Inactive
2d	Inactive	Inactive
2e	Inactive	Inactive
3a	98.62±0.43	0.52±0.009
3b	98.67±0.44	0.50±0.008
3c	98.71±0.45	0.48±0.005
3d	98.58±0.44	0.54±0.007
4a	98.94±0.84	0.39±0.003
4b	98.98±0.75	0.38±0.002
4c	99.03±0.84	0.36±0.003
4d	98.89±0.73	0.41±0.004
5a	99.58±0.23	0.23±0.001
5b	99.62±0.33	0.22±0.001
5c	99.67±0.23	0.21±0.001
5d	99.53±0.33	0.24±0.001
6a	99.26±0.55	0.30±0.004
6b	99.30±0.64	0.29±0.003
6c	99.35±0.55	0.28±0.003
6d	99.21±0.44	0.31±0.003
7a	Inactive	Inactive
7b	Inactive	Inactive
7c	Inactive	Inactive
8a	98.49±0.64	0.58±0.005
8b	98.53±0.53	0.56±0.006
8c	98.44±0.55	0.61±0.004
9a	98.80±0.54	0.44±0.006
9b	98.85±0.63	0.43±0.005
9c	98.76±0.56	0.46±0.005
10a	99.44±0.35	0.26±0.002
10b	99.49±0.24	0.25±0.002
10c	99.40±0.44	0.27±0.002
11a	99.12±0.84	0.34±0.003
11b	99.17±0.75	0.32±0.002
11c	99.08±0.93	0.35±0.004

IC_{50} data represent mean values for 8 separate experiments. Average and average ± SE, n = 8, Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

Anti-prostate cancer

All the tested compounds were screened as antitumor activities in different prostate cell lines namely, LNCaP-Rf, BM18, pRNS-1-1/ras, RC58T/hTERT and PPC-1. From the resulting data in Tables 3 and 4, all these compounds except derivatives **(1a-e, 2a-e)** and **(7a-c)** stopped the growth of the prostate cancer in these prostate cancer cell lines (Tables 3a and 3b). Anti-prostate cancer screening anti-androgenic bioassay in human prostate cancer cells were done for all the tested compounds depending on the light of the previous obtained data (Tables 1-3) and

calumniated on all these compounds except derivatives **(1a-e, 2a-e)** and **(7a-c)** were founded to be active (Table 4).

Table 3a: In Vitro antiproliferative activities of synthesized compounds 1-11

Comp. No	IC50 µM Tumor cell growth inhibition				
	LNCaP-Rf	BM18	pRNS-1-1/ras	RC58T/hTERT	PPC-1
Bicalutamide	0.02±2 $x10^{-10}$	0.03±1 $x10^{-10}$	0.04±4 $x10^{-10}$	0.04±5 $x10^{-10}$	0.098±5 $x10^{-10}$
1a	Inactive	Inactive	Inactive	Inactive	Inactive
1b	Inactive	Inactive	Inactive	Inactive	Inactive
1c	Inactive	Inactive	Inactive	Inactive	Inactive
1d	Inactive	Inactive	Inactive	Inactive	Inactive
1e	Inactive	Inactive	Inactive	Inactive	Inactive
2a	Inactive	Inactive	Inactive	Inactive	Inactive
2b	Inactive	Inactive	Inactive	Inactive	Inactive
2c	Inactive	Inactive	Inactive	Inactive	Inactive
2d	Inactive	Inactive	Inactive	Inactive	Inactive
2e	Inactive	Inactive	Inactive	Inactive	Inactive
3a	0.00017±6 $x10^{-10}$	0.00030±3 $x10^{-10}$	0.00080±3 $x10^{-10}$	0.00112±3 $x10^{-10}$	0.00260±3 $x10^{-10}$
3b	0.00017±5 $x10^{-10}$	0.00028±2 $x10^{-10}$	0.00076±4 $x10^{-10}$	0.00103±3 $x10^{-10}$	0.00236±4 $x10^{-10}$
3c	0.00017±4 $x10^{-10}$	0.00027±3 $x10^{-10}$	0.00071±3 $x10^{-10}$	0.00096±2 $x10^{-10}$	0.00215±3 $x10^{-10}$
3d	0.00018±5 $x10^{-10}$	0.00031±4 $x10^{-10}$	0.00085±4 $x10^{-10}$	0.00120±4 $x10^{-10}$	0.00286±4 $x10^{-10}$
4a	0.00015±2 $x10^{-10}$	0.00022±2 $x10^{-10}$	0.00053±3 $x10^{-10}$	0.00065±5 $x10^{-10}$	0.00133±4 $x10^{-10}$
4b	0.00015±3 $x10^{-10}$	0.00022±3 $x10^{-10}$	0.00050±3 $x10^{-10}$	0.00060±4 $x10^{-10}$	0.00121±3 $x10^{-10}$
4c	0.00015±3 $x10^{-10}$	0.00021±2 $x10^{-10}$	0.00047±4 $x10^{-10}$	0.00056±5 $x10^{-10}$	0.00110±3 $x10^{-10}$
4d	0.00015±2 $x10^{-10}$	0.00023±3 $x10^{-10}$	0.00057±3 $x10^{-10}$	0.00070±4 $x10^{-10}$	0.00147±3 $x10^{-10}$
5a	0.00011±4 $x10^{-10}$	0.00013±3 $x10^{-10}$	0.00024±4 $x10^{-10}$	0.00022±4 $x10^{-10}$	0.00035±3 $x10^{-10}$
5b	0.00011±3 $x10^{-10}$	0.00012±2 $x10^{-10}$	0.00022±5 $x10^{-10}$	0.00021±3 $x10^{-10}$	0.00032±4 $x10^{-10}$
5c	0.00011±2 $x10^{-10}$	0.00012±1 $x10^{-10}$	0.00021±4 $x10^{-10}$	0.00019±4 $x10^{-10}$	0.00029±4 $x10^{-10}$
5d	0.00012±4 $x10^{-10}$	0.00013±3 $x10^{-10}$	0.00025±3 $x10^{-10}$	0.00024±4 $x10^{-10}$	0.00039±4 $x10^{-10}$
6a	0.00013±3 $x10^{-10}$	0.00017±2 $x10^{-10}$	0.00035±2 $x10^{-10}$	0.00038±4 $x10^{-10}$	0.00068±6 $x10^{-10}$
6b	0.00013±3 $x10^{-10}$	0.00016±3 $x10^{-10}$	0.00033±3 $x10^{-10}$	0.00035±4 $x10^{-10}$	0.00062±5 $x10^{-10}$
6c	0.00013±4 $x10^{-10}$	0.00016±2 $x10^{-10}$	0.00032±4 $x10^{-10}$	0.00033±4 $x10^{-10}$	0.00057±5 $x10^{-10}$
6d	0.00013±2 $x10^{-10}$	0.00018±3 $x10^{-10}$	0.00038±3 $x10^{-10}$	0.00041±4 $x10^{-10}$	0.00075±5 $x10^{-10}$
7a	Inactive	Inactive	Inactive	Inactive	Inactive

Comp. No	IC50 µM Tumor cell growth inhibition				
	LNCaP-Rf	BM18	pRNS-1-1/ras	RC58T/hTERT	PPC-1
7b	Inactive	Inactive	Inactive	Inactive	Inactive
7c	Inactive	Inactive	Inactive	Inactive	Inactive
8a	0.00018±4 x10^{-10}	0.00033±2 x10^{-10}	0.00096±3 x10^{-10}	0.00141±4 x10^{-10}	0.00346±3 x10^{-10}
8b	0.00018±4 x10^{-10}	0.00032±3 x10^{-10}	0.00090±3 x10^{-10}	0.00130±5 x10^{-10}	0.00314±3 x10^{-10}
8c	0.00019±3 x10^{-10}	0.00035±3 x10^{-10}	0.00101±3 x10^{-10}	0.00152±3 x10^{-10}	0.00380±2 x10^{-10}
9a	0.00016±2 x10^{-10}	0.00025±3 x10^{-10}	0.00064±3 x10^{-10}	0.00082±2 x10^{-10}	0.00177±3 x10^{-10}
9b	0.00016±2 x10^{-10}	0.00024±2 x10^{-10}	0.00060±2 x10^{-10}	0.00076±3 x10^{-10}	0.00161±4 x10^{-10}
9c	0.00016±3 x10^{-10}	0.00026±2 x10^{-10}	0.00067±4 x10^{-10}	0.00089±3 x10^{-10}	0.00195±4 x10^{-10}
10a	0.00012±6 x10^{-10}	0.00015±2 x10^{-10}	0.00028±4 x10^{-10}	0.00028±4 x10^{-10}	0.00047±4 x10^{-10}
10b	0.00012±5 x10^{-10}	0.00014±2 x10^{-10}	0.00027±3 x10^{-10}	0.00026±4 x10^{-10}	0.00042±3 x10^{-10}
10c	0.00012±5 x10^{-10}	0.00015±1 x10^{-10}	0.00030±3 x10^{-10}	0.00030±4 x10^{-10}	0.00051±4 x10^{-10}
11a	0.00014±2 x10^{-10}	0.00019±3 x10^{-10}	0.00042±5 x10^{-10}	0.00048±4 x10^{-10}	0.00091±4 x10^{-10}
11b	0.00014±3 x10^{-10}	0.00018±4 x10^{-10}	0.00040±4 x10^{-10}	0.00044±4 x10^{-10}	0.00083±4 x10^{-10}
11c	0.00014±3 x10^{-10}	0.00020±2 x10^{-10}	0.00045±4 x10^{-10}	0.00052±4 x10^{-10}	0.00100±3 x10^{-10}

All data represent mean values for 8 separate experiments. Average and average ±SE, n = 8, Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

Table 3b: Selective Cytotoxicity Index SCI of synthesized compounds 1-11

Comp. No	Selective cytotoxicity index (SCI)				
	LNCaP-Rf	**BM18**	**pRNS-1-1/ras**	**RC58T/hTERT**	**PPC-1**
Bicalutamide	1345	1245	1234	1045	1421
3a	1359.869	11095	1899.686	1787.305	4054.279
3b	1361.229	13535.91	1975.674	1876.67	4459.707
3c	1347.691	1853.058	1334.694	1152.113	1719.41
3d	1349.039	2260.731	1388.082	1209.718	1891.351
4a	1350.388	2758.092	1443.605	1270.204	208.486
4b	1351.738	3364.872	1501.35	1333.714	228.535
4c	1353.09	4105.143	1561.404	1400.4	251.388
4d	1379.032	17941.6	3289.642	3538.741	156.12
5a	1380.411	2190.8	3421.228	3715.678	135.73
5b	1381.791	2672.7	3558.077	3901.462	8629.3
5c	1383.173	3260.3	3700.4	4096.535	492.23
5d	1368.049	365.6	2403.709	2395.159	182.402
6a	1369.417	4462	2499.858	2514.917	900.643
6b	1370.786	5421.03	2599.852	2640.663	690.707
6c	1372.157	6230.26	2703.846	2772.696	559.777
6d	1390.103	8837.7	4502.102	5228.332	3002.94

Comp. No	Selective cytotoxicity index (SCI)				
	LNCaP-Rf	BM18	pRNS-1-1/ras	RC58T/hTERT	PPC-1
8a	1391.493	1988	4682.186	5489.749	303.24
8b	1392.884	1485	4869.474	5764.236	333.56
8c	1394.277	2012	5064.253	6052.448	436.92
9a	1395.671	1955	5266.823	6355.07	483.61
9b	1397.067	2381	5477.496	6672.824	531.57
9c	1398.464	2909	5696.596	7006.465	586.73
10a	1399.863	3562	5924.459	7356.788	613.4
10b	1413.924	1719	8769.647	1193.43	1812.4
10c	1415.338	3197	9120.433	1252.6	1893.6
11a	1416.754	3853	9485.25	1321.73	201843
11b	1418.17	4701	9864.66	1387.32	2227.3
11c	1419.588	5735	1025.25	1456.94	244230

Table 4: Anti-prostate cancer activities of synthesized compounds 1-11

Compound No	Cytotoxicity IC_{50} (µM)	
	PC-3	LNCaP
Bicalutamide	$0.82\pm8\times10^{-14}$	$0.61900\pm6\times10^{-17}$
1a	Inactive	Inactive
1b	Inactive	Inactive
1c	Inactive	Inactive
1d	Inactive	Inactive
1e	Inactive	Inactive
2a	Inactive	Inactive
2b	Inactive	Inactive
2c	Inactive	Inactive
2d	Inactive	Inactive
2e	Inactive	Inactive
3a	$0.49\pm3\times10^{-14}$	$0.34250\pm5\times10^{-17}$
3b	$0.48\pm4\times10^{-14}$	$0.28542\pm4\times10^{-17}$
3c	$0.47\pm5\times10^{-14}$	$0.23785\pm3\times10^{-17}$
3d	$0.50\pm3\times10^{-14}$	$0.41100\pm4\times10^{-17}$
4a	$0.43\pm6\times10^{-14}$	$0.09559\pm4\times10^{-17}$
4b	$0.42\pm5\times10^{-14}$	$0.07965\pm3\times10^{-17}$
4c	$0.41\pm4\times10^{-14}$	$0.06638\pm4\times10^{-17}$
4d	$0.44\pm5\times10^{-14}$	$0.11470\pm5\times10^{-17}$
5a	$0.32\pm6\times10^{-14}$	$0.00744\pm5\times10^{-17}$
5b	$0.32\pm7\times10^{-14}$	$0.00620\pm4\times10^{-17}$
5c	$0.31\pm6\times10^{-14}$	$0.00517\pm5\times10^{-17}$
5d	$0.33\pm4\times10^{-14}$	$0.00893\pm6\times10^{-17}$
6a	$0.37\pm5\times10^{-14}$	$0.02668\pm3\times10^{-17}$
6b	$0.37\pm4\times10^{-14}$	$0.02223\pm4\times10^{-17}$
6c	$0.36\pm4\times10^{-14}$	$0.01853\pm5\times10^{-17}$
6d	$0.38\pm6\times10^{-14}$	$0.03201\pm4\times10^{-17}$
7a	Inactive	Inactive
7b	Inactive	Inactive

Compound No	Cytotoxicity IC_{50} (µM)	
	PC-3	LNCaP
7c	Inactive	Inactive
8a	$0.52\pm5\times10^{-14}$	$0.59184\pm3\times10^{-17}$
8b	$0.51\pm4\times10^{-14}$	$0.49320\pm3\times10^{-17}$
8c	$0.53\pm5\times10^{-14}$	$0.71021\pm3\times10^{-17}$
9a	$0.45\pm3\times10^{-14}$	$0.16517\pm5\times10^{-17}$
9b	$0.45\pm4\times10^{-14}$	$0.13764\pm6\times10^{-17}$
9c	$0.46\pm4\times10^{-14}$	$0.19821\pm4\times10^{-17}$
10a	$0.34\pm4\times10^{-14}$	$0.01286\pm8\times10^{-17}$
10b	$0.34\pm3\times10^{-14}$	$0.01072\pm7\times10^{-17}$
10c	$0.35\pm3\times10^{-14}$	$0.01544\pm6\times10^{-17}$
11a	$0.40\pm6\times10^{-14}$	$0.04610\pm6\times10^{-17}$
11b	$0.39\pm7\times10^{-14}$	$0.03841\pm5\times10^{-17}$
11c	$0.40\pm5\times10^{-14}$	$0.05532\pm5\times10^{-17}$

All data represent mean values for 8 separate experiments. Average and average ±SE, n = 8, Statistical comparison of the difference between control group and treated groups was done by one way ANOVA and Duncan's multiple comparison test *P < 0.05.

EGFR and VEGFR-2 kinase inhibitor activities

Aiming to clarification the anticancer activities both the EGFR and VEGFR-2 kinase activity assay by ELISA were done for all the newly synthesized compounds and calumniated on all these compounds except derivatives **(1a-e, 2a-e)** and **(7a-c)** were founded to be EGFR and VEGFR-2 kinase inhibitor activities (Table 5).

Table 5: Enzymatic inhibition (VEGFR-2/EGFR) of the synthesized compounds

Comp. No	Enzymatic inhibition (IC50/µM)	
	VEGFR-2	EGFR
1a	Inactive	Inactive
1b	Inactive	Inactive
1c	Inactive	Inactive
1d	Inactive	Inactive
1e	Inactive	Inactive
2a	Inactive	Inactive
2b	Inactive	Inactive
2c	Inactive	Inactive
2d	Inactive	Inactive
2e	Inactive	Inactive
3a	$0.08515\pm5\times10^{-4}$	$1.58235\pm7\times10^{-3}$
3b	$0.08348\pm4\times10^{-4}$	$1.52149\pm6\times10^{-3}$
3c	$0.08185\pm5\times10^{-4}$	$1.46297\pm5\times10^{-3}$
3d	$0.08686\pm6\times10^{-4}$	$1.64564\pm8\times10^{-3}$
4a	$0.07413\pm5\times10^{-4}$	$1.20245\pm8\times10^{-3}$
4b	$0.07268\pm4\times10^{-4}$	$1.15621\pm9\times10^{-3}$
4c	$0.07125\pm5\times10^{-4}$	$1.11174\pm7\times10^{-3}$
4d	$0.07561\pm6\times10^{-4}$	$1.25055\pm4\times10^{-3}$

Comp. No	Enzymatic inhibition (IC50/µM)	
	VEGFR-2	**EGFR**
5a	$0.05618\pm5\times10^{-4}$	$0.69439\pm3\times10^{-3}$
5b	$0.05508\pm4\times10^{-4}$	$0.66768\pm2\times10^{-3}$
5c	$0.05400\pm3\times10^{-4}$	$0.64200\pm1\times10^{-3}$
5d	$0.05731\pm6\times10^{-4}$	$0.72216\pm4\times10^{-3}$
6a	$0.06453\pm8\times10^{-4}$	$0.91377\pm6\times10^{-3}$
6b	$0.06327\pm7\times10^{-4}$	$0.87862\pm7\times10^{-3}$
6c	$0.06203\pm6\times10^{-4}$	$0.84483\pm8\times10^{-3}$
6d	$0.06583\pm7\times10^{-4}$	$0.95032\pm5\times10^{-3}$
7a	Inactive	Inactive
7b	Inactive	Inactive
7c	Inactive	Inactive
8a	$0.09036\pm7\times10^{-4}$	$1.77993\pm6\times10^{-3}$
8b	$0.08859\pm6\times10^{-4}$	$1.71147\pm7\times10^{-3}$
8c	$0.09217\pm6\times10^{-4}$	$1.85112\pm6\times10^{-3}$
9b	$0.07713\pm6\times10^{-4}$	$1.30057\pm3\times10^{-3}$
9a	$0.07867\pm7\times10^{-4}$	$1.35260\pm2\times10^{-3}$
9c	$0.08024\pm6\times10^{-4}$	$1.40670\pm4\times10^{-3}$
10a	$0.05962\pm4\times10^{-4}$	$0.78109\pm8\times10^{-3}$
10b	$0.05845\pm5\times10^{-4}$	$0.75105\pm7\times10^{-3}$
10c	$0.06081\pm5\times10^{-4}$	$0.81233\pm9\times10^{-3}$
11a	$0.06849\pm7\times10^{-4}$	$1.02786\pm5\times10^{-3}$
11b	$0.06714\pm6\times10^{-4}$	$0.98833\pm4\times10^{-3}$
11c	$0.06985\pm6\times10^{-4}$	$1.06898\pm6\times10^{-3}$
Delphinidin	5.09±0.0012	6.27±0.00076

All data represent mean values for 8 separate experiments. Average and average ±SE, n = 8, Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

Acute toxicity (LD_{50})

The LD_{50} of all compounds determined and indicating reasonable accepted safety margins (Table 6).

Table 6: Acute toxicity (LD_{50}) of the synthesized compounds 1-11.

Comp. No	LD_{50} [mg/kg]
1a	1440.87±8.5
1b	1485.54±7.6
1c	1531.59±8.7
1d	1579.07±9.8
1e	1628.02±9.8
2a	1678.49±9.7
2b	1730.52±9.8
2c	1784.17±9.7
2d	1839.48±9.7
2e	1896.50±8.6
3a	1236.89±8.8
3b	1199.70±9.7

3c	1163.63±9.8
3d	1275.24±9.7
4a	998.90±8.5
4b	968.86±7.6
4c	939.73±6.8
4d	1029.86±6.6
5a	651.48±8.7
5b	631.89±7.8
5c	612.89±9.9
5d	671.67±9.8
6a	806.70±9.5
6b	782.44±8.6
6c	758.92±9.5
6d	831.70±8.6
7a	1955.29±7.6
7b	2015.91±6.5
7c	2078.40±7.6
8a	1355.53±8.6
8b	1314.77±9.7
8c	1397.55±9.6
9a	1094.71±7.8
9b	1061.79±5.7
9c	1128.64±8.9
10a	713.96±7.7
10b	692.50±8.9
10c	736.10±8.6
11a	884.07±8.6
11b	857.49±9.5
11c	911.48±7.7

All data represent mean values for 8 separate experiments. Average and average ±SE, n = 8, Statistical comparison of the difference between control group and treated groups was done by one-way ANOVA and Duncan's multiple comparison test *P < 0.05.

4. Conclusion

The pyrazoline derivatives provided the highest androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **5, 6, 10** and **11**) (Table 5). N-Propinoyl pyrozline derivatives (**6** and **11**) were less active as androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities than the deacylated ones (**5** and **10**), the high activities of the later derivatives due to the free lone pair of electrons on the NH atom that capable of forming hydrogen bonding with the receptor sites. Careful examination of all the data obtained leads to the following facts and assumptions. Opening the pyrazoline ring decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **3,4,8** and **9**) due to conformational changes in molecule cage where the strain present in the pyrazoline moiety facilitate approach and binding with the receptor site due to small size and tight cage (derivatives **5,6,10** and **11** specially in **5** and **10**) while the open hydrazine part is slightly planner and spread over the receptor with no complete fitting characters, this hypothesis supported by the activities of the smaller size 16-methoxyl (derivatives **4** and **8**) were high than that of those of the biggest 16-ethoxyl (derivatives **3** and **9**) due to also the same reason where the methoxyl is less stereo hindered than the ethoxyl and permit approach and fitting to the receptor binding sit.

The 3β- trifluoroacetoxyl decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **2** to **6**) than the 3β-acetoxyl ones (derivatives **7** to **11**) due to high strict hindrance of the fluoride atom that make crowdedness that

hinders the approach and active fitting receptor site than the smaller hydrogen one. The fluoride atom on the aromatic moiety increases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities more than the bromide ones because the fluoride is of high inductive effect plus smaller in size some induces and permits approach and fitting to the active receptor sites.

The methoxyl group with +M decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities than that of the halide atom with –I, the same happens with slightly neutral group as methyl one due to charge accumulation with +M and charge separation with –I effects that play major role with attraction to the receptor clouds and neighboring (-I) and reputation with (+M). The arylidene derivatives **2** and **7** completely devoid from any androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities probably due to the absence of any nitrogen binding site and the remote cage effects that can it induces it.

Structure Activity Relationship (SAR)

- The pyrazoline derivatives provided the highest androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **5, 6, 10** and **11**).
- N-propionyl pyrozline derivatives (**6** and **11**) were less active as androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities than the deacylated ones (**5** and **10**).
- Opening the pyrazoline ring decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **3,4,8** and **9**) .
- The 3β- trifluoroacetoxyl decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities (derivatives **2** to **6**) than the 3β-acetoxyl ones (derivatives **7** to **11**).
- The fluoride atom on the aromatic moiety increases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities more than the bromide ones.
- The methoxyl group with +M decreases the androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities than that of the halide atom with –I, the same happens with slightly neutral group as methyl one due to charge accumulation with +M and charge separation with –I effects that play major role with attraction to the receptor clouds and neighboring (-I) and reputation with (+M).
- The arylidene derivatives (**2** and **7**) completely devoid from any androgen Receptor, anti-prostate cancer and EGFR and VEGFR-2 kinase inhibitor activities.

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-0172.

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