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**ARTICLE** *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · APRIL 2015

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# Cu (I) catalyzed alkyne-azide 1,3-dipolar cycloaddition (CuAAC): Synthesis of 17α-[1-(substituted phenyl)-1,2,3-triazol-4-yl]-19-nor-testosterone-17β-yl acetates targeting progestational and antipro-liferative activities

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ARTICLE INFO

*Article history:*
Received 4 December 2014
Received in revised form
30 March 2015
Accepted 22 April 2015
Available online 28 April 2015

*Keywords:*
Click chemistry
Aza steroids
Progestational activity
Docking
Anticancer activity

ABSTRACT

The progestational potency and selectivity of synthetic steroidal agonists can be enhanced by even larger chemical moieties at 17α-position of the steroid backbones. Hereby a series **5a-c** and **6a-c** of novel 17α-[1-(substituted phenyl)-1,2,3-triazol-4-yl]-19-nortestosterone-17β-yl acetates were designed and synthesized using click chemistry approach searching progestogenic derivatives with potential anticancer activity. Compounds **5a**,**b** and **6a**,**c** have affected to different extents the three histopatho-logical parameters considered for evaluation of their progestational activity. The compounds **5a**,**b** and **6a**,**c** showed modifications in rat uterus at 35.7–34.8 nM levels with privileged endometrial thickening effect and least change of uterine weight relative to NEA at 52.9 nM level. Up to 40 mg/kg dose compounds **5b** and **6c** were non-toxic. Molecular docking of the ligands in PR showed in the majority of cases a conformational fitting into the active site different from that of the reference steroid NEA. Compound **6b** revealed about 46.4% growth inhibition of CNS cancer SNB-75 cell line, 56% growth inhibition of renal cancer A498 cell line and 56.7% growth inhibition of prostate cancer PC-3 cell line which was mediated by cell cycle arrest. Drugability of the screened compounds showed tolerated results after being challenged to diverse physicochemical parameters.



## 1. Introduction

Steroidal derivatives in which ring D is modified with exo-heterocycles exhibit numerous forms of biological activity and are attractive for medicine. A large number of steroid derivatives containing five- or six-membered 17β-exo-heterocycles are known to cause the efficient inhibition of 17α-hydroxylase/C17, 20-lyase (P45017α), which can block androgen synthesis at an early stage, and may therefore be useful in the treatment of prostatic carcinoma. Also some steroid compounds are known to exert hormone receptor-independent antiproliferative activity via the inhibition of angiogenesis, tubulin polymerization and the up regulation of apoptotic pathways [1].

As previously reported progesterone receptor pockets can rearrange to accommodate different agonists [2]. Searching the progestational potency and selectivity of synthetic steroid agonists were enhanced by bulky chemical moieties at 17α-position of the steroid backbones [3].

In drug discovery 1,4-disubstituted-1,2,3-triazole moieties are attractive connecting units because they are stable to metabolic degradation and capable of hydrogen bonding, which can be favorable in the binding with biomolecular targets and can improve the solubility [4].

Cu (I) catalyzed alkyne-azide 1,3-dipolar cycloaddition (CuAAC) was exploited by many authors for the synthesis of 1,4-disubstituted 1,2,3-triazoles of antiproliferative [5–8], antiviral [9], antitubercular [10,11], antifungal [12–15] and antibacterial agents [16,17].

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http://dx.doi.org/10.1016/j.ejmech.2015.04.045
0223-5234/

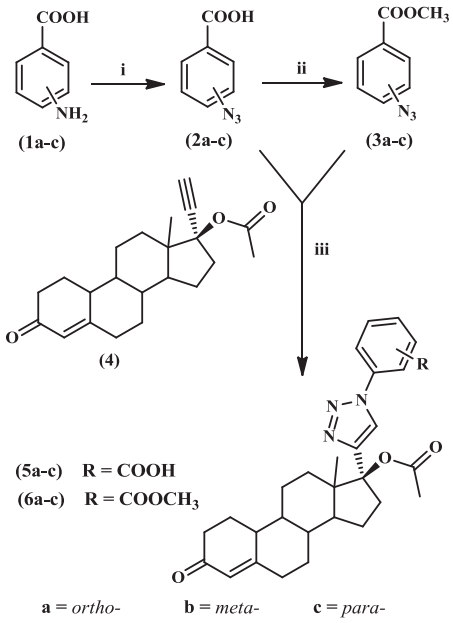
Our group applied CuAAC reaction for the condensation of the terminal ethynyl group of norethindrone enanthate and levonorgestrel with 3- and 4-substituted phenyl azides to prepare 1,4-disubstituted-1,2,3-triazole derivatives. In animal experiments the new derivatives disclosed potent progestational action at nM levels and marked decrease in uterine contraction [18].

In extension of our previous work we conjugated 17α-ethynyl group of norethindrone acetate (NEA) with three positional isomers of azidobenzoic acid and their methyl esters to prepare 17α-[1- (substituted phenyl)-1,2,3-triazol-4-yl]-19-nortesto- sterone-17β-yl acetates as potential candidates of progestational and antiproliferative activities. In the planned work we discussed the relation between the observed biological activity of the prepared isomers and *in silico* ligand receptor interaction abilities.

## 2. Results and discussion

### 2.1. Chemistry

Compounds **5a**–**c** and **6a**–**c** were synthesized by conventional methods outlined in Scheme 1. Different methods were reported for the synthesis of organic azides [19]. In our work 2-,3-, and 4-azidobenzoic acids **2a**–**c** were prepared by diazotization of the available 2-, 3-, and 4-aminobenzoic acids **1a**–**c**, followed by azidation of the resulting diazonium salts with sodium azide [20]. Methyl azidobenzoates **3a**–**c** were obtained via esterification of the corresponding azidobenzoic acids **2a**–**c** with methanol under reflux in presence of sulfuric acid as catalyst. It was observed that the *o*-azidobenzoic acid, **2a** and its methyl ester, **3a** showed the lowest yield (83 and 63% respectively) relative to the other two isomers that may be attributed to the steric hindrance of the vicinal moieties on the reacting substrates. The targeted derivatives of NEA, **5a**–**c** and **6a**–**c**, were prepared under click reaction conditions through 1,3-cycloaddition of the terminal ethynyl group of NEA, **4** and the respective azides, **2a**–**c** and **3a**–**c** catalyzed by Cu (I). The reactions were performed at room temperature under nitrogen and in presence of ten equivalents of sodium ascorbate in (1:1) aqueous




**Scheme 1.** Synthesis of the target compounds **5a**–**c** and **6a**–**c**. Reagents and conditions: (i) $H_2SO_4$, $NaNO_2$ then $NaN_3$ (ii) $CH_3OH$, $H_2SO_4$, Reflux,4 h; (iii) $CuSO_4.5H_2O$, Sodium ascorbate $H_2O$/t-BuOH (1:1), r.t.,$N_2$,8 h.

*t*-BuOH milieu. Trial to accelerate the synthesis of compound **5c** by heating the reaction mixture up to 60 °C was unsuccessful where unidentified products were obtained. Sensitivity of CuAAC reaction to steric hindrance was observed through the decreased yields (45% and 50%) of the triazoles **5a** and **6a** derived from *o*-azidobenzoic acid, **2a** and its methyl ester, **3a** relative to the other ligands prepared from the *m*- and *p*-isomers (55–75%). All of the synthetic compounds were characterized by IR, $^1$H NMR, and elemental analyses. $^{13}$C NMR for representative compounds was also carried out and was confirmed by DEPT experiments at 135°. All compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

### 2.2. Biological activity

#### 2.2.1. In vivo progestational activity

Development of X-ray crystal structure of the human progesterone receptor–ligand binding domain (PR-LBD) complexed with progesterone, norethisterone and other synthetic agonist and antagonist progestins paved the way for the development of novel clinically useful PR ligands. The binding of these ligands to the LBD subsequently induces conformational changes that lead to cascade of biological outcomes [21]. The preliminary *in vivo* progestational activity of the synthesized compounds **5a**–**c** and **6a**–**c** was evaluated using adult female Wistar rats. Induced uterine histopathological changes displayed by Table 1 and Fig. 1 were matched with the reference NEA and the vehicle DMSO as a control.

Differences observed in thickness of endometrium, myometrium and height of epithelial cells were correlated essentially with the hydrogen bonding interaction forces between the docked ligands and the progesterone receptor displayed by Table 2.

It is well documented that hydrophobic interaction is the main binding force of the steroid ligands with the PRs. Our docked ligands on PR showed contribution of 3–5 hydrophobic interaction forces more than NEA that cope with their relatively higher clog P values. Correlation between hydrophobic forces variations and the observed morphological modifications on the uterus cannot be perceived. On the other hand Cunha et al. reported hydrophilic interactions such as hydrogen bonding with the receptor, particularly in sites close to the C-17 hydroxyl function in the 19-nortestostenone series and at C-3 and C-20 in progesterone derivatives [22].

The effect of hydrogen bonding interaction forces was more influential either by their allowed number or position within the ligands.

The effect of structure variations in the prepared series on endometrial thickness can be directly correlated with the number of hydrogen bond interaction forces of ligands with the receptor. Thus **5a**, **b** and **6c** with the strongest endometrial thickening effect showed 1-2 hydrogen bonding interaction forces more than any one of **5c**, **6a**, **b** and NEA. The added number of hydrogen bonding was accompanied by decreased binding energies (ΔG) values.

The prepared compounds, **5a**–**c** and **6a**, **c** and the reference NEA showed varied magnitude of pronounced endometrial thickening than the control with maintained stronger effect than myometrial thickening. The reversed picture displayed by compound **6b** was an exception since its myometrial thickening effect was much more prominent than the endometrial. Molecular docking of **6b** in the PR (Table 2 and Fig. 2A and B) revealed the participation of the steroid C-3 and the *m*-benzoate ester carbonyls in hydrogen bonding with **Asn719** and **Arg766** respectively. Such orientation, associated with decreased ΔG score was different from that displayed by the docked *o*- and *p*-esters, **6a** and **6c** where their C-3 carbonyls were hydrogen bounded to the two amino acids **Gln725** and **Arg766** similarly involved by docked NEA (Fig. 2C). Compound **6c** showed an

**Table 1**
Impact of the electronic character, positional isomerism and type of phenyl substituents on the *in vivo* progestational activity of **5a**–**c**, **6a**–**c** and compounds cited in Ref. [18].

| Entry | Substituent on the phenyl ring | Hansh σ values | Endometrial thickness (μm) | Myometrial thickness (μm) | Epithelial cell height (μm) |
|---|---|---|---|---|---|
| **Vehicle** 20% DMSO | – | – | 154.47 ± 1.19 | 122.40 ± 2.37 | 57.80 ± 0.92 |
| **NEA** | – | – | 302.35 ± 2.2* | 228.60 ± 2.86* | 68.22 ± 0.71* |
| **5a** | *o*-COOH | NA | 452 ± 12.67*,** | 170.80 ± 8.87*,** | 45.50 ± 1.063*,** |
| **5b** | *m*-COOH | −0.1[b] | 375.5 ± 13.99*,** | 176 ± 10.27*,** | 43.60 ± 0.89*,** |
| **5c** | *p*- COOH | 0.00[b] | 277.5 ± 10.42* | 113.60 ± 3.78** | 39.60 ± 1.27*,** |
| **6a** | *o*-COOMe | NA | 343.7 ± 1.86*,** | 197.70 ± 7.92*,** | 64.60 ± 1.31* |
| **6b** | *m*-COOMe | 0.37 | 214.04 ± 2.21*,** | 319.80 ± 7.91*,** | 59.10 ± 0.75*,** |
| **6c** | *p*-COOMe | 0.45 | 417.6 ± 7.17*,** | 221.80 ± 2.56* | 29.60 ± 0.49*,** |
| **NET-EN**[a] | | | 300 ± 2* | 225 ± 7* | 67 ± 1* |
| **7**[a] | *p*-$CH_2COOH$ | −0.16[b] | 316 ± 1.5* | 239 ± 4* | 71 ± 2* |
| **8**[a] | *m*-$NO_2$ | 0.71 | 310 ± 4* | 229 ± 1* | 69 ± 0.8* |
| **9**[a] | *p*-$NO_2$ | 0.78 | 347 ± 24* | 241 ± 3* | 78 ± 1** |

*Significantly different from control group at $P < 0.05$.
**Significantly different from reference drug group at $P < 0.05$.
[a] Results cited in Ref. [18].
[b] Values of the ionized functions NA: not available.

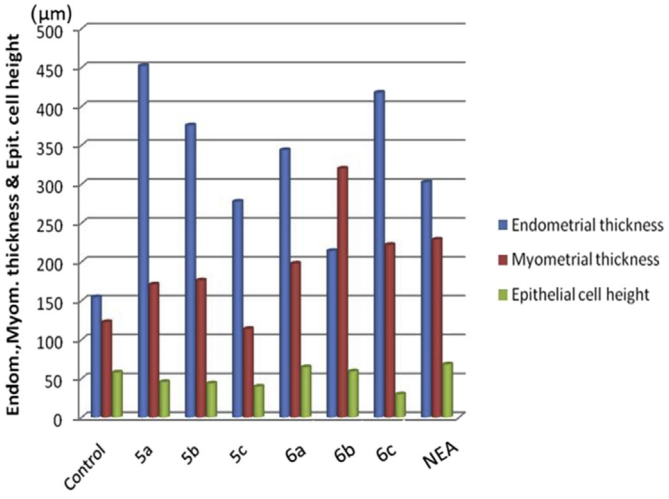



**Fig. 1.** Effect of compounds **5a**–**c**, **6a**–**c** and NEA on rat's uterus.

additional hydrogen bonding initiated between the ester carbonyl and **Asn719**. The evident differences between hydrogen bonding attachment sites of the C-3 and ester carbonyls revealed by **6a** (Fig. 2D) and **6c** on one side and those of **6b** can explain the anomalous thickening behavior of the latter.

According to the docking results, the presented interpretation cannot satisfy the three fold myometrial thickening effect of **6b** relative to the acidic **5c** showing the same ligand receptor interaction pattern. Here, other prevailing parameter than their identical binding mode to PR must be thought. Among relevant possibilities is the two fold cLog P values of the ester group **6a**–**c** relative to the carboxyl derivatives which correlates with the observed higher pattern of their myometrial thickening effect relative to those displayed by the acidic ones **5a**–**c**.

Furthermore the compounds **5a**–**c**, **6b**,**c** showed epithelial cell height values equal to or less than that of the control while **6a** is the one that showed significant higher value than the control and matching that of NEA. Actually **6a** is the single ligand in the series in which C-3 carbonyl was bound to **Gln725** and **Arg766** via two hydrogen bonds like in NEA with no further participation of other bonding interaction.

In the prepared series the substituted phenyl bridged via the 1,2,3-triazole moiety to the steroidal frame, have favorably altered interaction with the receptor. Substituents in **5a**,**b** and **6a**,**c** have affected to different extents the three histopathological parameters considered for evaluation of the progestational activity. Their role was disclosed through their ability to modify the hydrogen bonding interaction of the C-3 carbonyl group with the receptor amino acids in the active site. It was evident too that hydrogen bonding interaction of A**rg766** in the active site with either C-3 or the polar functions on the phenyl ring is essential for activity. On the other

**Table 2**
Docked Ligands/PR interactions.

| Comp. No. | ΔG (Kcal/mol) | Ligand binding groups/PR amino acids | | |
|---|---|---|---|---|
| | | Oxygen of C-3 carbonyl (distance Å) | Oxygen of carboxyl carbonyl (distance Å) | Oxygen of carboxyl hydroxy (distance Å) |
| **NEA** | −10.5131 | **Gln725** (2.73)<br>**Arg766** (2.67) | – | – |
| **5a** | −12.6971 | **Asn719** (2.82) | **Arg766** (2.49) | **Gln725** (2.77)<br>**Arg766** (2.56) |
| **5b** | −11.1299 | **Asn719** (2.69) | **Arg766** (2.72) | **Gln725** (2.72)<br>**Arg766** (2.41) |
| **5c** | −8.8336 | **Asn719** (2.65) | **Arg766** (2.31) | – |
| **6a** | −9.7881 | **Gln725** (2.78)<br>**Arg766** (2.46) | – | – |
| **6b** | −8.0234 | **Asn719** (2.43) | **Arg766** (2.31) | – |
| **6c** | −11.3694 | **Gln725** (2.52)<br>**Arg766** (3.24) | **Asn719** (2.63) | – |

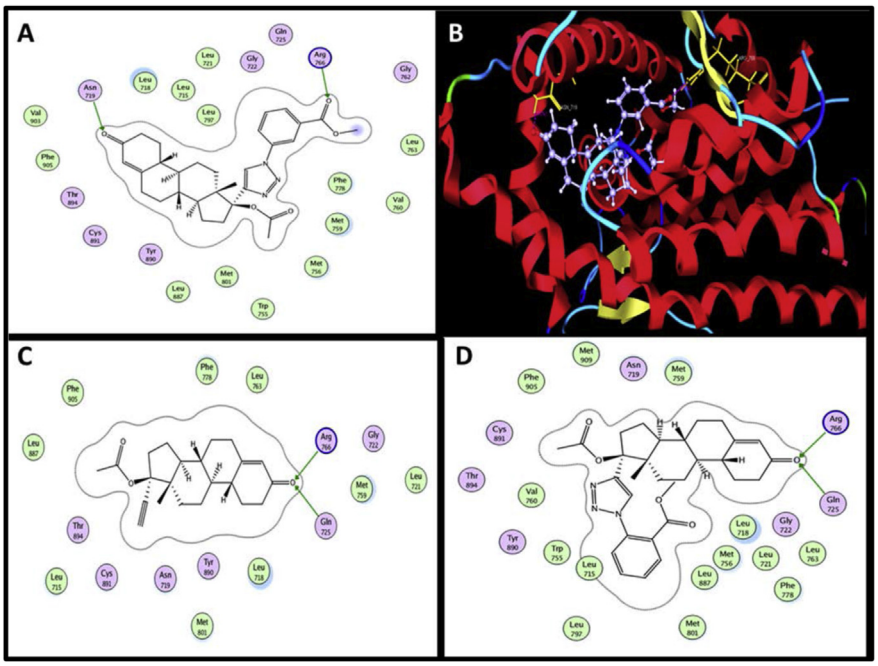



**Fig. 2.** (A) & (B) 2D & 3D representation of the binding mode of compound **6b** in the PR binding site. (C) 2D representation of the binding mode of NEA in the PR binding site. (D) 2D representation of the binding mode of compound **6a** in the PR binding site.

hand **Gln725** may be replaced or coupled with **Asn719** in the binding process to the ligands with at least conservation or enhancement of activity relative to the reference.

Relative uterine weights (RUW) of most of the screened compounds were changed within the range (1.3 ± 0.1: 2.0 ± 0.13) which is insignificantly different from that of the reference drug (1.7 ± 0.05). One exception **5c** was found to be significantly different (3.9 ± 0.35) in RUW relative to the references.

Distinct variations of the progestational activity of the prepared compounds and those cited in Ref. [18] (Fig. 3) are going to be correlated with the disparity of type and position of substituents on the phenyl ring.

Meta substitution on phenyl ring by the electron withdrawing groups $CO_2Me$ and $NO_2$ in the interties **6b** and **8** (Table 1), showed lower progestational activity in relation to **6c** and **9** isomers substituted in the para positions that was allied with relative higher Hansch electronic σ values of the para analogs [23].

On the other hand, the derivatives with ionizable carboxyls **5b**, **5c** and **7** did not show a correlation between the progestational activity and Hansch electronic σ values. It is worthy to note that separation of the COOH group by $CH_2$ distinctly ameliorated the activity of **7** relative to **5c**.

Under our experimental conditions **5a**,**b** and **6a**,**c** showed potent progestational activity (35.7–34.8 nM) with privileged endometrial thickening effect and least change of uterine weight relative to NEA (52.9 nM).

### 2.2.2. Acute toxicity and lethality ($LD_{50}$)

Acute toxicity of two active compounds **5b** and **6c** with prominent progestational activity was determined according to Buck et al. using albino mice [24]. The animals were grouped (three per group), separately received 5, 10, 15, 30 or 40 mg/kg dose and observed over 24 h for signs of toxicity and number of deaths. Control animals receiving the vehicle (10% DMSO) were kept under the same conditions without addition of **5b** or **6c**. All animals treated with the tested compounds up to 40 mg/kg dose were alive during the 24 h of observation and did not show visible signs of acute toxicity. Therefore these compounds were considered to be non-toxic.

### 2.2.3. In vitro anticancer activity

The newly synthesized 17α-(1-substituted-1,2,3-triazol- 4-yl)-19-nortestosterone acetates, **5a**–**c** and **6a**–**c** (Conc. $10^{-5}$ M) were screened for their anticancer activity according to NCI *in vitro* protocols [25] against a panel consisting of 60 human tumor cell lines. These cell lines were derived from eight cancer types: leukemia, non-small cell lung, colon, CNS, ovarian, renal, prostate and breast cancers. The most prominent activity results against the screened cancer cell lines are listed in Table 3 and the whole results are provided in the Supplementary data.

Krämer et al. studied the effect on the proliferation of HCC1500 cells incubated with estradiol and steroidal compounds in absence and in presence of growth factors. Norethendrone together with six progestins and testosterone showed to varying degrees antiproliferative effect [26]. Another study carried out by Xu et al. on the effects of progestogens in human breast cancer cells suggested that progestins exert different actions on estrogen-metaboilizing enzymes depending on the specific progestogen and regimen

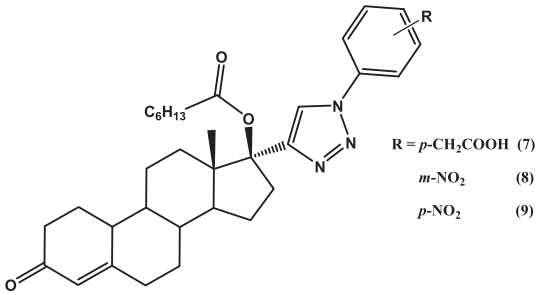



**Fig. 3.** Previously prepared Norethindrone enenthate (NET-EN) derivatives cited in Ref. [18].

used [27].

Our screened compounds showed varied growth inhibitory effects on breast, prostate and other cancer cell lines. No clear difference in anticancer activity can be correlated with the positional isomers of the carboxyl substituent. The attained best results was shown by compound **6b** which revealed 46.4% inhibition of SNB-75 cell line of CNS cancer and about 56% inhibition of both A498 cell line of renal cancer and PC-3 cell line of prostate cancer.

The effects of compound **6b** on cell cycle distribution of PC-3 cell line were evaluated by flow cytometry [28]. Treatment of PC-3 cell line with compound **6b** at the concentration $10^{-5}$ M resulted in accumulation of 31.76% of cells at S phase as compared to 27.87% in untreated cells (Fig. 4). On the contrary the cell population at Go-G1 phase was reduced to 53.94% compared to the untreated cells 59.01%, while the cell percentage of G2-M 14.75% was slightly increased compared to the untreated cells 13.12%. The obtained results imply that the anticancer activity of **6b** derivative could be preceded by the accumulation of cells in the S phase. It is clear that property of induction of cell cycle arrest make this series of 17α-[1-(substit-uted phenyl)-1,2,3-triazol-4-yl]-19-nortestosterone acetates promising progestins with anticancer potentials.

### 2.3. Physicochemical calculations and drugability

The effect of the structural modification on the physicochemical properties of norethindrone acetate was also attempted. Lipinski rule parameters, solubility (logS), the topological polar surface area (TPSA) and molecular volume calculated using Molecular Operating Environment (MOE®) version **10.2010** [29] and the molins-piration server [30].

Calculations displayed in Table 4 reveals: i) the planned structural modification of NEA had slightly increased the Lipinski parameters to the extent not affecting the drugability of these targets. ii) introduced modifications slightly diminished predicted water solubility relative to NEA, however the pendent ionization center carboxyl group provides the possibility of salt formation with bases that can enhance water solubility, iii) increased polar surface area of the target compounds was within the accepted limit [31] for orally active drugs that are transported passively by the transcellular route, iv) the increase in the molecular volume of the targeted compounds did not hamper interaction with the flexible PR active site that accommodate bulky ligands.

## 3. Conclusion

Anchored polar carboxyl moiety to NEA via 1,2,3-triazole ring provided the acid isomers **5a**,**b** and the esters **6a**,**c** with potent progestational activity at nM levels without exerting any toxicity up to 40 mg/kg dose. Molecular docking analysis of the bound conformers to PR revealed that the added moieties to the steroid nucleus strongly affected the binding modes which can be correlated with the biological activity. A number of the prepared compounds disclosed varied activities against certain cell lines of prostate, renal and CNS cancers. Compound **6b** exerted the most prominent cytotoxic activity which was mediated by cell cycle arrest. Our results strongly suggest that our agents may provide a promising new avenue for the development of progestogens with anticancer activity.

## 4. Experimental

### 4.1. General methods

All chemicals and reagents used in current study were of analytical grade. TLC was performed on 60 F254 precoated sheets 20 × 20 cm, layer thickness 0.2 mm (E. Merck, Germany) and visualized in UV light (254 nm). Column chromatography was performed using Fluka silica gel 60 (particle size 0.063–0.02 mm) eluting with ethyl acetate and hexane. Melting points (uncorrected) were determined on electrothermal apparatus (Stuart Scientific, England). IR spectra were recorded as KBr disk using Shimadzu IR 400-91527 Spectrophotometer (Shimadzu Corp., Kyoto, Japan). All the $^{1}$H NMR spectra were recorded on Varian EM-360L NMR Spectrophotometer (60 MHz) (USA) and JEOl-JNM-LA400 FT-NMR Spectrometer (400 MHz) (Japan) in $CDCl_3$ and DMSO-$d_6$. Chemical shifts were reported in $\delta$ (ppm) with TMS as internal standard for protons and solvent signals. $^{13}$C NMR spectra were performed on JEOl-JNM-LA400 FT-NMR Spectrometer (400 MHz) (Japan) in $CDCl_3$. Elemental analyses were performed on Perkin Elmer 2400 CHN elemental analyzer.

### 4.2. General method of synthesis of azidobenzoic acids (*2a–c*) [32]

A solution of sodium nitrite (1.06 g, 15.4 mmol) in cold water (5 °C) (3 mL) was added portion wise to a stirred cold mixture of

**Table 3**
*In vitro* anticancer activity for compounds **5a–c** and **6a–c**.

| Cell line | | Growth percentage (%) ($10^{-5}$ M) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5a | 5b | 5c | 6a | 6b | 6c |
| **Leukemia** | **RPMI-8226** | 94.11 | 91.60 | 83.16 | 77.11 | 70.41 | 90.00 |
| | **SR** | 102.31 | 102.35 | 103.61 | 92.69 | 72.24 | 87.71 |
| **Non-Small Cell Lung cancer** | **A549/ATCC** | 95.55 | 95.73 | 84.99 | 92.02 | 68.57 | 87.89 |
| | **NCI-H522** | 90.41 | 117.20 | 66.54 | 72.64 | 75.69 | 87.48 |
| **Colon cancer (HT29)** | | 102.06 | 110.02 | 93.50 | 97.72 | 79.16 | 100.28 |
| **CNS Cancer** | **SNB-75** | 76.79 | 72.11 | 68.32 | 66.46 | 53.60 | 73.88 |
| | **SF-295** | 104.44 | 103.90 | 92.85 | 89.47 | 74.94 | 93.66 |
| | **SNB-19** | 91.28 | 98.03 | 96.41 | 90.77 | 74.93 | 82.72 |
| **Ovarian cancer (OVCAR-4)** | | 98.07 | 95.43 | 77.20 | 92.60 | 69.60 | 78.59 |
| **Renal cancer** | **A498** | 94.79 | 77.5 | 156.59 | 72.61 | 43.93 | 82.76 |
| | **UO-31** | 82.69 | 80.60 | 83.16 | 62.22 | 62.09 | 73.58 |
| | **CAKI-1** | 93.74 | 96.31 | 96.25 | 86.45 | 67.79 | 92.57 |
| | **RXF393** | 100.35 | 122.63 | 107.34 | 96.84 | 69.13 | 114.66 |
| **Prostate cancer (PC-3)** | | 94.83 | 83.89 | 79.39 | 65.99 | 43.30 | 73.54 |
| **Breast cancer** | **MCF7** | 109.54 | 88.82 | 99.69 | 97.48 | 76.87 | 96.84 |
| | **MDA-MB-468** | 95.48 | 108.97 | 90.65 | 82.67 | 73.27 | 88.15 |

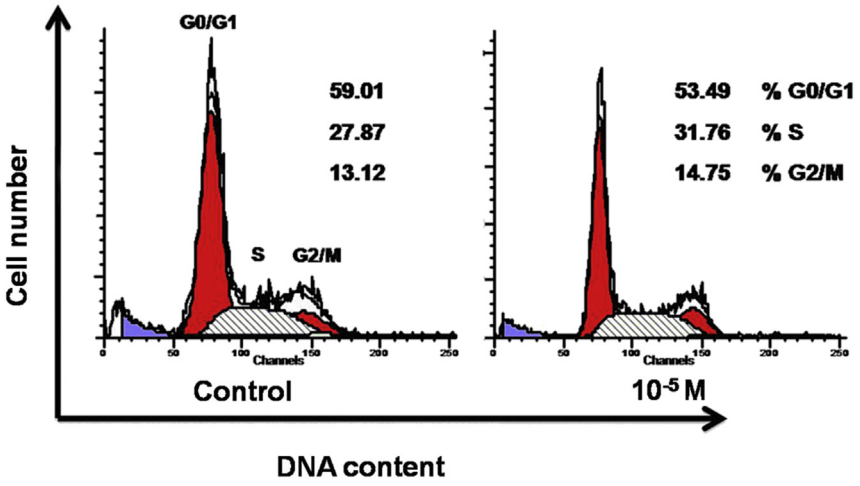



**Fig. 4.** Effect of compound **6b** on cell cycle distribution in PC-3 cell line compared with untreated cells.

**Table 4**
Calculated properties of the synthesized compounds **5a**–**c**, **6a**–**c** and NEA.

| Compound | M.wt. | cLog P(O/W) | Lip-don | Lip-acc | Volume | TPSA | LogS |
|---|---|---|---|---|---|---|---|
| **NEA** | 340.4630 | 3.7070 | 0 | 3 | 333.749 | 43.376 | −5.4856 |
| **5a** | 503.5990 | 3.7990 | 1 | 8 | 458.363 | 111.393 | −6.1313 |
| **5b** | 503.5990 | 3.8380 | 1 | 8 | 458.363 | 111.393 | −6.1313 |
| **5c** | 503.5990 | 3.8010 | 1 | 8 | 458.363 | 111.393 | −6.1313 |
| **6a** | 517.6260 | 4.0630 | 0 | 8 | 475.891 | 100.399 | −6.5436 |
| **6b** | 517.6260 | 4.1020 | 0 | 8 | 475.891 | 100.399 | −6.5436 |
| **6c** | 517.6260 | 4.0650 | 0 | 8 | 475.891 | 100.399 | −6.5436 |

**M.wt.:** Molecular weight, **Lip-don**: Number of Lipinski hydrogen bond donors, **Lip-acc**: Number of Lipinski hydrogen bond acceptors, **Volume**: Molecular volume, **TPSA**: Total polar surface area, **Log S**: Aqueous solubility.

the respective amino benzoic acid **1a**–**c** (2 g, 14.5 mmol), water (10 mL) and concentrated sulfuric acid (3 mL). To the resultant clear mixture, a solution of sodium azide (1.20 g, 18.45 mol) in water (3 mL) was then added with vigorous stirring. A white product was precipitated; the stirring was continued for further 10 min. The precipitate was washed thoroughly with water, filtered under suction, crystallized from the proper solvents to afford the pure products **2a**–**c**.

*4.2.1. 2-Azidobenzoic acid (**2a**)*

White, crystallized from n-hexane, yield: 83% mp: 141–143 °C; IR (KBr, $cm^{-1}$): 3670–3060 (OH); 2100 ($N_3$); 1684 (C=O); $^1$H NMR (60 MHz, $CDCl_3$, $\delta$ ppm, J Hz): 7.09–7.42 (m, 2H, H3,5); 7.42–7.81 (m, 1H, H4); 8.03–8.29 (d, 1H, J = 9, H6); 11.21 (br.s, exchangable, 1H, COOH).

*4.2.2. 3-Azidobenzoic acid (**2b**)*

White, crystallized from n-hexane, yield: 89% mp: 163–165 °C; IR (KBr, $cm^{-1}$): 3625–3380 (OH); 2345 ($N_3$); 1647 (C=O); $^1$H NMR (60 MHz, $CDCl_3$, $\delta$ ppm): 7.13–8.09 (m, 4H, H2, 4–6); 9.16 (s, exchangable, 1H, COOH).

*4.2.3. 4-Azidobenzoic acid (**2c**)*

White, crystallized from water/ethanol, yield: 95% mp: 188–191 °C; IR (KBr, $cm^{-1}$): 3755–3090 (OH); 2095 ($N_3$); 1668 (C=O); $^1$H NMR (60 MHz, $CDCl_3$, $\delta$ ppm, J Hz): 7.03–7.33 (d, 2H, J = 8, H3,5); 7.93–8.29 (d, 2H, J = 8, H2,6); 8.63 (s, exchangable, 1H, COOH).

*4.3. General method for synthesis of methyl azidobenzoates (**3a**–**c**)*

A mixture of the respective azidobenzoic acid **2a**–**c** (2 g, 0.0122 mol), absolute methanol (5 mL, 0.125 mol) and concentrated sulfuric acid (0.36 g, 0.2 mL) was refluxed for 4 h. The solvent was evaporated under reduced pressure and allowed to cool. The product was extracted with chloroform (2 × 50 mL) and the organic layer washed with sodium bicarbonate solution (20%) until effervescence ceases, then with water, and dried over anhydrous magnesium sulfate. The chloroform was evaporated under vacuum and the product was collected and used without further purification.

*4.3.1. Methyl 2-azidobenzoate (**3a**)*

Dark red oil Yield: 63%; IR (KBr, $cm^{-1}$): 2955 (–C–H); 2095 ($N_3$); 1713 (C=O); $^1$H NMR (60 MHz, $CDCl_3$, $\delta$ ppm): 3.89 (s, 3H, $CH_3$); 6.93–8.00 (m, 4H, H3-6).

*4.3.2. Methyl 3-azidobenzoate (**3b**)*

Dark red oil Yield: 75%; IR (KBr, $cm^{-1}$): 2985 (–C–H); 2090 ($N_3$); 1715 (C=O); $^1$H NMR (60 MHz, $CDCl_3$, $\delta$ ppm): 3.86 (s, 3H, $CH_3$); 6.83–7.73 (m, 4H, H2, 4–6).

*4.3.3. Methyl 4-azidobenzoate (**3c**)*

Reddish white crystals, Yield: 80%; mp: 38 °C IR (KBr, $cm^{-1}$): 2100 ($N_3$); 1710 (C=O); $^1$H NMR (60 MHz, DMSO-$d_6$, $\delta$ ppm, J Hz): 3.86 (s, 3H, $CH_3$); 7.00–7.33 (d, 2H, J = 8.5, H3, 5); 7.76–8.06 (d, 2H, J = 8.5, H2, 6).

*4.4. General method for synthesis of 17-α-(1-substituted-1,2,3-triazol-4-yl)-19-nortestosterone acetates (**5a**–**c**) and (**6a**–**c**)*

Norethindrone acetate (**4**) (250 mg, 0.73 mmol) and the respective azide **2a**–**c**, and **3a**–**c** (2.92 mmol) were suspended in a mixture 1:1 of water and t-butyl alcohol (12 mL). An aqueous solution of sodium ascorbate (14.5 mg, 0.073 mmol, water 300 μL) was added, followed by copper (II) sulfate pentahydrate solution

(1.8 mg, 0.0073 mmol, in water 100 μL). The heterogeneous mixture was stirred vigorously under nitrogen overnight, at which point it cleared and TLC monitoring (hexane/ethyl acetate) indicated complete consumption of the steroid. The reaction mixture was diluted with water (50 mL), extracted with ethyl acetate (3 × 20 mL (, dried via filtering over anhydrous magnesium sulfate. The organic layer was evaporated under vacuum, and the residues were purified by column chromatography using gradient elution of (hexane/ethyl acetate) to afford the target products.

*4.4.1. 2-[4-(17-Acetoxy-13-methyl-3-oxo 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-17-yl)-1H-1,2,3-triazol-1-yl] benzoic acid (**5a**)*

White, Yield: 45%, mp: 145–148°C; IR (KBr, $cm^{-1}$): 2590–3695 (OH); 1718 (C=O acetyl); 1651 broad (C=O ketonic overlapped with COOH carbonyl); $^1$H NMR (400 MHz, $CDCl_3$, δ ppm, J Hz): 8.03–8.01 (d, 1H, Ar H6); 7.68–7.64 (m, 1H, Ar H4); 7.58–7.52 (m, 3H, Ar H3,5, triazole H5); 5.81 (s,1H, steroidal C4-H); 3.05–0.64 (unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons); Anal. Calcd for $C_{29}H_{33}N_3O_5$: C, 69.17; H, 6.60; N, 8.34. Found: C, 69.21; H, 6.58; N, 8.49.

*4.4.2. 3-[4-(17-Acetoxy-13-methyl-3-oxo-2,3,6,7,8, 9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-17-yl)-1H-1,2,3-triazol-1-yl]benzoic acid(**5b**)*

White, Yield: 70%, mp: 151–153°C; IR (KBr, $cm^{-1}$): 2530–3435(OH); 1718(C=O acetyl); 1651 broad (C=O ketonic and COOH carbonyl); $^1$H NMR (400 MHz, $CDCl_3$, δ ppm, J Hz): 8.34 (s,1H, Ar H2); 8.14–8.12 (m, 2H, Ar H4,6); 7.84 (s,1H, triazole H5); 7.64–7.60 (t,1H, J = 8, Ar H5); 5.82 (s,1H, steroidal C4-H); 3.13–0.66 (unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons); $^{13}$C NMR (400 MHz, $CDCl_3$): 14.82 (C18); 21.73 (O=C–$CH_3$); 24.07 (C11); 25.91(C15); 26.37 (C7); 30.70 (C6); 32.90 (C16); 35.47 (C2); 36.33 (C1,C12); 40.72 (C8); 42.41 (C10); 46.36 (C14); 47.61 (C13); 48.65 (C9); 88.11 (C17); 119.39 (Ar C2); 121.15 (Ar C4); 124.50 (Ar C5); 125.33 (C4); 130.02 (triazole C5); 130.11 (Ar C6); 131.15 (triazole C4); 137.12 (Ar C1); 150.58 (Ar C3); 166.86 (C5); 170.51 ($OCOCH_3$, COOH); 200.37 (C3); Anal. Calcd for $C_{29}H_{33}N_3O_5$: C, 69.17; H, 6.60; N, 8.34. Found: C, 69.28; H, 6.69; N, 8.57.

*4.4.3. 4-[4-(17-Acetoxy-13-methyl-3-oxo-2,3,6,7,8, 9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-17-yl)-1H-1,2,3-triazol-1-yl]benzoic acid(**5c**)*

White, Yield: 55%, mp: 184–186 °C; IR (KBr, $cm^{-1}$): 2565–3455 (OH); 1714 (C=O acetyl); 1645 (C=O ketonic); 1615 (C=O carboxyl); $^1$H NMR (400 MHz, $CDCl_3$, δ ppm, J Hz): 8.23–8.21 (d, 2H, J = 8.8, Ar H2,6); 7.89–7.86 (d, 2H, J = 8.8, Ar H3,5); 7.82 (s, 1H, triazole H5); 5.81 (s, 1H, steroidal C4-H); 3.75–0.65 (unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons); Anal.Calcd for $C_{29}H_{33}N_3O_5$: C, 69.17; H, 6.60; N, 8.34. Found: C, 69.24; H, 6.71; N, 8.62.

*4.4.4. Methyl 2-[4-(17-acetoxy-13-methyl-3-oxo 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradeca hydro-1H-cyclopenta[a] phenanthren -17-yl)-1H-1,2,3-triazol-1-yl] benzoate (**6a**)*

White, Yield: 50%, mp: 93–96 °C; IR (KBr, $cm^{-1}$): 1722 strong (acetyl and ester carbonyl group); 1656 (C=O ketonic); $^1$H NMR (400 MHz, $CDCl_3$, δ ppm, J Hz): 7.98–7.94 (m,1H, Ar H6); 7.64–7.49 (m, 4H, Ar H3,4,5+ triazole H5); 5.77 (s,1H, steroidal C4-H); 3.67–3.60 (s,1H, $COOCH_3$); 3.01–0.68 (unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons); $^{13}$C NMR (400 MHz, $CDCl_3$): δ 14.52 (C18); 21.55 ($OCOCH_3$); 24.07 (C11); 26.02 (C15); 26.57 (C7); 29.45 (C6); 30.83 (C16); 32.77 (C2); 35.47 (C12); 36.44 (C1); 40.75 (C8); 42.45 (C10); 46.13 (C14); 48.91 (C9,C13); 52.53 ($COOCH_3$); 88.07 (C17); 118.32 (Ar C3); 123.47 (Ar C1); 124.57 (C4); 126.92 (Ar C6); 129.83 (Ar C5,triazole C5); 131.27 (triazole C4); 132.63(Ar C4); 150.95 (Ar C2); 166.46 ($COOCH_3$); 195.03 ($OCOCH_3$), 218.26 (C3); Anal.Calcd for $C_{30}H_{35}N_3O_5$: C, 69.61; H, 6.82; N, 8.12. Found: C, 69.68; H, 6.85; N, 8.27.

*4.4.5. Methyl 3-[4-(17-acetoxy-13-methyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradeca hydro-1H-cyclopenta[a] phenanthren-17-yl)-1H-1,2,3-triazol-1-yl] benzoate(**6b**)*

White, Yield: 75%, mp: 97–99 °C; IR (KBr, $cm^{-1}$): 1719 strong (acetyl and ester carbonyl group); 1657 (C=O ketonic); $^1$H NMR (400 MHz, DMSO-$d_6$, δ ppm, J Hz): 8.84 (s,1H, Ar H2); 8.44 (m,1H, triazole H5); 8.24–8.22 (m,1H, Ar H6); 8.03–8.02 (d, J = 7.6,1H, Ar H4); 7.76–7.71 (m, 1H, Ar H5); 5.67 (s, 1H, steroidal C4-H); 3.90 (s, 3H, $COOCH_3$); 3.40–0.54 (unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons). Anal.Calcd for $C_{30}H_{35}N_3O_5$: C, 69.61; H, 6.82; N, 8.12. Found: C, 69.70; H, 6.88; N, 8.32.

*4.4.6. Methyl 4-[4-(17-acetoxy-13-methyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradeca hydro-1H-cyclopenta[a] phenanthren-17-yl)-1H-1,2,3-triazol-1-yl] benzoate(**6c**)*

White, Yield: 60%, mp: 119–122°C; IR (KBr, $cm^{-1}$): 1715 strong (acetyl and ester C=O group); 1655 (C=O ketonic); $^1$H NMR (400 MHz, DMSO-$d_6$, δ ppm, J Hz): 8.11–8.09 (d, 2H, J = 8.8, Ar H2,6); 8.08–8.05 (d, 2H, J = 8.8, Ar H3,5); 8.78 (s,1H, triazole H5); 5.63 (s, 1H, steroidal C4-H); 3.83 (s, 1H, $COOCH_3$); 3.33–1.03(unresolved multiplets of cycloaliphatic protons of the steroidal nucleus in addition to acetyl and C18 methyl protons). Anal.Calcd for $C_{30}H_{35}N_3O_5$: C, 69.61; H, 6.82; N, 8.12. Found: C, 69.57; H, 6.95; N, 8.33.

### *4.5. In vivo progestational activity*

#### *4.5.1. Animals*

Adult female Wistar rats (weighing 200–215 g) were housed into 8 groups 6 per cage at 20–22 °C under controlled conditions of light, with free access to rat chow and tap water. Rats showing regular estrous cycle length (4–5 days). The phases of estrous cycle were determined by observing the vaginal smear in the morning. Only those rats showing at least two consecutive 4-days estrous cycles were used. For all experiments the treatment was started when the animals were in estrus phase.

#### *4.5.2. Reference standard*

Norethindrone acetate was obtained as a gift from Hi pharm pharmaceutical company, El Obour City, Egypt.

#### *4.5.3. Morphometric measurements*

Calculations were carried out using Lieca Qwin 500 Image Analyzer in Pathology Department, National Research Centre, Cairo, Egypt. All the *in vivo* investigational studies were carried out in Pharmacology and Pathology Departments, National Research Centre, Cairo, Egypt.

#### *4.5.4. Methodology*

Initial body weight before treatment and final body weight at the time of sacrifice were recorded. Then 1 mL solution of the tested compounds **5a**–**c**, **6a**–**c** or reference standard (Norethindrone acetate) in DMSO (0.018 mg/mL) was injected subcutaneously daily for 8 days to rat groups, while the control group received an equivalent amount of the vehicle. Twenty-four hours after the final

dose, rats were killed, and their uteri were carefully excised, trimmed of extraneous tissue, blotted filter paper to remove excess fluid, weighed to calculate the uterus weight as the following:

$$\text{Relative organ weight (kg)} = [\text{organ weight (g)}/\text{body weight (g)}] \times 1000$$

The uteri were fixed and stained, paraffin sections were evaluated for histological changes. The thickness endometrium, myometrium and the uterine epithelial cell heights were measured using an objective lens of magnification 10, and eye lens 10, the total magnification was 100 times. Ten fields were chosen in each specimen and the mean values were taken.

#### 4.5.5. Statistical analysis

The data were expressed as mean ± **SEM** and analyzed using SPSS statistical software. One way analysis of variance (ANOVA) was used to assess the variation of the means among the treatments. If the variation was greater than expected by chance alone, Tukey multiple comparison tests were performed to compare each treatment group with the control and standard groups. Significance was established when the p value was less than 0.05.

#### 4.5.6. Evaluation of the acute toxicity and lethality ($LD_{50}$) for **5b** and **6c**

*Test solutions*: aliquots of the tested compounds were dissolved separately in DMSO and diluted with water to the required concentration to give 10% final dilution of DMSO.

*Doses*: the animals were injected subcutaneously with a dose of 5, 10, 15, 30, 40 mg/kg mice.

*Control animals*: received the vehicle (10% DMSO).

*Procedure*: Groups of adult albino mice (25–30 g) of either sex, each of three animals (six in case of 40 mg/kg dose) were injected with graded doses of the tested compounds solutions and the vehicle as control. Animals were observed per dose for 24 h and signs of toxicity and number of deaths were recorded.

### 4.6. In vitro anticancer activity

#### 4.6.1. Screening for anticancer activity

Newly synthesized 17α-(1-substituted-1, 2,3-triazol- 4-yl)-19-nortestosterone acetates **5a**–**c** and **6a**–**c** (conc. $10^{-5}$ M) were screened for their anticancer activity according to NCI *in vitro* protocols, against a panel consisting of 60 human tumor cell lines.

#### 4.6.2. Cell cycle analysis for compound **6b**

Prostate cancer cells PC-3 from the treated ($10^{-5}$ M of **6b**) and control cells were collected after 48 h. Cell cycle distribution of the cell population was analyzed using Cycle TEST TMPLUS DNA Reagent kit (BD Biosciences, USA). Cells were fixed with 70% ice-cold ethanol, washed and the pellet was suspended in trypsin buffer and left for 10 min at room temperature. 1% RNAase buffer was added after addition of trypsin inhibitor and incubated for 10 min, followed by the addition of 100 μg/mL propidium iodide. Samples were incubated in the dark for 30 min at 4 °C. Distribution of cell-cycle phases with different DNA contents was determined using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA, USA). This study was carried out at Cancer biology department, National Cancer Institute, Cairo, Egypt.

### 4.7. Docking simulations

The X-ray crystallographic structures of progesterone receptor complexed with norethindrone (PDB ID: 1SQN) was obtained from the Protein Data Bank through the internet (http://www.rcsb.org). All the molecular modeling calculations and docking simulation studies were performed using Molecular Operating Environment (MOE®) version **10.2010**, Chemical Computing Group (CCG) Inc., Montreal, Canada.

## Acknowledgment

We would like to acknowledge The National Cancer Institute (NCI) for the *in vitro* anticancer activity screening.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.045.

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